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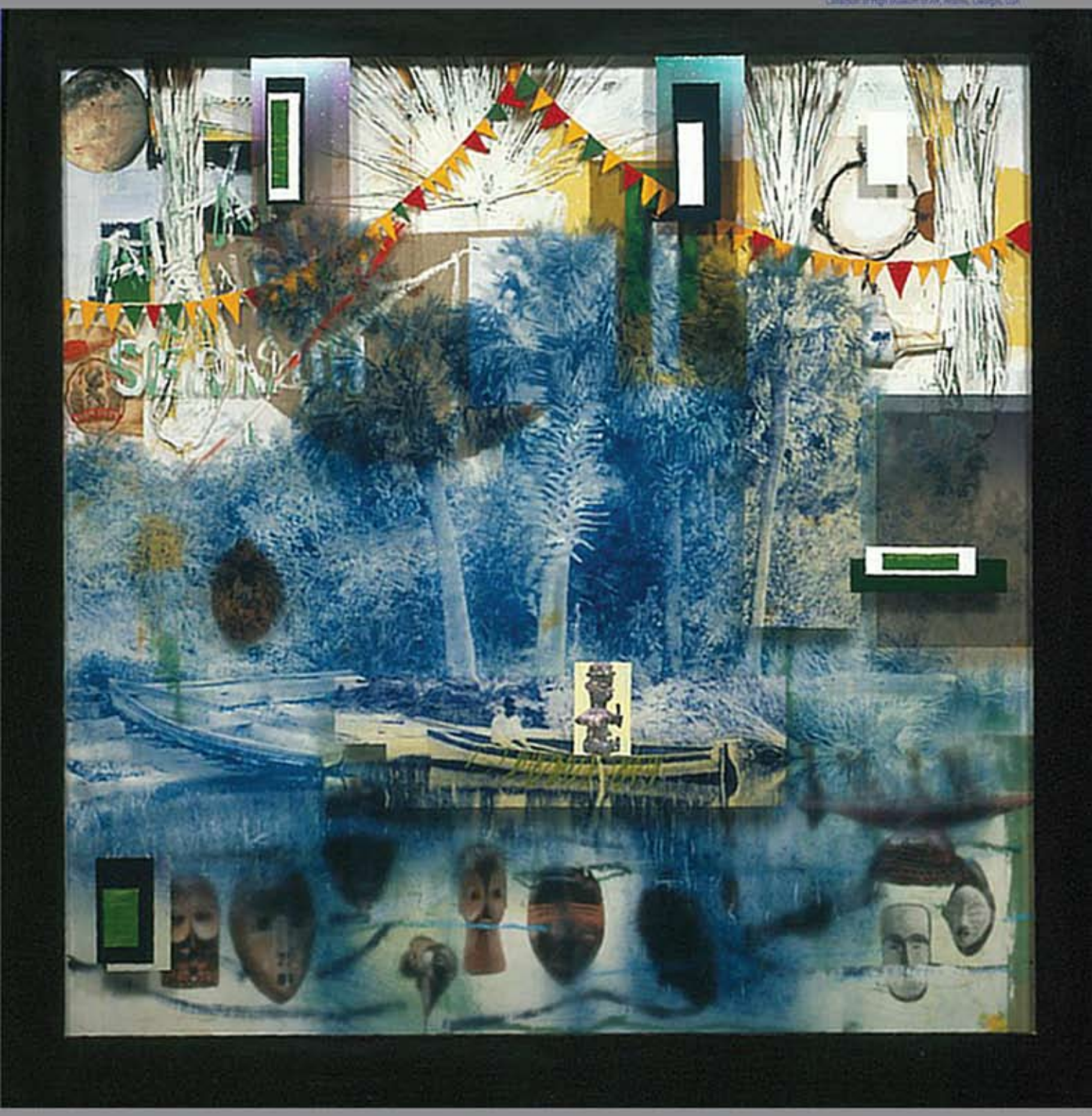
February 2012

Infections New and Old



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Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30333, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

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EMERGING INFECTIOUS DISEASES

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On the Cover

Radcliffe Bailey (b. 1968)
***En Route* (2005)**
Photograph on Plexiglas, coconut palms, felt, acrylic, and wood
(172.7 cm × 172.7 cm × 14 cm)
Collection of High Museum of Art, Atlanta, Georgia, USA

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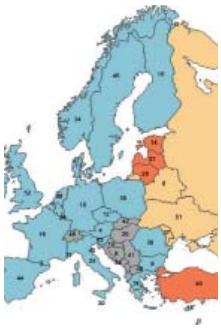

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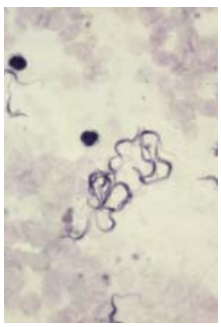

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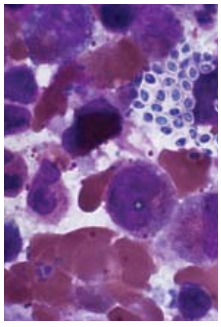

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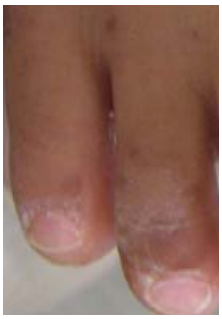

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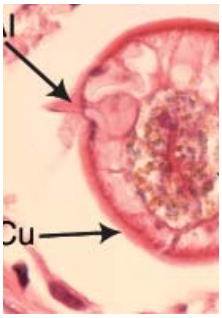

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Pathogenic Responses among Young Adults during the 1918 Influenza Pandemic

G. Dennis Shanks and John F. Brundage

Of the unexplained characteristics of the 1918–19 influenza pandemic, the extreme mortality rate among young adults (W-shaped mortality curve) is the foremost. Lack of a coherent explanation of this and other epidemiologic and clinical manifestations of the pandemic contributes to uncertainty in preparing for future pandemics. Contemporaneous records suggest that immunopathologic responses were a critical determinant of the high mortality rate among young adults and other high-risk subgroups. Historical records and findings from laboratory animal studies suggest that persons who were exposed to influenza once before 1918 (e.g., A/H3Nx 1890 pandemic strain) were likely to have dysregulated, pathologic cellular immune responses to infections with the A/H1N1 1918 pandemic strain. The immunopathologic effects transiently increased susceptibility to ultimately lethal secondary bacterial pneumonia. The extreme mortality rate associated with the 1918–19 pandemic is unlikely to recur naturally. However, T-cell–mediated immunopathologic effects should be carefully monitored in developing and using universal influenza vaccines.

The influenza pandemic of 1918–19 was the most deadly single event in recorded history. Because of its unique severity and global effects, it is the prototype of a global natural disaster. In recent years, fears of recurrence of an influenza pandemic similar to that in 1918 have motivated planning, preparations, and allocations of resources by public health and other government agencies, nongovernmental organizations, medical care providers, pharmaceutical and medical device manufacturers, medical researchers, private businesses, and persons worldwide (*1*).

Because of severe consequences and current relevance of the 1918 pandemic, it is essential to review its events and effects, determine their underlying causes, and assess likelihood of a recurrence. These tasks are difficult because the 1918 pandemic occurred at the end of World War I, before influenza viruses were discovered and before influenza vaccines, antiviral and antibacterial drugs, and intensive medical care were available. Fortunately, abundant and detailed written records exist of clinical, laboratory, and epidemiologic events during the pandemic period (*2*–*6*). In addition, isolates of the virus that caused the lethal second wave of the pandemic (in the fall of 1918 in most locations) have been reconstructed from preserved remains of patients who died (*7*). These isolates have been used to determine the genetic relationships between the 1918 pandemic influenza strain and subsequent seasonal and pandemic A/H1N1 strains (*8*). Genetic relationships between the 1918 pandemic strain and strains that caused the clinically mild first wave of epidemics in 1918 and pandemics before 1918 remain undefined (*9*–*11*).

It is commonly believed that the 1918 pandemic resulted from the sudden emergence and worldwide spread of an inherently hypervirulent influenza strain. However, this view is inconsistent with several well-documented characteristics of the pandemic. In this report, we review unique, poorly understood, or unexplained clinical and epidemiologic characteristics of the 1918 pandemic. Also, we present hypotheses that are scientifically credible, consistent with the historical record, and account for epidemiologic and clinical manifestations of the pandemic. Finally, we discuss implications of our hypotheses regarding pandemic influenza preparedness and research and development of universal influenza vaccines (*12*).

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DOI: http://dx.doi.org/10.3201/eid1802.102042

Unique and Unexplained Characteristics of the 1918 Pandemic

Mortality and Case-Fatality Rates

Because the 1918 pandemic spread worldwide and caused unprecedented numbers of deaths, it is often presumed that the pandemic strain was unusually transmissible and that infection with the virus was inherently lethal (i.e., direct effects of the virus routinely and rapidly caused death). During the 1918 pandemic, influenza infection rates were similar to those during other pandemics of the last century; and in most affected populations, overall mortality rates were <1%, and case-fatality rates were <3% (*4*,*13*). Thus, the 1918 pandemic strain was not unusually transmissible compared with other pandemic strains (*13*); and even without definitive treatments (e.g., antiviral and antibacterial drugs) or modern life-preserving measures (e.g., mechanical ventilation, medical intensive care), most infected persons survived (*10*).

Deaths Caused by Secondary Bacterial Pneumonia

In 1918, most pandemic-related deaths were not caused by primary influenza-related pneumonia or acute respiratory distress syndrome, and relatively few deaths occurred within the first few days after illness onset (*11*). Most deaths occurred ≥7 days after illness onset and were the result of secondary bacterial pneumonia caused by common colonizers of the respiratory tract, e.g., *Haemophilus influenzae*, *Streptococcus pneumoniae*, *S. pyogenes*, and *Staphylococcus aureus* (*3–5*,*14*). Clinical and pathologic records suggest that lethal secondary bacterial pneumonias often followed dysregulated immune responses to infections with influenza (*15*,*16*).

Increased Mortality Rate in Young Adults (W-shaped Mortality Curve)

In general, during the 1918–19 influenza pandemic period, illness rates were highest among children of school age. However, mortality rates were highest among infants, young adults, and the elderly (Figure) (*17*). The W-shaped relationship between mortality rate and age is a unique and unexplained characteristic of the 1918 pandemic. The lack of correspondence between illness and mortality rate in relation to age belies the common views that direct pathologic effects of the virus were independently and invariably life threatening and that the usual clinical course after infection was rapid deterioration of respiratory function terminating in death.

In 1889–90, pandemic influenza (Russian flu) spread rapidly throughout the world, and from 1890 through the winter of 1900–01, widespread epidemics of influenza-like illness recurred (*4*,*18*). The 1890–91 and subsequent epidemic waves likely were caused by variants of the 1889–90 pandemic strain (*19*). Thus, before 1918, most members of the 1875–1900 birth cohorts had been exposed to the 1889–90 pandemic influenza strain. These persons were 18–43 years old, the age groups at highest mortality risk, during the lethal second wave of the 1918 pandemic (*20*).

Timing and Characteristics of Epidemic Waves

During 1918–19, three distinct influenza epidemic waves occurred. The first wave (mid-1918 in most locations) caused widespread illness but few deaths (3-day fever). The second wave (fall of 1918 in most locations) caused widespread illness and high mortality rates (*14*). The third wave (winter 1919) caused widespread illness but affected fewer persons and caused fewer deaths than the second wave.

The sharp contrast in the clinical expressions of infections during the first and second waves suggests that they were caused by different influenza virus strains. If the first 2 epidemic waves of the 1918–19 pandemic were caused by the same or immunologically cross-reactive influenza A (H1N1) viruses, persons affected during the first wave should have been protected from infection and, in turn, illness, secondary pneumonia, and death during

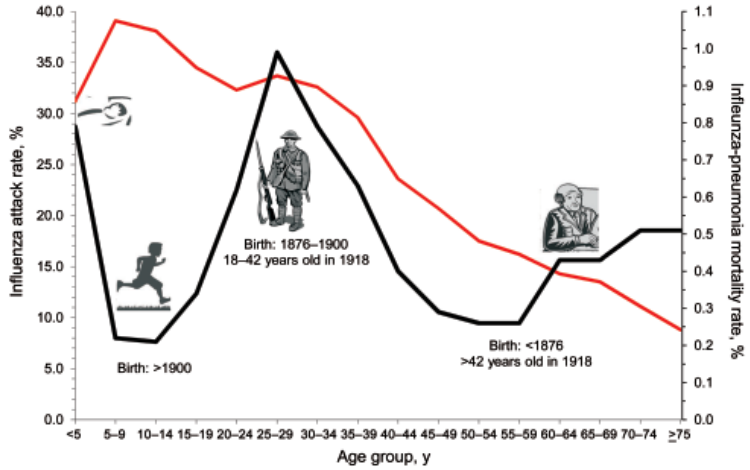

Figure. Illness attack rate (red line) and overall mortality rate (black line) for influenza-related pneumonia, by age groups of selected US populations, during the 1918 influenza pandemic period.

the second wave. Protection from infection would have derived from neutralizing antibodies against the same or similar viral surface antigens (e.g., hemagglutinin).

There are conflicting reports regarding the immunologic susceptibility to infection during the second wave among persons who were infected during the first wave. Several reports of the experiences of localized groups (e.g., students, prisoners, military units) suggest that illness during the first wave protected from influenza during the second wave (*3*,*9*). However, our review of the medical records of all persons who served in the Australian Imperial Forces in Europe and the Middle East in 1918 documents that persons affected during the first wave were as likely to become ill, but were much less likely to die, from influenza–pneumonia during the second wave (*12*). Together, the findings suggest that infections during the first wave altered immune responses to the pandemic strain during the second wave. In turn, persons infected during the first wave had milder clinical expressions and lower mortality rates when infected with the pandemic strain during the second wave.

Mortality Rates among Nurses and Medical Officers

During the 1918 pandemic period, military nurses and medical officers were intensively and repeatedly exposed to the influenza A (H1N1) pandemic strain in clinics, in ambulances, and on crowded open wards. However, during the lethal second wave, nurses and medical officers of the Australian Army had influenza-related illness rates similar to, but mortality rates lower than, any other occupational group (*12*). Similar observations were made in other groups of military and civilian health care workers (*21*). These findings suggest that the occupational group with the most intensive exposure to the pandemic strain had relatively low influenza-related pneumonia mortality rates during the second wave (*12*).

Mortality Rates among Military Members with Least Service

During the fall of 1918, all 40 large mobilization/training camps throughout the United States and Puerto Rico were affected by influenza epidemics (*13*,*22*). During the camp epidemics, influenza–pneumonia mortality rates were inevitably highest among the soldiers with the least military service. In the US Army overall, 60% of those who died of influenza-related pneumonia were soldiers with <4 months of military service (*13*,*14*,*22*).

Among Australian soldiers in Europe and the Middle East in the fall of 1918, persons with the least military service also had the highest influenza-related pneumonia mortality rate (*12*). In general, in deployed Australian Army formations, soldiers were not segregated by time in military service. Thus, the nature or intensity of soldiers' exposures to the pandemic strain likely did not vary in relation to their length of military service. During epidemics of the second wave, most soldiers were likely exposed to the same influenza A (H1N1) strain, and most of those affected were treated in the same military medical system as their counterparts regardless of seniority. Thus, the sharp differences in mortality rates in relation to length of service, in mobilization camps in the United States and deployed settings in Europe, likely reflected differences in host immune responses to the pandemic strain.

Mortality Rates among Passengers and Crews on US Troop Transport Ships

Influenza illness rates were similar on US troop transports. However, case-fatality rates were sharply higher among soldiers who had recently congregated on the ships than among permanently assigned crewmen (*23*).

Mortality Rates among Residents and Soldiers from Urban and Rural Areas

In the United States during the pandemic period, the influenza-related mortality rate was higher among residents of urban areas than among residents of rural areas. In contrast, the mortality rate was higher among soldiers from rural than among those from urban areas (*24*).

Mortality Rates among Island Populations

When pandemic influenza attacked island populations, mortality rates were often high, sometimes extraordinarily so, e.g., Western Samoa (22%) and Nauru (16%) (*25*–*28*). However, some island populations had relatively low mortality rates during pandemic-related epidemics, e.g., the Philippines (1%), Puerto Rico (1%), and Hawaii (0.5%) (*25*,*29*). On other islands, mortality rates varied widely among different groups of island residents, e.g., Chamorrans (12%) versus Caroline Islanders (0.4%) on Saipan (*26*); indigenous Fijians (5.7%) versus Europeans (1.4%) on Fiji (*4*); and indigenous (Maori) (4.2%) versus European (0.5%) residents of New Zealand (*30*). Thus, on some islands, subgroups of residents who shared the same microbiological and environmental exposures had markedly different influenza-related mortality rates. Because the same influenza strain likely caused all pandemic-related island epidemics, the wide variability in mortality rates across island populations suggests that host immune factors were determinants (perhaps with other factors such as poverty, overcrowding, and malnutrition) of the clinical courses and outcomes of infections with the pandemic influenza strain.

Clinical Expression of Infections with Similar A/H1N1 Strains in 2009 and 1918

The 1918 influenza A (H1N1) pandemic strain is genetically similar to the novel pandemic (H1N1) 2009

strain. However, clinical expressions of infections in 2009 were much less severe than in 1918, e.g., mortality rates in 1918 were >100× higher than in 2009 (*31*).

Hypotheses

Unique and unexplained characteristics of the 1918 pandemic suggest that the risk for lethal secondary bacterial pneumonia after influenza infections depended on the nature, timing, and intensity of immune responses to the pandemic strain; and subsequently on the likelihood of exposure during transient periods of increased susceptibility to bacterial strains against which affected persons had no protective antibodies. In 1918, nearly all humans were immunologically susceptible to infection with the A/H1N1 pandemic strain; not surprisingly, the pandemic spread rapidly worldwide. The rapid spread of the pandemic with high illness attack rates in most age groups indicates that an influenza virus antigenically similar to the pandemic strain did not widely circulate among humans within at least several decades before 1918.

We hypothesize that in 1918 many persons had second lifetime exposures to an immunodominant T-cell epitope that was conserved on an internal protein of the 1918 pandemic strain and a heterosubtypic other strain (e.g., 1889 pandemic strain). When persons were reexposed to the identical epitope in 1918, epitope-specific memory CD8+ T-cells produced excessive cytokines, chemokines, immune cell activation, and epithelial cell necrosis. The immunopathologic effects of the dysregulated T-cell response transiently increased susceptibility of infected hosts to respiratory bacterial strains against which they lacked protective antibodies.

In contrast, persons who were first exposed in 1918 to the immunodominant T-cell epitope of hypothesized concern may have had primary T-cell responses that controlled virus replication without increasing susceptibility to bacterial invasion of the lower respiratory tract. Persons who had multiple prior exposures to influenza viruses and other respiratory infectious agents before 1918 had diversely partitioned memory CD8+ T-cell repertoires and extensive portfolios of bacterial strain–specific antibodies. Their immune responses to infection with the 1918 pandemic strain may have controlled virus replication without increasing their susceptibility to bacterial invasion.

Modern genetic analyses have estimated that 3 distinct variants of influenza A (H1N1) viruses co-circulated in the early 1900s (*8*,*32*). These variants were the respective prototypes of all pandemic, seasonal, and classical swine influenza A (H1N1) viruses since 1918. The first epidemic wave of the 1918 pandemic may have been the last wave of the 1889–90 Russian flu pandemic. If so, the first wave spread widely and rapidly in the face of background immunity to an influenza strain that had been circulating among humans for nearly 3 decades (*4*,*18*).

Alternatively, the first wave may have been caused by an antigenically distinct seasonal strain of influenza A (H1N1) (*8*,*32*). If so, antibodies against hemagglutinin of the seasonal strain did not provide complete protection against infection with the pandemic strain. However, because many internal proteins of human influenza viruses are conserved and strongly immunogenic (e.g., matrix 2, nucleoprotein [NP]), antibodies and memory CD8+ T lymphocytes that were produced during the first wave may have altered clinical expressions, decreased susceptibility to secondary bacterial pneumonia, and reduced deaths during the second wave (*33*).

The peak of mortality rates among young adults (W-shaped mortality curve) remains a unique and unexplained characteristic of the 1918 pandemic (*20*). Before World War I, there was relatively little global interconnectedness. Even in the most industrialized countries, many persons lived their entire lives in their birth communities and had relatively little exposure to outsiders. The situation sharply and permanently changed with the social disruptions and population dislocations precipitated by worldwide armed conflict.

Persons born before 1901 (the last year of widespread circulation of the 1890 Russian flu pandemic strain) and after 1875 (the first year after widespread epidemics of a poorly characterized influenza-like illness) were 18–43 years of age in 1918. Worldwide, members of these birth cohorts had high influenza attack rates and were likely to die from secondary bacterial pneumonia during the 1918 pandemic period (Figure).

Persons born before 1875 were >43 years of age in 1918. Before the 1918 pandemic period, they likely had been exposed to more heterosubtypes of influenza A and more respiratory bacterial strains than their younger counterparts. In general, during the pandemic period, middle-age and elderly adults had lower influenza attack rates and were less likely to die than their younger counterparts (Figure).

Also in 1918, persons born after 1901 were less likely than those older to have been exposed to the 1890 pandemic strain or displaced by war-related activities. During the pandemic period, persons <17 years of age had relatively high influenza attack rates but relatively low mortality rates (except for infants) (Figure).

We hypothesize that, soon after infection with the 1918 pandemic influenza strain, infected persons experienced a transient increase in susceptibility of the lower respiratory tract to invasion by bacteria to which they were immunologically naive. Thus, for example, those who were relatively new to their living or work environments (e.g., military recruits, soldiers on troop

ships, patients on hospital wards) at the time they were infected with the 1918 pandemic strain had a relatively high risk of death from secondary bacterial pneumonia (*12*). During the course of their influenza illnesses, such persons were likely to be exposed to bacterial strains to which they lacked protective antibodies (*10*). Thus, for example, residents of rural areas were relatively unlikely to be exposed to novel strains of bacteria while recovering from influenza, and they had low pandemic-related mortality rates. In contrast, military recruits from the same rural areas were likely to be exposed to novel strains of bacteria while recovering from influenza, and they had relatively high pandemic-related mortality rates (*24*).

This interpretation explicates the somewhat counterintuitive finding that nurses, medical officers, and the crews of troop ships had high influenza attack rates but relatively low mortality rates during the lethal second wave of the 1918 pandemic. Before being infected with the pandemic influenza strain, these persons were often exposed in their occupational settings to high concentrations of diverse strains of respiratory infectious agents. Because of their extensive portfolios of respiratory bacteria strain–specific antibodies, they were naturally immune to and protected from secondary pneumonia caused by these agents (*12*,*23*,*24*).

The hypotheses presented here are consistent with the historical record and scientifically plausible. For example, studies in humans have identified an immunodominant NP-derived CD8+ T-cell epitope that is consistently presented by high frequency HLA class I molecules and recognized by cytotoxic T lymphocytes. The epitope is present on the NP of the 1918, 1976, and 2009 human pandemic strains and on most swine strains, but not on most other human strains of the past century (*34*).

Studies in pigs suggest that the NP of most swine influenza strains contains a strongly immunogenic CD8+ T-cell epitope. For example, pigs that were primed with a DNA vaccine that expresses NP, and subsequently challenged with an influenza A strain with the same NP, had dysregulated, pathogenic immune responses (*35*). Also, pigs that were primed with an inactivated swine influenza A vaccine (A/swine/Iowa/15/1930 H1N1) and subsequently challenged with a later generation swine influenza A strain with markedly different surface proteins (A/swine/Minnesota/00194/2003 H1N2) showed development of enhanced (immunologically potentiated) pneumonia that were not observed after challenge with the homologous strain (*36*). The findings have been reproduced by using pandemic (H1N1) 2009 virus as the challenge strain and adding a recombinant matrix 2 protein to the vaccine construct (*37*).

Studies in mice have documented that T-cell–mediated immunopathologic responses can contribute to severe pneumonitis when mice are exposed to a highly glycosylated influenza virus and subsequently infected with a poorly glycosylated strain. Infection with a recent seasonal influenza virus (H1N1), followed by infection with pandemic (H1N1) 2009 virus, elicited severe immunopathogenic responses (*38*). Finally, studies in ferrets have documented that those infected with pneumococci after acquiring influenza, but not before, showed development of lethal secondary pneumonia and other invasive complications (*39*).

In summary, we hypothesize that mortality risk after infection with the 1918 pandemic influenza A (H1N1) strain depended on the number, nature, and diversity of prior infections with influenza virus and respiratory bacteria. Specifically, mortality rates during the lethal second wave were highest among persons with prior exposures to heterosubtypic influenza strains that enhanced immunopathogenic effects when a person was infected with the 1918 pandemic strain and had limited exposures to other respiratory infectious agents. In such persons, infection with the pandemic strain caused high viral loads, dysregulated and pathogenic cell mediated immune responses, and transient increases in susceptibility to invasive bacterial infections. If such influenza virus–infected hosts were subsequently exposed to bacterial strains to which they had no protective antibodies, they were at high risk of acquiring life-threatening secondary bacterial pneumonia.

The unique circumstances that enabled the unprecedented mortality rates of the 1918 pandemic no longer exist on a global scale. For example, in modern times, even the most isolated communities (e.g., Pacific islanders, indigenous populations of North America, Australia, and New Zealand) are interconnected through myriad commercial and sociopolitical activities. As a result, most populations are exposed to annual seasonal influenza viruses, and most young adults are exposed to numerous viral and bacterial respiratory pathogens. Thus, compared with the situation in 1918, adults in modern communities have more diversified immune repertoires against influenza strains and bacterial respiratory pathogens. The hypotheses presented may explain at least in part the relatively low mortality rate associated with pandemic (H1N1) 2009 virus. During the 2009 pandemic, many persons who died had underlying medical conditions, including obesity, asthma, cardiovascular diseases, diabetes, and pregnancy; histopathologic changes consistent with diffuse alveolar damage; and evidence of bacterial co-infections. (*40*)

Finally, the findings of this report are relevant to the research and development of a universal influenza vaccine. Candidate vaccines that contain antigens that are highly conserved across influenza A strains and strongly immunogenic must be closely monitored to ensure that

T-cell–mediated immune responses to future seasonal and pandemic strains are protective but not pathogenic.

Dr Shanks is director of the Australian Army Malaria Institute in Enoggera, Queensland, Australia. His research interests include infectious diseases, malaria chemoprophylaxis and treatment, and influenza epidemiology.

Dr Brundage is a physician/epidemiologist at the Armed Forces Health Surveillance Center in Silver Spring, Maryland. His research interests include epidemiologic and clinical effects of interactions among co-circulating infectious agents.

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Invasive Pneumococcal Disease and Pandemic (H1N1) 2009, Denver, Colorado, USA

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Learning Objectives

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

- Evaluate the epidemiology of pandemic (H1N1) 2009 in cases of invasive pneumococcal disease (IPD).
- Distinguish variables associated with IPD during the period of circulating pandemic (H1N1) 2009.
- Assess patterns of vaccination and antiviral treatment among patients with IPD.
- Analyze the severity of IPD during the period of circulating pandemic (H1N1) 2009.

Editor

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Disclosures: ***George E. Nelson, MD; Kenneth A. Gershman, MD, MPH; David L. Swerdlow, MD; Bernard W. Beall, PhD;*** *and* ***Matthew R. Moore, MD, MPH, have*** *disclosed no relevant financial relationships.*

Pneumococcal pneumonia was a complication during previous influenza pandemics but was not evident initially during pandemic (H1N1) 2009. During October 2009 in Denver, Colorado, USA, invasive pneumococcal disease (IPD) and pandemic (H1N1) 2009 peaked simultaneously, which suggests a link. We compared cases of IPD in October 2009 with cases in February 2009, the most recent peak month of seasonal influenza. During October 2009, we observed 58 IPD cases, which was 3× the average number of IPD cases that usually occur in October in Denver. Patients with IPD in October 2009 were younger and more likely to have chronic lung disease than patients who had IPD in February 2009; a total of 10/47 patients had influenza, and 33/53 patients had influenza-like illness. Thus, ≈17% to 62% of cases of IPD may have been associated with pandemic (H1N1) 2009. Pneumococcal disease prevention strategies should be emphasized during future influenza pandemics.

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DOI: http://dx.doi.org/10.3201/eid1802.110714

Pneumonia caused by *Streptococcus pneumoniae* (pneumococci) was a frequent complication of influenza during previous pandemics. In 1 autopsy series, ≈20% of deaths during the 1918 influenza pandemic were associated with pneumococci (*1*). Pandemic (H1N1) 2009 was the first pandemic in which pneumococcal and influenza vaccines and antiviral drug treatment had the potential to change the interaction between pneumococcal infection and influenza.

Among early cases of pandemic (H1N1) 2009, pneumococcal complications were rarely reported (*2*–*4*). However, in October 2009, the Colorado Department of Public Health and Environment identified a substantial increase in cases of invasive pneumococcal disease (IPD) in the Denver metropolitan area, concurrent with a peak in pandemic (H1N1) 2009–associated hospitalizations, raising the question of the role of this pandemic (H1N1) 2009 virus. We evaluated the IPD cases in October 2009 in terms of age, prevalence of concurrent conditions, severity of illness, evidence of co-infection with pandemic (H1N1) 2009 virus, use of antiviral drugs, and influenza and pneumococcal vaccination. We also assessed the possible contribution of changes in laboratory practices to the increase in reported IPD cases.

Methods

Epidemiologic Investigation

Classification as IPD required isolation of *S. pneumoniae* from a normally sterile site, such as blood or cerebrospinal fluid. IPD cases were identified through the Active Bacterial Core surveillance (ABCs), a population- and laboratory-based system run continuously since 2000 in the 5-county Denver metropolitan area (population 2.4 million). To evaluate the magnitude of the apparent increase in IPD cases during October 2009, we compared the number of cases during that month to the mean number of cases occurring each October during 2004–2008 because the decrease in IPD rates that followed introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) for US infants stabilized by 2004 (*5*).

To evaluate if IPD cases in October 2009 were epidemiologically different from IPD cases in previous, nonpandemic years, we compared the October 2009 cases to IPD cases in February 2009, the most recent local peak in seasonal influenza (H1N1). If the October 2009 cases were associated with pandemic (H1N1) 2009 and if this influenza affected IPD risk differently than seasonal influenza, we would expect the epidemiology of October 2009 IPD cases to differ from a month with predominant nonpandemic, seasonal influenza (H1N1) circulation. Data analyzed included epidemiologic information from the standard ABCs case report form (www.cdc.gov/abcs/files/ABCs_case_report_form_2009.pdf). More detailed information on initial symptoms, diagnostic testing, clinical laboratory information, and clinical management was collected by chart abstraction.

Chart abstraction included review of physician notes, consultation reports, and laboratory results included in patients' medical records. For October 2009 cases, supplementary information was obtained from interviews with patients or their surrogates. For comparisons of underlying conditions and basic demographics, we ensured consistent methods across time by comparing data derived only from the ABCs case report form. Most October case-patients had pneumonia; for these, severity of illness was assessed by using the Pneumonia Severity Index, a well-established scoring system that incorporates concurrent conditions, laboratory findings, and vital signs at clinical presentation (*6*). Additional data available for only October 2009 cases included vaccination, antiviral drug treatment, serologic test results, and intensive care unit (ICU) admission.

Influenza-associated hospitalizations were identified by using the Colorado Emerging Infections Program. This program defines laboratory-confirmed influenza infection as any positive rapid test or pandemic (H1N1) 2009 virus–specific real-time PCR result in a hospitalized resident of the surveillance area.

To identify the upper limit of potential pandemic (H1N1) 2009 cases among the October 2009 IPD cases, we used 2 approaches. First, because recent data (*7*,*8*) suggest that the sensitivity of PCR for pandemic (H1N1) 2009 virus decreases 5 days after symptom onset, patients with influenza-like illness (ILI) (fever plus cough or sore throat) and negative or unknown PCR results >5 days before the date of pneumococcal culture were considered to be potentially associated with pandemic (H1N1) 2009. Second, all IPD cases in excess of 2 SD above the mean number of IPD cases in October during 2004–2008 were defined as cases that may have been associated with pandemic (H1N1) 2009. Health care providers and the Colorado Immunization Information System were contacted to verify vaccination status for PCV7, pneumococcal polysaccharide vaccine (PPV23), and seasonal influenza and pandemic (H1N1) 2009 vaccines.

Laboratory Methods

S. pneumoniae isolates were serotyped by using type-specific antisera and observation of the Quellung reaction at the Streptococcus Laboratory (Centers for Disease Control and Prevention, Atlanta, GA, USA). For analysis, pneumococcal serotypes were grouped as follows: PCV7 serotypes (4, 6B, 6A, 9V, 14, 18C, 19F, and 23F); PPV23 serotypes (PCV7 serotypes plus 1, 2, 3, 5, 7F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 19A, 20, 22F, and 33F); and all other serotypes and nontypeable pneumococci (not included in either vaccine). Serotype 6A was included in

PCV7 serotypes because of cross-reactivity of 6A and 6B (*9*). Serotype 6C strains were identified within serogroup 6 by using PCR (*10*). To determine whether IPD cases were caused by an outbreak of a single pneumococcal strain, we compared serotypes causing IPD in the Denver area in October 2009 with those causing IPD in previous years.

Influenza diagnostic methods investigated were real-time reverse transcriptase PCR, rapid influenza test, direct or indirect fluorescent antibody, serologic analysis, and viral culture. Test results were separated by virus identification (A, B, or both) and influenza A subtype (H1, H3, pandemic [H1N1] 2009, unsubtypeable, or other). We surveyed 13 clinical laboratories serving the 16 reporting hospitals to assess total numbers of IPD cases and total positive blood cultures in September–October 2009 and September–October 2008, and to identify if there had been any changes in laboratory procedures related to blood culturing.

Statistical Analysis

Data were analyzed with SAS version 9.2 (SAS Institute, Cary, NC, USA). The χ^2 test was used to compare proportions. Medians were compared by using the Wilcoxon ranked-sum test; p values <0.05 were considered significant.

Results

Descriptive Epidemiology

Fifty-eight cases of IPD were identified in the Denver Metropolitan Area during October 2009, which was >3× the October average (mean ± SD 18.4 ± 4.7) during 2004–2008 (Figure). Cases were reported from 16 of 20 Denver area acute care hospitals; these hospitals were distributed throughout the 5-county area. Forty-five cases occurred in February 2009.

Medical records were abstracted for all 58 cases-patients with IPD (Table 1). Compared with February case-patients, October case-patients were younger (median age 45 years vs. 54 years; p = 0.02), and the proportion of nonelderly case-patients (age <60 years) was higher (45 [78%] of 58 vs. 26 [58%] of 45; p = 0.03). After adjusting for different age distributions of the 2 groups, we found that October 2009 case-patients were more likely to have concurrent conditions, specifically, chronic lung disease (Table 1).

Severity of Illness

Fifty-four (93%) of 58 IPD case-patients were hospitalized, and 47 (81%) had pneumonia. Seven case-patients died (12%), and 19 (33%) were admitted to the ICU. All patients who died were >40 years of age (Table 2). There were no major differences between the October 2009 and February 2009 case-patients in the proportion hospitalized or the case-fatality rate. A high (6/7, 86%) proportion of IPD case-patients 20–39 years of age who were hospitalized in October 2009 were admitted to the ICU. All 42 October pneumonia patients had Pneumonia Severity Index scores ≥2; the 7 case-patients who died all had pneumonia and scores of 4 or 5, which indicated severe illness and recommended hospitalization.

Influenza-associated IPD

Of 58 case-patients with IPD, interviews were completed with 46 (79%). Among the IPD case-patients, 47 (81%) had a documented influenza test during their illness; 4 were evaluated before hospitalization and 43 were tested on or after admission. Nine (19%) were evaluated with rapid tests alone, 8 (17%) with PCR alone, and 26 (55%) with both methods. The type of test used for the remaining 4 case-patients could not be determined. Of 47 case-patients tested for influenza, 10 (21%) had documented influenza virus infection. Therefore, ≥17% (10/58) of the IPD cases were associated with pandemic (H1N1) 2009.

Of the 10 positive case-patients, 2 were given a diagnosis before admission (1 by PCR and 1 by PCR and rapid test) and 8 were given a diagnosis at admission (all patients had both a rapid test and PCR). For those patients given a diagnosis at admission, only 2 (25%) of the rapid test results were positive and all 8 PCR test results were positive. Of the 7 patients for whom influenza A virus subtype analysis was conducted, 6 had pandemic (H1N1) 2009 virus; the other virus was not subtypeable.

Fifty-three of 58 IPD case-patients had sufficient information to evaluate for preceding ILI. Of these case-patients, 33 (62%) reported symptoms consistent with ILI >5 days before date of the culture that yielded pneumococci. All 10 influenza-positive case-patients reported ILI symptoms. Overall, 31 (94%) of the 33 case-patients with ILI were tested for influenza virus.

On the basis of IPD surveillance in Denver during 2004–2008, the maximum expected number of IPD cases during October of nonpandemic years was 28 (mean ± 2 SD 18 ± 5). If all remaining 30 IPD cases were considered to be excess cases associated with pandemic (H1N1) 2009, then 52% of all cases of IPD in October 2009 may have been associated with pandemic (H1N1) 2009.

Role of Vaccination

All 9 children had been vaccinated with PCV7 at appropriate ages. Of 38 adults 18–64 years of age, 29 (76%) had indications for PPV23 vaccination and 25 (86%) of them had available vaccination records; of these persons, 3 (12%) were vaccinated. The proportion of persons ≥65 years of age (universal PPV23 vaccination recommendation) with available vaccination records who were vaccinated was 67% (4/6).

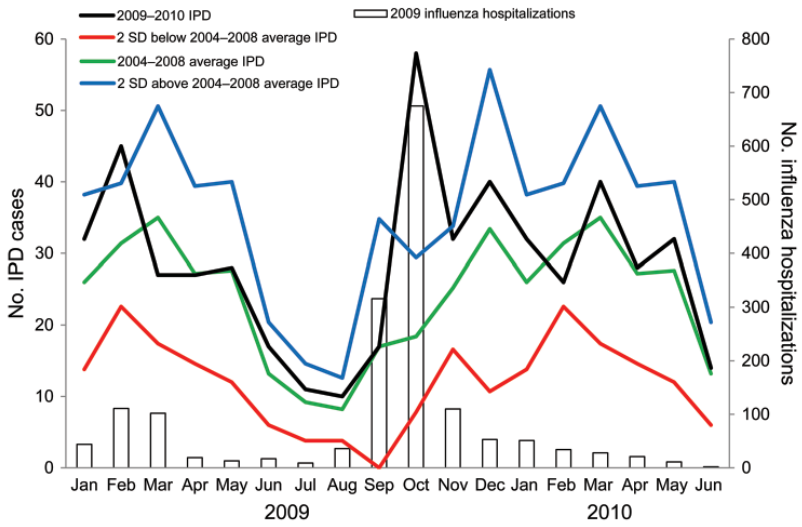

Figure. Influenza hospitalizations and invasive pneumococcal disease (IPD) cases, Denver, Colorado, USA, 2009–2010 vs. 5-year average (2004–2008). The 2004–2008 average IPD data line is repeated in 2010.

Antiviral Drug Treatment

Thirty-one (53%) of 58 IPD case-patients received antiviral medications during their illnesses; 20 (61%) of 33 patients who reported ILI symptoms received antiviral drugs and 9 (90%) of the 10 patients who had a positive influenza test result received antiviral drugs. According to medical records, the most common reason for not prescribing antiviral drugs was the duration of time since symptom onset. The 1 patient who had confirmed pandemic (H1N1) 2009–associated IPD who died received antiviral drug treatment 72 hours after admission and 48 hours after confirmatory influenza test results.

Serotypes of Pneumococci Causing IPD

Among the 47 (81%) cases with available isolates, 75% were caused by serotypes included in PPV23 and 4% by those included in PCV7. Serotype 7F was the predominant serotype identified, and it accounted for 34% of the October cases. Serotype distribution was consistent with overall epidemiology of IPD in Colorado during nonpandemic periods. After adjusting for age, we found that the proportion of all case-patients in Denver with IPD caused by serotype 7F during 2004–2008 increased from 3% to 25%. This increase was observed when analyzing only October (0%–34%; $p = 0.025$) and all other months (2%–23%; $p<0.0001$). Among the 9 adult influenza-positive case-patients, 7 of the isolates were serotypes contained in PPV23. Five (71%) of 7 persons with an indication for PPV23 had a serotype covered by PPV23, and only 1 (20%) had received PPV23.

Laboratory Survey

Among 13 surveyed laboratories, 4 implemented changes in blood culture practices over the preceding year. Two hospitals increased the number of times that blood cultures were evaluated for growth, 1 hospital adopted a different automated culture system, and 1 hospital began using a different skin antiseptic. Eleven laboratories saw a higher number of blood cultures submitted during September–October 2009 than in September–October 2008 (overall increase of 16%). The proportion of cultures positive for pneumococci increased at 9 laboratories by an average of >4 additional isolates, which was a relative increase of 50% over the previous year (Table 3).

Discussion

An investigation of IPD cases in Denver during October 2009 showed 3× the average number of IPD cases identified during October of the previous 5 years and a notable association with pandemic (H1N1) 2009 virus infection. Our findings do not prove a causal relationship between pandemic (H1N1) 2009 and IPD. However, we confirmed that 10 (17%) of 58 case-patients had influenza, and 2 estimates of the maximum proportion of IPD cases that may have been associated with pandemic (H1N1) 2009 showed that proportion was 52%–62%. Increases in testing for pneumococcal infection were modest and could not account for the magnitude of the increase in IPD incidence that we observed. However, the reported increase by area clinical laboratories in blood culture positivity for pneumococci supports a true increase in IPD incidence.

This investigation highlighted factors that may be distinct to IPD cases associated with pandemic (H1N1) 2009. Previous influenza pandemics have implicated secondary bacterial infection as a complication and cause of serious illness and death (*1*). These studies were based largely on autopsy series and histologic confirmation, but were limited in their ability to evaluate clinical presentation, symptoms, and onset that may be distinct to IPD cases identified during nonpandemic influenza seasons.

Table 1. Epidemiologic characteristics of patients with invasive pneumococcal disease, Denver, Colorado, USA, February and October 2009*

Characteristic	Patients with pandemic (H1N1) 2009, Oct 2009, n = 58	Patients with seasonal influenza, Feb 2009, n = 45	p value
Age, y			
0–<5	6 (10)	0 (0)	NC
5–19	3 (5)	3 (7)	NC
20–39	10 (17)	6 (13)	NC
40–59	26 (45)	17 (38)	NC
≥60	13 (22)	19 (42)	NC
Median age (range 2 mo–91 y)	45	54	0.02
Sex			0.120
M	38 (66)	26 (58)	NC
F	20 (34)	16 (36)	NC
Unknown	0	3 (7)	NC
Race†			0.012
White	31 (53)	11 (24)	NC
African American	4 (7)	3 (7)	NC
Asian	0 (0)	2 (4)	NC
Unknown	23 (40)	29 (64)	NC
Ethnicity†			<0.0001
Hispanic	14 (24)	7 (16)	NC
Non-Hispanic	25 (43)	4 (9)	NC
Unknown	19 (33)	34 (76)	NC
Concurrent condition‡			NC
Any	40 (69)	17 (38)	<0.0001
Chronic lung disease	17 (29)	3 (7)	0.0002
Diabetes	5 (9)	6 (13)	0.852
HIV infection	2 (3)	0 (0)	0.247
Liver disease	5 (8.6)	2 (4)	0.276
Smoking	15 (26)	10 (22)	0.669
Hospitalized			
Yes	54 (93)	36 (80)	0.536
No	4 (7)	2(4)	NC
Unknown	0	7 (16)	NC
Tested for influenza§	47 (81)	NA	NC
Outpatient setting	8	NA	NC
Inpatient setting	43	NA	NC
Positive test result for influenza§	10	NA	NC

*Values are no. (%) unless otherwise indicated. NA, not available; NC, not calculated.
†Race and ethnicity data required additional patient consent.
‡Underlying concurrent condition comparisons were adjusted for age.
§Includes data from supplemental case report form not available for prior years.

During the 2009–10 influenza season, the predominant circulating influenza virus was pandemic (H1N1) 2009 virus (*11*). IPD cases mirrored the epidemiology of pandemic (H1N1) 2009, peaked at the same time, and affected younger persons. Because some cases of IPD occur every October and because some cases of IPD are likely attributable to seasonal influenza (*12*), we compared cases detected during October 2009 with IPD cases seen during a peak month of seasonal influenza activity. Attack rates for pandemic (H1N1) 2009 were likely higher than those for seasonal influenza (H1N1) (*13*). Thus, the October IPD cases were more likely to mirror the epidemiology of pandemic (H1N1) 2009.

Studies in animal models demonstrated that influenza strains during previous pandemics have different abilities to predispose persons to secondary pneumococcal infections (*14*). If pandemic (H1N1) 2009 contributed to the increase in IPD cases during October 2009, we would expect a shift from the baseline IPD epidemiology toward a younger population with conditions known to increase the risk for infection with pandemic (H1N1) 2009 virus. In addition, the prevalence of underlying conditions (especially chronic lung disease) is consistent with a causal association between pandemic (H1N1) 2009 and October 2009 IPD cases.

October 2009 cases were not more severe than February 2009 cases, although our statistical power to identify a significant difference was limited. However, for persons 20–39 years of age, a high proportion of IPD hospital admissions required ICU (there were no prepandemic data for comparison). Data gathered during the pandemic from

Table 2. Severity of illness for patients with invasive pneumococcal disease, by age group, Denver, Colorado, USA, February and October 2009*

Characteristic	No. (%) patients with pandemic (H1N1) 2009, Oct 2009, n = 58	No. (%) patients with seasonal influenza, Feb 2009, n = 45	p value
Age 0–19 y			
Total	9	3	
Hospitalized†	8 (89)	2 (67)	0.461
Admitted to ICU‡§	1 (13)	NA	NC
Died	0	0	NC
Age 20–39 y			
Total	10	6	
Hospitalized	7 (70)	5 (83)	0.551
Admitted to ICU	6 (86)	NA	NC
Died	0	0	NC
Age 40–59 y			
Total	26	17	
Hospitalized	26 (100)	13 (76)	0.168
Admitted to ICU	8 (31)	NA	NC
Died	3	1	0.532
Age >60 y			
Total	13	19	
Hospitalized	13 (100)	16 (84)¶	NC¶
Admitted to ICU	4 (31)	NA	NC
Died	4	3	0.354
Total			
Hospitalized	54 (93)	36 (80)	0.536
Not hospitalized	4 (7)	2 (4)	
Data missing	0	7 (16)	
Admitted to ICU	19 (33)	NA	NC
Died	7 (12)	4 (9)	0.602

*ICU, intensive care unit; NA, not available; NC, not calculated.
†Denominator for percentage of hospitalized cases is total case-patients for each age group.
‡Percentage of those admitted to ICU is percentage of hospitalized case-patients for each age group.
§Includes data from supplemental case report form, not available for prior years.
¶Three case-patients with unknown hospitalization information. Of known case-patients, 100% were hospitalized.

domestic and international sites (*4*,*15*,*16*) suggested that pandemic (H1N1) 2009–associated IPD was not unique to Denver. However, there were no widespread levels of IPD that were greater than expected. At the time of our investigation in Denver, whether increases in IPD in other ABCs sites were statistically or epidemiologically significant was not clear. Since that time, increases have become apparent in other sites, although these increases were consistently more modest than those observed in Denver and were not as thoroughly investigated. In contrast to the previous 5 years, Denver experienced a peak in IPD in October 2009, which was likely attributable to pandemic (H1N1) 2009, and a second peak in December, which likely represented endemic disease.

Prevention of IPD during future influenza pandemics should focus on vaccination and prompt diagnosis. This influenza pandemic was the first in which pneumococcal vaccines and antiviral drug treatment were available. Among adults with IPD in Denver, vaccination rates for persons 18–64 years of age with indications for vaccination were less than the national rate, and vaccination rates for persons >65 years of age were similar to national estimates (*17*). The increase in IPD cases during October, the peak month of hospitalizations of persons with pandemic (H1N1) 2009, might have been minimized if adults at highest risk for IPD had received the recommended polysaccharide vaccine. Increasing PPV23 coverage in populations with increased risk for IPD is a key prevention measure, especially in anticipation of influenza pandemics.

Introduction of PCV7 into routine childhood immunization programs in the United States resulted in dramatic reductions in rates of pneumococcal-related diseases and major changes in the epidemiology for all age groups (*5*,*18*–*23*). In Denver during October 2009, we identified only 2 cases of IPD caused by serotypes included in PCV7, both in adults.

Whether vaccine against pandemic (H1N1 2009, which became available in Denver during late October, could have reduced the number of pandemic (H1N1) 2009–associated IPD cases is unknown. Antiviral drug administration was sometimes delayed or withheld despite national guidance for treatment even if >48 hours had elapsed from onset of illness (*24*), and such withholding may have changed the clinical course of some of the IPD cases.

Table 3. Blood culture practices of 13 laboratories for samples from patients with invasive pneumococcal disease, Denver, Colorado, USA, September–October 2008 and 2009*

Laboratory ID	Change in procedures	Sep–Oct 2008		Sep–Oct 2009		Change in cultures prepared, no. (%)	% change for SPN+
		No. cultures	No. (%) SPN+	No. cultures	No. (%) SPN+		
1	None	935	3 (0.3)	1,095	3 (0.3)	160 (17)	0
2	None	5,634	11 (0.2)	6,515	5 (0.1)	881 (16)	–50
3	None	141	0	297	6 (2)	156 (111)	NC
4	None	2,301	3 (0.1)	2,454	6 (0.2)	153 (7)	100
5	BC on all 3 shifts	2,007	4 (0.2)	2,480	8 (0.3)	473 (24)	50
6	BC on all 3 shifts	1,233	0	1,917	2 (0.1)	684 (55)	NC
7	2009 Feb 1: new blood culture system	2,008	4 (0.2)	2,391	9 (0.4)	383 (19)	100
8	None	2,072	5 (0.2)	2,188	4 (0.2)	116 (6)	0
9	None	1,538	1 (0.1)	1,768	2 (0.1)	230 (15)	0
10	2009 Oct: changed antiseptic	2,423	4 (0.2)	2,407	15 (0.6)	–16 (–1)	200
11	None	820	4 (0.5)	1,079	10 (0.9)	259 (32)	80
12	None	1,456	6 (0.4)	2,178	6 (0.3)	722 (50)	–25
13	None	3,405	5 (0.1)	3,390	9 (0.3)	–15 (0)	200
Total		25,973	50 (0.2)	30,159	85 (0.3)	4,186 (16)	50

*ID, identification; SPN+, positive for *Streptococcus pneumoniae*; NC, the percentage increase from 0 to 2 specimens could not be calculated; BC, blood culture.

Outbreaks of *S. pneumoniae* have occurred in many settings (*25–39*), and individual serotypes have been implicated in localized outbreaks (*27–29*,*31*,*39*). The variety of serotypes identified in this outbreak indicates that the increase in IPD was not attributable to enhanced transmission of a single serotype. To address whether the increase in October 2009 reflected a clonal outbreak of 7F, we analyzed the proportion of IPD cases in Denver that were serotype 7F during 2004–2010. During 2004–2009, the proportion of 7F increased (from 3% to 25%). When we evaluated all months except October during 2004–2009, the proportion of 7F still increased (from 2% to 23%). The proportion of IPD caused by 7F has been increasing in Denver over time and cannot be attributed to an increase in October 2009 alone or the pandemic. Furthermore, the distribution of serotypes was similar to serotype distributions in national (*5*) and Denver-specific IPD cases, which suggested that if pandemic (H1N1) 2009, was causally associated with this outbreak, it facilitated pneumococcal infection without a predilection for any particular serotype.

Our investigation had limitations. Low numbers of cases may have limited our ability to identify differences in the epidemiology of IPD during October 2009 and peak months of seasonal influenza activity. We were also unable to ascertain PPV23 vaccination histories for all cases, which may have underestimated PPV23 use. Of 47 influenza tests ordered, 9 (19%) were only rapid tests. The sensitivity of rapid tests for detecting pandemic (H1N1) 2009 ranged from 20% to 40% (*40*). Twenty-four (41%) of 58 IPD case-patients were not tested for pandemic (H1N1) 2009 by PCR (the standard for detection), which may have underestimated the number of confirmed influenza-associated IPD cases. Some patients with negative test results may have been infected with influenza virus but were tested too late in the course of their illness. Finally, ILI does not capture all influenza cases and cases with influenza within 5 days of pneumococcal culture and not tested samples would not be included for a possible influenza-associated IPD case. In addition, ILI includes symptoms that occur frequently with signs and symptoms of pneumococcal pneumonia and may be a result of the symptom course of IPD rather than preceding influenza infection.

In conclusion, up to two thirds of IPD cases in Denver during October 2009 may have been associated with pandemic (H1N1) 2009. Pandemic influenza may have altered the epidemiology of IPD and shifted the age distribution to younger persons and to persons 18–64 years of age with an increased prevalence of underlying conditions. Missed opportunities for PPV23 vaccination were common. During future influenza pandemics, public health officials should increase awareness of the association between IPD and influenza among persons of greatest risk for influenza-associated IPD. Prevention efforts should include use of pneumococcal vaccines and vaccines for directly preventing influenza infection.

Acknowledgments

We thank Sema Mandal, Nancy Williams, Andrew Boyd, Deborah Aragon, Denise Woods-Stout, Jennifer Sadlowski, Shaun Cosgrove, and Karen Gieseker for assistance during the field investigation and data collection and Felicita David and Carolyn Wright for data management.

This study and ABCs were supported by the Centers for Disease Control and Prevention Emerging Infections Programs.

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Diphtheria in the Postepidemic Period, Europe, 2000–2009

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Diphtheria incidence has decreased in Europe since its resurgence in the 1990s, but circulation continues in some countries in eastern Europe, and sporadic cases have been reported elsewhere. Surveillance data from Diphtheria Surveillance Network countries and the World Health Organization European Region for 2000–2009 were analyzed. Latvia reported the highest annual incidence in Europe each year, but the Russian Federation and Ukraine accounted for 83% of all cases. Over the past 10 years, diphtheria incidence has decreased by >95% across the region. Although most deaths occurred in disease-endemic countries, case-fatality rates were highest in countries to which diphtheria is not endemic, where unfamiliarity can lead to delays in diagnosis and treatment. In western Europe, toxigenic *Corynebacterium ulcerans* has increasingly been identified as the etiologic agent. Reduction in diphtheria incidence over the past 10 years is encouraging, but maintaining high vaccination coverage is essential to prevent indigenous *C. ulcerans* infections and reemergence of *C. diphtheriae*.

In 1994, following success of widespread vaccination programs earlier in the century, diphtheria was proposed as a candidate for elimination in the World Health Organization (WHO) European Region; the goal was for elimination of indigenous diphtheria by 2000 (*1*). However, during the 1990s, when this goal seemed within sight, several factors caused a resurgence of diphtheria to epidemic proportions in the newly independent states of the former Soviet Union. There were a large number of unnecessary contraindications to vaccination in guidance for these countries at that time, which led to reductions in adequate vaccination coverage in children. This problem was exacerbated by mistrust in vaccinations among health professionals and the public and by use of low-dose formulation vaccine for primary vaccinations. Waning immunity in the adult population, large-scale population movements caused by breakup of the former Soviet Union, disruptions in health services, and lack of adequate supplies of vaccine and antitoxin for prevention and treatment in most affected countries provided conditions under which diphtheria could spread (*2*,*3*). At the peak of the epidemic in 1995, there were >50,000 cases reported in the WHO European Region (*2*). Intensive vaccination strategies brought the disease under control in most countries, but some endemic transmission still continues.

Clinical diphtheria is caused by toxin-producing corynebacteria. Three species (*Corynebacterium diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*) can potentially produce diphtheria toxin. *C. diphtheriae* is the most common of potentially toxigenic species and is associated with epidemic diphtheria and person-to-person spread. The organism has 4 biovars (gravis, mitis, intermedius, and belfanti). *C. ulcerans* is historically associated with cattle or raw dairy products, and, although it is rarely reported, its incidence has increased slightly in some countries in western Europe and in the United States in recent years (*4*–*6*). *C. pseudotuberculosis* rarely infects humans and is typically associated with farm animals (*7*).

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DOI: http://dx.doi.org/10.3201/eid1802.110987

[1]Additional members of the Diphtheria Surveillance Network who contributed data are listed in online Technical Appendix 1 (wwwnc.cdc.gov/EID/pdfs/11-0987-Techapp1.pdf).

Currently, no direct evidence has been found of person-to-person spread of *C. ulcerans* or *C. pseudotuberculosis*.

Classical respiratory diphtheria is characterized by formation of a gray-white pseudomembrane in the throat that is firmly adherent (*8*). A swollen, bull-neck appearance caused by inflammation and edema of soft tissues surrounding lymph nodes is associated with severe illness and higher death rates (*8*). In progressive disease, the toxin can bind to cardiac and nerve receptors and cause systemic complications. Milder respiratory disease may manifest as a sore throat, most commonly seen in patients who are fully or partially vaccinated. In some tropical areas, cutaneous symptoms, characterized by rolled-edge ulcers, are more common. Patients may have both cutaneous and respiratory disease. The purpose of this study was to analyze diphtheria data for Europe during 2000–2009.

Methods

Case-based diphtheria surveillance data from each of 25 Diphtheria Surveillance Network (DIPNET) member countries (Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Ireland, Italy, Latvia, Lithuania, the Netherlands, Norway, Poland, Portugal, Romania, Slovenia, Spain, Sweden, Turkey, and the United Kingdom) for 2000–2007 were submitted retrospectively to the coordinating center in the United Kingdom during 2008. Data for 2008 and 2009 were obtained in August 2009 and September 2010 from the DIPNET online database, which was launched in September 2007.

We analyzed cases meeting the DIPNET case definition (isolation of a toxigenic strain or clinically compatible case with an epidemiologic link to a laboratory-confirmed case) (online Technical Appendix 2, wwwnc.cdc.gov/EID/pdfs/11-0987-Techapp2.pdf). In addition, 48 cases without laboratory confirmation and pseudomembrane (mild diphtheria/severe pharyngitis) and 5 cases with unknown manifestations were included for Latvia because these cases had been recorded in the national dataset. For most cases, toxigenicity was confirmed by using the Elek phenotypic test (*9*). However, in some cases, toxigenicity was evaluated only by detection of the toxin gene with PCR. We assumed that all cases in this dataset were toxigenic (toxin producing) because the number of cases without Elek confirmation was small and referred to symptomatic cases. Data fields collected included year; organism; biovar; and patient age, sex, clinical manifestations, vaccination status, veterinary contact, risk group, and outcome. Further strain characterization (ribotyping) was available for a limited number of isolates as part of a screening study in 10 DIPNET countries (*10*).

Cases were assigned to 5 clinical manifestation groups. These groups were classic respiratory diphtheria with pseudomembrane (the most serious form of the disease); mild diphtheria/severe pharyngitis (respiratory symptoms without the pseudomembrane); cutaneous (toxigenic organism isolated from skin lesions); other (e.g., toxigenic organism isolated from blood); and asymptomatic (carriers of toxigenic organisms, usually contacts of a confirmed case-patient).

Additional information concerning countries in the WHO European Region that are not DIPNET member countries was provided by the WHO Regional Office for Europe. Twenty-five of 53 member states of the WHO European Region are members of DIPNET. WHO European Region countries (including DIPNET members) report total cases annually to the WHO Regional Office for Europe through the WHO/United Nations Children's Fund Joint Reporting Form, which is the global annual data survey of WHO member states for vaccine-preventable diseases and immunization program indicators. In addition, 16 countries in 2003 (Figure 1) were asked to prospectively participate in monthly surveillance and provide more detailed information (e.g., pathogen biovar; patient age, sex, and outcome; and carriers among contacts). Twelve countries currently provide monthly reports to WHO Regional Office for Europe through this system. The only major source of cases that has not participated in the monthly reporting system (but

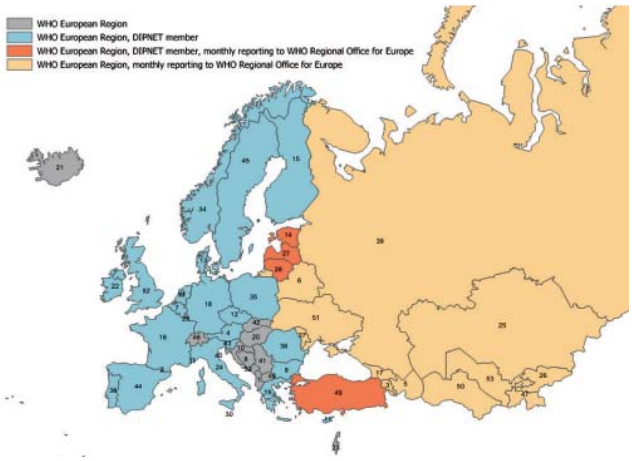

Figure 1. Diphtheria Surveillance Network (DIPNET) and World Health Organization (WHO) European Region countries. 1, Albania; 2, Andorra; 3, Armenia; 4, Austria; 5, Azerbaijan; 6, Belarus; 7, Belgium; 8, Bosnia and Herzegovina; 9, Bulgaria; 10, Croatia; 11, Cyprus; 12, Czech Republic; 13, Denmark; 14, Estonia; 15, Finland; 16, France; 17, Georgia; 18, Germany; 19, Greece; 20, Hungary; 21, Iceland; 22, Ireland; 23, Israel (neighboring countries not shown); 24, Italy, 25; Kazakhstan; 26, Kyrgyzstan; 27, Latvia; 28, Lithuania; 29, Luxembourg; 30, Malta; 31, Monaco; 32, Montenegro; 33, the Netherlands; 34, Norway; 35, Poland; 36, Portugal; 37, Republic of Moldova; 38, Romania; 39, Russian Federation; 40, San Marino; 41, Serbia; 42, Slovakia; 43, Slovenia; 44, Spain; 45, Sweden; 46, Switzerland; 47, Tajikistan; 48, Former Yugoslav Republic of Macedonia; 49, Turkey; 50, Turkmenistan; 51, Ukraine; 52, United Kingdom (Great Britain and Northern Ireland); 53, Uzbekistan.

does report annually) is the Russian Federation. Rates per 1 million person-years were calculated by using population estimates derived from the Population Division of Economic and Social Affairs of the United Nations Secretariat (*11*).

Statistical Analyses

Proportions were compared by using χ^2 or Fisher exact tests, as appropriate, in Stata statistical software version 7.0 (StataCorp LP, College Station, TX, USA). For assessment of a trend for variables in ordered groups (vaccinated, partially vaccinated, unvaccinated) and severity of disease (classic respiratory, mild diphtheria/severe pharyngitis, asymptomatic), the Wilcoxon test for trend in Stata (*12*) was used. This test enabled nonparametric analysis across these groups.

Results

Overall, across the WHO European Region, the number of cases of diphtheria has substantially decreased since the epidemic in the 1990s (Figure 2). Data on clinically confirmed cases and toxigenic isolates of *C. diphtheriae* and *C. ulcerans* reported to DIPNET during 2000–2009 are shown in Tables 1 and 2, respectively. Member countries that are not listed reported no isolates. Data are analyzed separately for Latvia, where diphtheria is endemic.

Diphtheria-Endemic Countries in WHO European Region

During 2000–2009, Latvia reported the highest annual incidence rate of diphtheria in the European Region each year and a 10-year incidence rate of 23.8 cases/1 million person-years. This rate was ≈7× higher than in countries with the next highest 10-year incidence: i.e., Georgia (3.5), Ukraine (3.3), and the Russian Federation (3.0). However, during this time, 4,304 (>61%) of 7,032 cases in the WHO European Region were reported from the Russian Federation, and 2 countries, the Russian Federation and Ukraine, accounted for 83% of all cases.

Over the past 10 years, diphtheria incidence decreased by >95% across the region (from 1.82/1 million population in 2000 to 0.07/million in 2009), including in Latvia (from 111.22/million in 2000 to 2.67/million in 2009). In 2009, Latvia was the only country in the region that had not yet achieved the elimination benchmark of an incidence <1 case/million population (Figure 2).

Most cases reported to WHO through the monthly surveillance system were in teenagers and adults. However, the major risk groups for death have been infants (too young for complete primary vaccination) and adults ≥40 years of age (unvaccinated or with waning immunity). Although risk did not differ by sex in cases in children, during 2002–2009, ≈2× as many cases were reported in women ≥20 years of age than in men (510 [64%] vs. 292 [36%], respectively). Most (75%) case-patients reported in the European Region were at least partially vaccinated, but most (74%) case-patients and (93%) infants who died were unvaccinated). *C. diphtheriae* biovar gravis was the predominant strain (60%–80%). Of isolates from Latvia (Table 1), 355 (99%) of 358 with a known biovar were gravis and 3 (1%) were mitis.

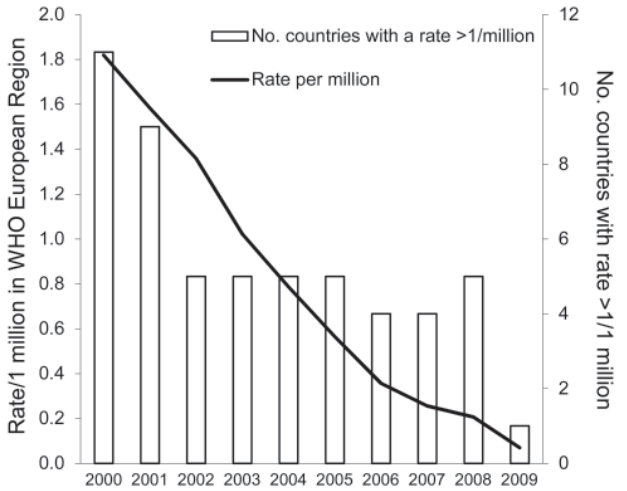

Figure 2. Diphtheria cases per 1 million population in the World Health Organization (WHO) European Region and number of countries with a rate >1 cases/1 million population, 2000–2009.

Clinical manifestations and vaccination status for cases from Latvia (all *C. diphtheriae*) reported to DIPNET are shown in Table 3. Most (340/341) case-patients with symptoms had respiratory manifestations, and 141 (41%) of 340 respiratory case-patients had classic diphtheria symptoms. Vaccination showed a significant protective effect with respect to severity of infection ($p<0.001$ by test for trend).

For symptomatic cases for 2002–2009 (excluding the military outbreak in 2000 and cases from 2001 for which limited information was available) the highest overall incidences were in children 0–4 and 5–15 years of age and adults 45–64 years of age; lower incidence rates were observed in other age groups (Figure 3). Most (123/196, 63%) symptomatic cases during those years were in female patients.

The second most common risk factor (after military service) identified among symptomatic case-patients in Latvia was unemployment (60 case-patients). Information was not available regarding connections of case-patients to other countries of the former Soviet Union.

Non–Disease-Endemic Countries (DIPNET)

Clinical manifestations and immunization status for case-patients with toxigenic *C. diphtheriae* and *C. ulcerans*

Table 1. Toxigenic *Cornyebacterium diphtheriae* isolates and epidemiologically linked cases and deaths reported by DIPNET member countries, Europe, 2000–2009*

Characteristic	Patient description†	No. toxigenic isolates or clinical cases with epidemiologic link (no. deaths)									
		2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Country											
Estonia	Symptomatic	2	2	0	0	0	0	0	0	0	0
	Asymptomatic	1	7	0	0	0	0	0	0	0	0
	Total	3	9	0	0	0	0	0	0	0	0
Finland	Total	0	2 (1)	0	0	0	0	0	0	0	0
France	Total	0	0	1	0	1	0	1	1 (1)	1	0
Germany	Total	1	2	4	0	0	1	0	0	0	2
Latvia	Symptomatic	145	0	45	26	20	20	32	18	29	6
	Asymptomatic	61	24	15	22	2	2	11	5	12	3
	Not known	119	91	0	0	0	0	0	0	0	0
	Total	325 (9)	115 (5)	60 (3)	48 (2)	22 (1)	22 (2)	43 (6)	23 (1)	41 (2)	9 (1)
Lithuania	Symptomatic	2	0	4	0	0	0	0	0	2	0
	Asymptomatic	0	0	1	0	0	0	0	0	2	0
	Total	2	0	5 (1)	0	0	0	0	0	4 (1)	0
Norway	Symptomatic	0	0	0	0	0	0	0	0	3	0
	Asymptomatic	0	0	0	0	0	0	0	0	1	0
	Total	0	0	0	0	0	0	0	0	4	0
Sweden	Total	0	0	0	0	0	0	0	0	0	1
Turkey	Symptomatic	1	3	1	0	0	0	0	0	0	0
	Asymptomatic	2	0	0	0	0	0	0	0	0	0
	Not known	1	4	1	1	0	0	0	0	0	0
	Total	4 (1)	7 (3)	2 (1)	1	0	0	0	0	0	0
United Kingdom	Total	1	0	6	3	0	0	1	0	2 (1)	2
Total known symptomatic patients	NA	152	9	61	29	21	21	34	19	37	11
Total (all countries)	NA	336 (10)	135 (9)	78 (5)	52 (2)	23 (1)	23 (2)	45 (6)	24 (2)	52 (4)	14 (1)
Total known symptomatic patients, excluding Latvia	NA	7	9	16	3	1	1	2	1	8	5
Total, excluding Latvia	NA	11 (1)	20 (4)	18 (2)	4	1	1	2	1 (1)	11 (2)	5

*DIPNET, Diphtheria Surveillance Network; NA, not applicable. A total of 89 cases were clinically diagnosed without microbiological confirmation (76 in Latvia, 11 in Turkey, and 2 in Lithuania).
†If only total is displayed for a country, all patients were symptomatic.

isolates and epidemiologically linked cases reported by 24 DIPNET member countries, excluding Latvia, during 2000–2009 are shown in Table 4. Vaccination had a significant protective effect with respect to severity of infection ($p = 0.001$ by test for trend).

C. diphtheriae Isolates

Isolates of *C. diphtheriae* were sporadically reported in the 24 DIPNET member countries, excluding Latvia. Each year, 0–6 symptomatic cases of toxigenic *C. diphtheriae* infection were reported by each country (53 cases during 2000–2009). For each case-patient, 0–4 asymptomatic contacts were reported (14 in the 10-year period). Of 60 isolates with a biovar recorded during 2000–2009, a total of 32 were gravis and 28 were mitis. Seventeen cutaneous cases, 35 respiratory (24 classic respiratory) cases, and 1 case with other manifestations were reported. Most (15/17, 88%) cutaneous cases were caused by biovar mitis, and most (17/28, 61%) respiratory cases with a known biovar were caused by biovar gravis. Sixteen of 17 patients with cutaneous disease had recently returned from traveling, had contact with travelers, or were recent immigrants from a disease-endemic area, as was the situation for 12 of 35 patients with respiratory disease. One case-patient with bacterial endocarditis had contact with a relative who had recently traveled to Pakistan. For case-patients with *C. diphtheriae* symptomatic infection, sex distribution was even. A higher incidence rate was observed in male patients 0–4 years of age (Figure 3), but this finding was influenced by 6 cases reported in Turkey during 2001–2003.

C. ulcerans Isolates

A total of 4–8 isolations of toxigenic *C. ulcerans* were reported by DIPNET member countries each year (53 [50 symptomatic] during 2000–2009). Of these cases, 51% were reported by the United Kingdom, 19% by Germany, and 17% by France. Of the symptomatic cases for which patient sex/age group were known, 38 (78%) of 49 were in female patients and 29 (59%) of 49 were in patients ≥45 years of age. Incidence rate was higher in female patients than in than male patients (0.014/1 million person-years vs.

Table 2. Isolates of toxigenic *Corynebacterium ulcerans* and patient deaths reported by DIPNET member countries, Europe, 2000–2009*

Characteristic	Patient description†	No. toxigenic isolates (no. deaths)									
		2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Country											
France	Total	0	1	0	1	3	0	2	1	0	1
Germany	Total	1	1 (1)	0	0	1	2	1	2	0	2
Italy	Total	0	0	1	0	0	0	0	0	0	0
The Netherlands	Total	0	1	0	0	0	0	0	1	0	0
Romania	Asymptomatic	0	0	1	0	0	0	0	0	0	0
	Total	0	0	1	0	0	0	0	0	0	0
Sweden	Symptomatic	0	0	0	0	0	0	0	0	1	0
	Not known	0	0	0	0	1	0	1	0	0	0
	Total	0	0	0	0	1	0	1	0	1	0
United Kingdom	Total	7 (1)	3	2	2	1	2	2 (1)	3	3	2
No. symptomatic patients	NA	8	6	3	3	5	4	5	7	4	5
No. isolates	NA	8 (1)	6 (1)	4	3	6	4	6 (1)	7	4	5

*DIPNET, Diphtheria Surveillance Network; NA, not applicable
†If only total is shown for a country, all patients were symptomatic.

0.004/1 million person-years). Eleven cutaneous cases, 38 respiratory (14 classic respiratory) cases, and 1 case with other manifestations were reported. Ninety-four percent of case-patients for which information was available had contact with domestic animals. Traditional risk factors such as consumption of raw milk products were not reported, and no patients had a recent history of travel. One of the 2 case-patients infected with *C. ulcerans* who died in the United Kingdom had an identical strain of *C. ulcerans* to that isolated from a dog with which the patient had been in contact (*14*). A similar finding was observed in France for a nontoxigenic case reported in 2003 (*5*,*15*). In 2007, identical strains were isolated from a patient infected with *C. ulcerans* and her pig in Germany (*16*).

C. pseudotuberculosis Isolates

Four case-patients with diphtheria caused by toxigenic *C. pseudotuberculosis* were reported: 1 in France in 2005 and 1 in 2008, 1 in Germany in 2004, and 1 in United Kingdom in 2008. Three of these patients had cutaneous manifestations (1 was unvaccinated, 2 had an unknown vaccination status) and 1 (partially vaccinated) had bacterial endocarditis. To our knowledge, none of these infected patients died. Animal contact (with a calf) was recorded for only 1 patient (1 had no history of animal contact and 2 had an unknown history of animal contact).

Deaths Caused by Diphtheria

During 2000–2009, a total of 32 deaths caused by diphtheria were reported in Latvia, and 13 deaths (10 caused by *C. diphtheriae* and 3 caused by *C. ulcerans*) (Tables 1, 2) were reported by the remaining 24 DIPNET countries. Overall, patients with respiratory disease and a pseudomembrane had a significantly higher case-fatality rate (CFR) than patients with respiratory disease without a pseudomembrane (14.6% vs. 1.3%; $p<0.001$). For case-patients in Latvia, the CFR was 5% for patients with any respiratory symptom (including classic manifestations) and 12% for patients with classic respiratory symptoms. Of 18 case-patients in Latvia who died, 14 were ≥40 years of age and 4 were ≤7 years of age; all were unvaccinated.

Nine of 13 patients who died of diphtheria in DIPNET countries excluding Latvia had classic respiratory diphtheria symptoms, and 2 had severe pharyngitis (2 had unknown manifestations). All 3 deaths caused by *C. ulcerans* (2 in the United Kingdom and 1 in Germany) were in elderly (>75 years of age) patients (unvaccinated or vaccination status unknown). Two of the patients infected with *C.*

Table 3. Vaccination status of case-patients and clinical manifestations of toxigenic *Corynebacterium diphtheriae* infections and epidemiologically linked cases without laboratory confirmation, Latvia, Europe, 2000–2009*

Vaccination status	Classic diphtheria (with membrane)	Mild diphtheria/ severe pharyngitis	Cutaneous	Asymptomatic	Not known	Total
Full	64†	118	0	71	0	253
Partial	1	3	0	5	0	9
Unvaccinated	74	70	1	18	0	163
Not known	2	8	0	63	210	283
Total	141	199	1	157	210	708

*$p<0.001$ by test for trend (vaccination status and disease severity).
†Includes 52 fully vaccinated case-patients with classic respiratory diphtheria (with membrane) from an outbreak in the military in 2000. The outbreak comprised 145 symptomatic case-patients and 25 asymptomatic contacts. A total of 96% of these case-patients and contacts were 18–23 years of age at the time of diagnosis. Spread of disease was traced to use of a communal drinking cup (*13*).

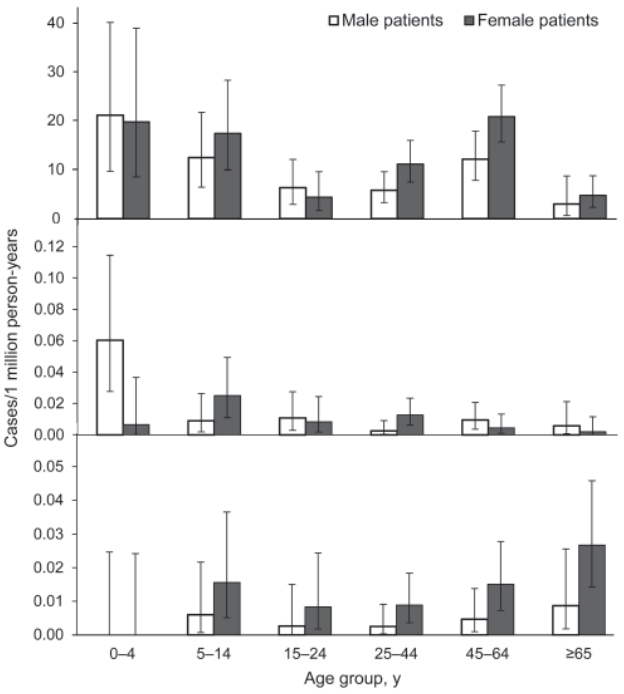

Figure 3. Diphtheria incidence per 1 million person-years for Latvia (*Corynebacterium diphtheriae*, 2002–2009) and the remaining 24 Diphtheria Surveillance Network (DIPNET) countries (*C. diphtheriae* and C*. ulceran*s, 2000–2009). Error bars indicate 95% CIs. The period 2002–2009 excludes the military outbreak in 2000 and cases from 2001 for which limited information was available.

diphtheriae who died were unvaccinated infants (1 from Mayotte and 1 from Finland). The infant in Finland died at 3 months of age in 2001 after recent contact with visitors from Russia (*17*). Six other children died: an unvaccinated school age child in the United Kingdom (*18*) and 5 children <7 years of age in Turkey (vaccination status unknown). Two adults in Lithuania (ages 45–64 years; vaccination status unknown) also died. The CFR for patients with any respiratory symptoms reported for patients infected with toxigenic *C. diphtheriae* or *C. ulcerans* in regions where diphtheria was not endemic was 15%; CFR was 24% among patients with classic respiratory diphtheria.

The difference between CFRs for respiratory diphtheria cases in Latvia and member countries excluding Latvia (5% and 15%, respectively) was significant (p = 0.002). The difference between CFRs for classic respiratory diphtheria in Latvia and the member countries excluding Latvia (12% and 24%, respectively) showed borderline significance (p = 0.06).

Any case-patients without symptoms recorded who died likely had respiratory diphtheria. However, because symptoms were also not available for several surviving case-patients for whom clinical manifestations were less certain, all case-patients for whom clinical manifestations were unavailable were excluded from analysis.

Discussion

Substantial progress has been made in controlling diphtheria across Europe since the epidemic in the 1990s, but diphtheria has not disappeared as a serious public health threat. After major disruption to a mass vaccination program, recovery time is lengthy, and pockets of unvaccinated persons can remain because recovery is not necessarily homogeneous.

The protective effect of vaccination in preventing progression to severe disease is clear. However, 64 patients in Latvia recorded as fully vaccinated had classic respiratory diphtheria symptoms. Most of these patients were infected during a military outbreak in 2000 and would have been scheduled for primary vaccinations during the 1980s, when changes in vaccines, vaccination policy, medical practice, and public acceptance led to less intensive vaccination of children in the former Soviet Union. Beginning in 1980, Soviet vaccination recommendations enabled use of an alternative primary vaccination schedule against diphtheria that recommended 3 doses of a lower-potency vaccine (*19*). The classification of fully/partially vaccinated relies on specific interpretation of a country. Since the 2000 outbreak, greater attention has been given to checking vaccination records of new recruits into the Latvian military, and booster vaccinations are given where appropriate.

Lower CFRs for respiratory diphtheria in disease-endemic areas compared with those in nonendemic areas highlight how lack of familiarity with a rare disease can affect diagnosis and treatment. As the incidence of diphtheria has decreased, so has the practice of routine laboratory screening (*20*). No DIPNET member country routinely screens all throat swab specimens for corynebacteria, although sentinel screening of all throat swab specimens is conducted in Denmark, Ireland, and the United Kingdom. All other DIPNET countries (and outside sentinel screening areas) perform screening only at the request of the clinician or if the laboratory identifies particular criteria for screening from information accompanying a swab specimen (DIPNET, unpub. data). This practice has resulted in a loss of laboratory expertise and the opportunity for infections to go undetected because only clinically indicated swab specimens are tested; thus, milder cases or those with unusual manifestations may be missed.

A recent DIPNET external quality assurance evaluation of 6 simulated throat specimens found that only 6 of 34 international centers produced acceptable results for all 6 specimens; many centers could not isolate the target organism (*21*). In some poor countries, screening can be limited by cost of laboratory reagents, and problems

Table 4. Vaccination status of case-patients and clinical manifestations of toxigenic *Corynebacterium diphtheriae* and *C. ulcerans* infections and epidemiologically linked cases without laboratory confirmation, DIPNET cases excluding Latvia, Europe, 2000–2009*

Vaccination status	Classic respiratory diphtheria (with membrane)	Mild respiratory diphtheria/severe pharyngitis	Cutaneous	Other	Asymptomatic	Not known	Total
Full	4	17	2	1†	2	0	26
Partial	5	3	7	0	0	0	15
Unvaccinated	14	3	4	0	1	0	22
Not known	15	10	15‡	1§	12	11¶	64
Total	38	33	28	2	15	11	127

*DIPNET, Diphtheria Surveillance Network. p = 0.001 by test for trend (vaccination status and disease severity).
†Bacterial endocarditis (*C. diphtheriae*, fully vaccinated)
‡One cutaneous case-patient also had a sore throat.
§Isolation from blood (*C. ulcerans*, vaccination status not known).
¶Includes 2 case-patients infected with *C. diphtheriae* who died and are assumed to have respiratory symptoms without specific details available.

have also occurred in obtaining Elek reagents and media (*21*). During a recent screening study across 10 countries in Europe, toxigenic organisms were isolated in Latvia and Lithuania (*10*). At least one of these cases in Lithuania would not have been correctly diagnosed in the absence of the screening study. In addition to the potential for missed or late diagnoses, in areas where diphtheria is not endemic, diphtheria antitoxin treatment is not always available, which can have serious consequences. A recent international survey highlighted global shortages of diphtheria antitoxin (*22*). Information about administration and timing of antitoxin treatment was not collected for this analysis, but studying such timing in relation to differing CFRs would be useful.

Higher incidence rates of *C. diphtheriae* among women in disease-endemic countries could be caused by several factors. Women more commonly work as caregivers in domestic and health care settings, consultation rates are usually higher among women, and men are more likely to have received diphtheria vaccine during military service.

Although the United Kingdom, France, and Germany regularly report isolations of toxigenic *C. ulcerans*, it is unlikely that this organism is present only in these countries. The ability to detect *C. ulcerans* could indicate the capability of a country to detect potentially toxigenic organisms and provide an indicator of good surveillance. Detection of mild diphtheria cases (any toxigenic organism) is another potential indicator of good surveillance. *C. ulcerans* appears to have a wide host range and has been isolated from many domestic and wild animals, including the killer whale and lion (nontoxigenic strain) (*23*). During 2002 and 2003, toxigenic *C. ulcerans* strains isolated from domestic cats in the United Kingdom were found to have the predominant ribotypes observed among human clinical isolates, which suggests that cats could be a potential reservoir for human infection (*24*). Identical *C. ulcerans* strains have been isolated from diphtheria patients and dogs in France and the United Kingdom (*14*,*15*). The presence of this organism reinforces the need to maintain high vaccination levels in all countries. Higher incidence of infection among elderly women could be related to pet ownership habits, in combination with low or waning immunity.

Vaccination coverage for diphtheria is assessed annually in many countries in Europe by using a range of methods, including computerized vaccination registers, survey methods, administrative methods, or a combination (*25*). These methods will provide varying degrees of accuracy in coverage estimates, which makes countries difficult to compare. Coverage for vaccination with diphtheria-tetanus-pertussis 3 vaccine (third dose of diphtheria, tetanus, pertussis vaccine) in early childhood in 2009 was >90% for most (85%) countries in the European Region, and 66% of countries (including Latvia, Lithuania, Turkmenistan, and the Russian Federation) reported coverage ≥95% (*26*). Coverage in Ukraine decreased from 98% in 2006 and 2007 to 90% in 2008 and 2009. Austria, Denmark, Georgia, and Moldova recorded diphtheria-tetanus-pertussis 3 vaccine coverage <90%. Azerbaijan and Malta had the lowest coverage (73% for both countries) in the European Region in 2009.

Following high-profile vaccine-scare stories in some countries in eastern Europe, such as the Russian Federation and Ukraine, anti-vaccination groups have gained strength by using television, the Internet, and other media for publicity (*27*); this activity could seriously affect vaccination coverage. Adult diphtheria immunity can be increased through scheduled booster vaccinations every 10 years (e.g., as in Austria, Belgium, Bulgaria, Cyprus, Estonia, Finland, France, Germany, Greece, Latvia, Norway, Portugal, and Romania) or as part of a combined tetanus and low-dose diphtheria vaccine given for tetanus-prone injuries. In Latvia, annual adult vaccination coverage surveys are undertaken, but in most countries adult coverage is rarely assessed. Seroprevalence studies have indicated that many adults in some countries have immunity levels below the protective threshold (*28*). Gaps in immunity in the adult population contributed to the resurgence of diphtheria in eastern Europe during the 1990s.

Trends in diphtheria cases in Europe are encouraging, but continued striving for improved vaccination coverage is essential. Diphtheria has a socioeconomic component; outbreaks are typically seen in marginalized groups. In the current economic climate, more socially deprived groups that are vulnerable to infection will emerge. The economic crisis may also threaten supplies of vaccine and antitoxin and delivery of immunization programs. Because reductions in finances can limit the capacity for surveillance, decreases in case reporting need to be interpreted with caution. Every effort must be made to maintain high diphtheria vaccination coverage.

Acknowledgments

We thank all members of DIPNET for submitting data for analysis and for helpful comments on the draft manuscript and Nick Andrews for assistance with the statistical analyses.

DIPNET is supported by the European Commission (DG SANCO agreement no. 2005210).

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Declining Guillain-Barré Syndrome after Campylobacteriosis Control, New Zealand, 1988–2010

Michael G. Baker, Amanda Kvalsvig, Jane Zhang, Rob Lake, Ann Sears, and Nick Wilson

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Release date: January 26, 2012; Expiration date: January 26, 2013

Learning Objectives

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

- Distinguish the infection most closely associated with GBS
- Analyze the temporal relationship between campylobacteriosis and GBS
- Assess differences in the association between campylobacteriosis and GBS based on age
- Evaluate the effect of infection-control measures on rates of campylobacteriosis and GBS

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P. Lynne Stockton, VMD, MS, ELS(D), Technical Writer/Editor, *Emerging Infectious Diseases. Disclosure: P. Lynne Stockton, VMD, MS, ELS(D), has disclosed no relevant financial relationships*.

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Infection with *Campylobacter* spp. commonly precedes Guillain-Barré syndrome (GBS). We therefore hypothesized that GBS incidence may have followed a marked rise and then decline in campylobacteriosis rates in New Zealand. We reviewed records for 1988–2010: hospitalization records for GBS case-patients and campylobacteriosis case-patients plus notifications of campylobacteriosis. We identified 2,056 first hospitalizations for GBS, an average rate of 2.32 hospitalizations/100,000 population/year. Annual rates of hospitalization for GBS were significantly correlated with rates of notifications of campylobacteriosis. For patients hospitalized for campylobacteriosis, risk of being hospitalized for GBS during the next month was greatly increased. Three years after successful interventions to lower *Campylobacter* spp. contamination of fresh poultry meat, notifications of campylobacteriosis had declined by 52% and hospitalizations for GBS by 13%. Therefore, regulatory measures to prevent foodborne campylobacteriosis probably have an additional health and economic benefit of preventing GBS.

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DOI: http://dx.doi.org/10.3201/eid1802.111126

Guillain-Barré syndrome (GBS) is an autoimmune condition that affects the peripheral nervous system. Patients typically describe ascending weakness and sensory disturbance that evolve over several days; during this acute phase, approximately one third of patients require ventilatory support. The condition is generally self-limiting, but for 3%–10% of patients, it is fatal (*1*).

An estimated 40%–70% of patients with GBS had an infection before GBS onset; for 6%–39% of these patients, the infection affected the gastrointestinal system (*2*). Campylobacteriosis is the most commonly identified antecedent infection; several studies have shown that in industrialized countries (Europe, North and South America, Japan, and Australia), *Campylobacter* spp. infection preceded GBS for 20%–50% of patients (*3*,*4*).

During 1980–2006 in New Zealand, incidence of campylobacteriosis steadily increased. The notification rate in 2006 (379 cases/100,000 population) remains the highest national rate reported in the literature (*5*,*6*). In 2006, in response to this high incidence, New Zealand introduced an array of voluntary and regulatory interventions to reduce contamination of poultry with *Campylobacter* spp. (*7*). By 2008, the rate of campylobacteriosis notifications had dropped to 157 cases/100,000 population, a decrease of 59% over 2 years (*7*); this decline has persisted (*8*). Given the known association between *Campylobacter* spp. infection and GBS and the marked recent changes in reported rates of campylobacteriosis in New Zealand, we examined GBS hospitalization data for evidence of responsiveness to trends in campylobacteriosis incidence.

Methods

Identification of GBS Incidence

Because GBS is a serious illness that nearly always results in hospitalization, hospitalization data provided the most accurate available measure of GBS incidence. We obtained national hospital discharge data for the 23-year period 1988–2010 in New Zealand. To estimate the case-fatality proportion, we also obtained data on deaths from GBS for 1988–2008 (the most recent year available). Both datasets are collated and maintained by the New Zealand Ministry of Health.

Although hospitalization data are available for earlier years, we used 1988 as the starting point because that is when use of unique patient identifiers, the National Health Index (NHI), became universal in New Zealand. Use of the NHI enables identification and removal of repeat GBS hospitalizations for the same patient, thereby identifying the first GBS hospitalization for each case (hereafter called GBS hospitalization), which provides an estimate of the number of incident cases of GBS.

We selected all cases from 1988 on that had International Classification of Diseases, 9th and 10th Revisions, Clinical Modification and Australian Modification, codes for GBS (ICD-9 CM 357.0 and ICD-10 AM G61.0) recorded as the principal or additional diagnosis. Records of patients who had been transferred between hospitals were merged to create 1 hospitalization event. We identified repeat hospitalizations for the current year and for previous years, i.e., case-patients with the same NHI number previously admitted in the same or a previous year. Some patients were readmitted before universal use of the NHI in 1988, so the calculation needed to take these estimated repeat hospitalizations into account. (See online Technical Appendix Tables 1, 2, wwwnc.cdc.gov/EID/pdfs/11-1126-Techapp.pdf, for a description of how estimated repeat hospitalizations and incident cases were calculated.)

Identification of Campylobacteriosis Incidence

Since 1980, campylobacteriosis has been a notifiable disease in New Zealand. Medical practitioners are required to report all identified and suspected cases to the local medical officer of health. These data are in turn collated nationally by the Institute of Environmental Science and Research for the New Zealand Ministry of Health. We used published annual totals of notifications (*9*) as well as anonymized datasets of notified cases. Most cases were culture confirmed (>96% during 1997–2008 [*7*]), although the case definition also allows for cases epidemiologically linked to a confirmed case.

Hospitalizations for campylobacteriosis are recorded in hospital discharge data, which are electronically available for a similar period. However, a specific diagnostic code for *Campylobacter* spp. infection was not introduced until July 1995. Hospitalizations for campylobacteriosis were defined as those with ICD-9 CM code 008.43 from July 1995 on and ICD-10 AM code A04.5 from July 1999 on. To create a dataset of incident cases, we included principal or additional diagnoses, merged records for those transferred with records from preceding hospitalizations, and removed repeat hospitalizations in the current and previous years.

Analysis of Hospitalizations for GBS after Campylobacteriosis

To assess the association between the 2 conditions, we investigated the incidence of GBS among patients hospitalized for campylobacteriosis. Because campylobacteriosis was only specifically identified in hospitalization data from July 1995, this analysis focused on the period starting in July 1995. To allow a follow-up period for GBS cases to emerge, we continued the inclusion period through December 2008.

For those cases identified, we first analyzed the time from hospital admission for campylobacteriosis to

admission for GBS. For epidemiologic purposes, the risk period for GBS after *Campylobacter* spp. infection is ≈2 months (*10*); neurologic signs of GBS usually develop 1–3 weeks after a preceding infection (*3*). In our dataset, a clear trend was seen toward a close temporal association between hospitalization dates: for most (34/35, 97.1%) patients, hospitalizations for GBS and campylobacteriosis were concurrent (patients were discharged with a diagnosis of both), or hospitalization for GBS occurred within 1 month of hospitalization for campylobacteriosis.

To assess the risk for GBS associated with campylobacteriosis, we calculated GBS hospitalization rates for comparison conditions, notably other infections that might be associated with an elevated risk for GBS. We used the GBS rate in the total New Zealand population as our reference rate for calculating age-standardized rate ratios for GBS after campylobacteriosis and other conditions of interest.

We also evaluated which age groups might be more vulnerable to development of GBS. To do so, we compared the age distributions of all patients hospitalized for GBS and those associated with campylobacteriosis with the age distributions for those with campylobacteriosis alone (hospitalized or with notified case).

Statistical Analyses

Because of marked changes in campylobacteriosis disease incidence and some changes in case identification during the 23-year study period, some outcomes were measured over a shorter time. The periods associated with implementation of the *Campylobacter* spp. control interventions used a baseline period similar to that used in a previous study (*7*).

Data were analyzed by using Stata version 11.0 (StataCorp LP, College Station, TX, USA) and SAS version 9.1 (SAS Institute, Cary, NC, USA). CIs are given at the 95% level throughout. We used well-documented methods for calculating adjusted rates, rate ratios (RRs), and 95% CIs (*11*). Rates were calculated by using mean population estimates published by Statistics New Zealand (www.stats.govt.nz/browse_for_stats/population/estimates_and_projections/national-pop-estimates.aspx) as denominators. To calculate age-standardized rates, we used the population age structure determined by the New Zealand 2006 Census of Population and Dwellings (www.stats.govt.nz/Census/2006CensusHomePage/classification-counts-tables/about-people/age.aspx).

Results

GBS Incidence

This study identified 2,056 first hospitalizations for GBS that occurred during 1988–2010, resulting in an average rate of 2.32 hospitalizations/100,000 population/year (online Technical Appendix Table 1). Incidence was not stable over the period of the study (Figure). The minimum recorded rate was 1.53 hospitalizations/100,000 population/year in 1989; the maximum was 2.93 in 2005. During 1989–2008, a total of 56 deaths from GBS were recorded; case-fatality proportion (56 deaths/1,873 cases) was 3.0%.

Changes in GBS and Campylobacteriosis Incidence

For 1988–2010, there was a significant direct correlation between annual rates of hospitalization for GBS and annual rates of notification of campylobacteriosis cases (Spearman $\rho = 0.52$, $p = 0.012$). During 1988–2006, incidence of campylobacteriosis notifications and of GBS hospitalizations increased (Figure; online Technical Appendix Table 3). Subsequently, campylobacteriosis notifications then decreased markedly, and GBS hospitalizations decreased, although less dramatically. The fall in campylobacteriosis notifications followed the introduction of countrywide campylobacteriosis control measures focused on reducing contamination levels in fresh poultry meat (*7*).

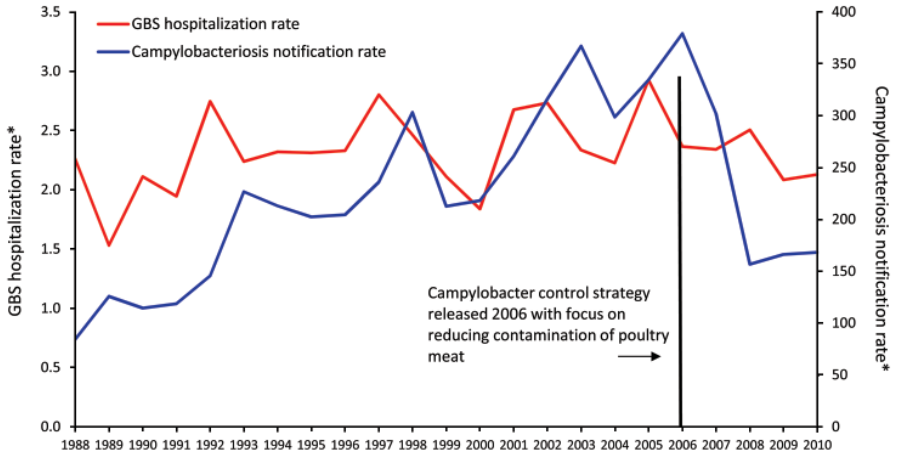

Figure. Guillain-Barré syndrome (GBS) hospitalization rates and campylobacteriosis notification rates, by year, New Zealand, 1988–2010. *Per 100,000 population.

Table 1 summarizes the changes between the 2 periods: 1) 2002–2006, the baseline period, when rising campylobacteriosis rates became an urgent public health concern, and 2) 2008–2010, the postintervention period, after implementation of wide-ranging control measures. The transition year, 2007, was excluded.

During the postintervention period, notifications and hospitalizations decreased by ≈50% (online Technical Appendix Tables 3, 4). Incidence of GBS declined by 13%, which was statistically significant (RR 0.87, 95% CI 0.81–0.93), suggesting that ≈25% of GBS was caused by preceding campylobacteriosis.

GBS among Patients Hospitalized for Campylobacteriosis or Other Conditions

During 1995–2008, among the 8,448 patients hospitalized for campylobacteriosis, 35 were also hospitalized for GBS. The frequency distribution of time delays is shown in Table 2. These data show that most (29) of these 35 patients had diagnoses of GBS and campylobacteriosis at time of hospital discharge. Another 5 patients were hospitalized for GBS within 4 weeks of being hospitalized for campylobacteriosis. The time difference for the remaining patient was >1,500 days (this patient was excluded from subsequent analyses). This striking distribution further supports a causative association between campylobacteriosis and GBS in New Zealand.

We calculated the rate of GBS hospitalizations among the cohort of patients hospitalized for campylobacteriosis and compared this with rates of GBS hospitalization among other patient cohorts hospitalized for infectious diseases (Table 3). This analysis used the overall rate of GBS hospitalizations among the New Zealand population as a reference for calculating age-standardized RRs.

The age-standardized rate of GBS was 810.0 hospitalizations/100,000 person-years (95% CI 41.4–1,578.7) in the month after hospitalization for campylobacteriosis. The RR, compared with the rate of GBS hospitalizations among the New Zealand population, was 319.4 (95% CI 201.5–506.4). This rate was markedly higher than rates for the other patient cohorts examined (Table 3).

Patients with GBS (median age 52.5 years) were significantly older than those hospitalized for campylobacteriosis (median 41 years), who in turn were significantly older than those with campylobacteriosis notifications (median 31 years) (Tables 4, 5). The age of the subpopulation of patients with GBS associated with campylobacteriosis was similar (median 54 years) to that of the total population with GBS.

Discussion

This study shows how the incidence of an acute infectious disease, campylobacteriosis, can influence incidence of a serious neurologic condition, GBS. At the population level, hospitalizations for GBS were significantly correlated with notifications of campylobacteriosis for the same year. At the individual level, compared with rates for the New Zealand population as a whole, hospitalizations for campylobacteriosis were associated with an almost 320-fold increased risk for subsequent hospital admission for GBS in the next month.

Results also show that food safety measures to reduce contamination of fresh poultry meat with *Campylobacter* spp. not only reduced incidence of campylobacteriosis but also were associated with reduced incidence of GBS. In the 3 years after introduction of these control measures, campylobacteriosis notifications and hospitalizations decreased by ≈50%, and GBS hospitalizations dropped by 13%. These findings suggest that in New Zealand, *Campylobacter* spp. infection may be responsible for ≈25% of GBS cases, which is consistent with data from other industrialized countries (*3*).

A recent systematic review (*12*) summarized attempts to quantify the association between campylobacteriosis and GBS incidence. There is general agreement that measuring GBS population rates is useful, for example, for monitoring vaccine adverse effects (*13*,*14*). However,

Table 1. Incidence of campylobacteriosis and GBS before and after intervention to reduce *Campylobacter* spp. in poultry, New Zealand 2002–2010*

	Before intervention, 2002–2006			After intervention, 2008–2010†			Change	
Incident condition	Total no.	Average/year	Rate‡	Total no.	Average/year	Rate‡	Rate ratio (95% CI)	p value
Campylobacteriosis notifications§	69,207	13,841	339.4	21,217	7,072	163.8	0.48 (0.48–0.49)	<0.0001
Campylobacteriosis hospitalizations¶	4,669	934	23.2	1,603	534	12.2	0.53 (0.51–0.54)	<0.0001
GBS hospitalizations¶	513	103	2.6	290	97	2.2	0.87 (0.81–0.93)	0.0496

*GBS, Guillain-Barré syndrome.
†Excludes 2007, which was a transitional year.
‡Annual no. cases/100,000 person-years at risk. Denominator populations based on mean population estimates published by Statistics New Zealand (www.stats.govt.nz/browse_for_stats/population/estimates_and_projections/national-pop-estimates.aspx). Campylobacteriosis and Guillan-Barré syndrome hospitalizations used age-standardized rates based on the age structure of the New Zealand 2006 Census of Population and Dwellings (www.stats.govt.nz/Census/2006CensusHomePage/classification-counts-tables/about-people/age.aspx).
§Published campylobacteriosis notification data (*9*).
¶Hospitalization data from New Zealand Ministry of Health.

Table 2. Hospitalization for Guillain-Barré syndrome during or after hospitalization for campylobacteriosis, New Zealand, July 1995–December 2008

Interval, d	No. (%) persons hospitalized	Cumulative %
Concurrent	29 (82.9)	82.9
1–7	2 (5.7)	88.6
8–28	3 (8.6)	97.1
1,524 (4.2 y)	1 (2.9)	100
Total	35 (100)	Not applicable

to our knowledge, no similar population-based analysis of the relationship between GBS and campylobacteriosis has been conducted for other countries, probably because few countries collect similarly detailed national-level hospitalization data. An earlier population-based study in New Zealand did not show an association between notifications for campylobacteriosis and GBS incidence (*15*). However, that study was over a shorter period and did not use a correction factor to account for undetected repeat hospitalizations in the early years of the observation period, which would have made it harder to detect an association between incidence rates for the 2 conditions.

Compared with global estimates, rates of GBS in New Zealand are high. In a review of reported GBS rates during 1980–2000, worldwide incidence varied between 1.0 and 1.8 cases/100,000 population/year (*2*). The average reported rate for New Zealand during this period was at the upper end of this range (1.8/100,000). A more recent study from the United States estimated that annual hospitalization rates for GBS varied between 1.65 and 1.79/100,000 during 2000–2004 (*16*). In New Zealand during the same period, the annual hospitalization rates varied between 1.8 and 2.7/100,000.

The 320-fold increased risk for GBS in the month after hospitalization for campylobacteriosis found in this study is higher than that previously reported. In a case–control study of GBS and potential antecedent infections in the United Kingdom, Tam et al. reported that persons with *Campylobacter* enteritis had a 38-fold increased risk that GBS would develop in the next 2 months (*17*). However, when they added a correction factor to account for under-ascertainment of campylobacteriosis, the risk increased to 60-fold. Similarly, a population-based study in Sweden estimated that patients with laboratory-confirmed *C. jejuni* infection had a 100-fold increased risk that GBS would develop in the next 2 months (*10*). We used a 1-month risk period because the GBS cases we identified subsequent to hospitalizations for campylobacteriosis were confined to this period. Using a 2-month risk period would have halved our estimated age-standardized RR, but the elevated risk would still be higher than that reported elsewhere.

The proportion of GBS cases attributable to preceding *Campylobacter* spp. infection estimated for New Zealand (≈25%) is within the range described elsewhere. Studies from other countries and regions have reported serologic evidence of previous *C. jejuni* infection in 13%–72% of GBS case-patients (*18*). A systematic review, based on 32 eligible studies, estimated that 31% of GBS cases were attributable to *Campylobacter* spp. infection (*12*).

Table 3. Incidence of GBS after hospitalization for campylobacteriosis and other infectious diseases compared with total population incidence rate for GBS, New Zealand, July 1995–December 2008*

Initial hospitalization condition	ICD-9 codes	ICD-10 codes	Denominator population†	Subsequent GBS hospitalizations (concurrent hospitalizations)‡	Crude rate§	Age-standardized rate¶ (95% CI)	Age-standardized rate ratio (95% CI)
Infectious diseases (ICD chapter 1)	001–139	A00–B99	732,254	56 (273)	90.7	87.0 (56.9–116.4)	34.3 (29.2–40.3)
Pneumonia and influenza	480–488	J09–J18	250,399	19 (82)	91.1	96.2 (25.1–167.3)	37.9 (26.5–54.3)
Enteric diseases#	001–002 004–008.42 008.44–009.3	A00–A01 A03–A04.4 A04.6–A09	77,793	6 (21)	93.3	132.0 (1.2–262.7)	52.0 (32.2–84.2
Campylobacteriosis	008.43	A04.5	8,448	5 (29)	710.2	810.0 (41.4–1,578.7)	319.4 (201.5–506.4)
Salmonellosis	003	A02	2,148	0 (0)	0	0	0
New Zealand population GBS rate	NA	NA	53,617,400	1,320	2.5	2.5 (2.4–2.7)	Referent

*GBS, Guillain-Barré syndrome; ICD, International Classification of Diseases; ICD-9, ICD 9th Revision; ICD-10, ICD 10th Revision; NA, not applicable.
†Denominator population based on either 1) incident hospitalizations for specific condition (number of acute and arranged first overnight hospitalizations as principal or additional diagnosis); or 2) total New Zealand population person-years for July 1995– December 2008 for calculating the New Zealand population GBS rate.
‡First hospitalization of GBS either 1) among those with a previous hospitalization in the preceding 30 d and excluding those with concurrent diagnoses (numbers in parentheses); or 2) in the total New Zealand population for July 1995–December 2008.
§Rate per 100,000 person-years at risk. For GBS hospitalizations after specific conditions, monthly rate has been multiplied by 12 to convert to annual rate.
¶Standard population is population of New Zealand according to the New Zealand 2006 Census of Population and Dwellings (www.stats.govt.nz/Census/2006CensusHomePage/classification-counts-tables/about-people/age.aspx).
#Excluding campylobacteriosis and salmonellosis.

Table 4. Distribution of campylobacteriosis and GBS cases, by age, New Zealand, July 1995–December 2008*

Age group, y	Campylobacteriosis notifications		Camplyobacteriosis hospitalizations		GBS hospitalizations		GBS hospitalizations associated with camplylobacteriosis	
	No. (%)	Rate‡	No. (%)	Rate‡	No. (%)	Rate‡	No. (%)	Rate‡
<5	15,232 (11.7)	442.5	538 (6.4)	13.9	45 (3.4)	1.2	0	0
5–9	6,295 (4.9)	176.9	200 (2.4)	5.0	33 (2.5)	0.8	2 (5.9)	0.1
10–19	14,481 (11.2)	203.7	965 (11.4)	12.2	113 (8.6)	1.4	3 (8.8)	0
20–29	25,063 (19.3)	385.9	1,509 (17.9)	20.6	115 (8.7)	1.6	1 (2.9)	0
30–39	19,511 (15.0)	270.7	935 (11.1)	11.5	146 (11.1)	1.8	3 (8.8)	0
40–49	16,572 (12.8)	237.0	747 (8.8)	9.6	149 (11.3)	1.9	5 (14.7)	0.1
50–59	14,311 (11.0)	261.9	778 (9.2)	13.0	226 (17.1)	3.8	6 (17.7)	0.1
60–69	9,559 (7.4)	255.7	824 (9.8)	19.9	209 (15.8)	5.0	7 (20.6)	0.2
70–79	6,174 (4.8)	235.9	1,046 (12.4)	35.9	200 (15.2)	6.9	5 (14.7)	0.2
≥80	2,712 (2.1)	190.8	906 (10.7)	57.9	84 (6.4)	5.4	2 (5.9)	0.1
Total	129,910 (100.0)	270.4	8,448 (100.0)	15.8	1,320 (100.0)	2.5	34 (100.0)	0.1

*GBS, Guillain-Barré syndrome.
†Association with hospitalization for camplylobacteriosis. Includes subsequent and concurrent hospitalizations (campylobacteriosis and GBS diagnoses at time of hospital discharge).
‡Average annual no./100,000 population.

The strength of the association with GBS may vary geographically, according to the neuropathic propensity of local *Campylobacter* strains. We would also expect the percentage contribution of preceding *Campylobacter* spp. infection to vary according to the incidence of this infection in the population and the incidence of other causal infections and exposures.

The results of our study suggest that risk for GBS may not be uniform for different degrees of campylobacteriosis severity. Our study found that risk for GBS was ≈1 in 1,690 (5 in 8,448) among patients hospitalized for campylobacteriosis and that ≈25% of GBS cases were caused by campylobacteriosis. On the basis of an annual incidence of ≈100 GBS cases, these data suggest that ≈42,000 cases of campylobacteriosis occur each year in New Zealand. Current estimates of total campylobacteriosis incidence are higher. Annual notifications remain at ≈7,000 cases. A study from the United Kingdom estimated that 9.3 cases of campylobacteriosis occurred in the community for every notified case (*19*); a study from Australia estimated this number to be 10 (*20*). Applied to New Zealand, these multipliers suggest an incidence among the population of 65,000 to 70,000 cases per year. These findings suggest that the causal association between campylobacteriosis and GBS is probably weaker for patients with less severe infections, who do not require hospitalization.

Analysis of the age distribution of patients with campylobacteriosis and GBS suggests that older age is a major risk factor for more severe outcomes (hospitalization and GBS) from this enteric infection. The rising incidence of GBS with increasing age in New Zealand is consistent with incidence observed in other countries (*21*).

One strength of this study is that it has been able to monitor a natural experiment in which campylobacteriosis incidence decreased by 50% within a few months, providing an unusual opportunity to assess the effect of this change on incidence of GBS. New Zealand's comprehensive recording of national hospitalization data and use of a unique patient number also provided us with a consistent base for estimating population rates of GBS over a prolonged period. Although the spectrum of GBS includes extremely mild cases, studies elsewhere indicate that only ≈3.0%–5.8% of patients with GBS are not hospitalized (*22*,*23*). In addition, patients with *Campylobacte*r-associated GBS are believed to experience more severe disease (*24*,*25*), which would minimize the number of *Campylobacter*-associated GBS cases missed by this investigation.

One limitation of this study is the group used to compare risk for GBS: the total New Zealand population. A variety of conditions and events have been identified as possible GBS triggers (*1*,*24*,*26*–*29*). Consequently, because it is not possible with current knowledge to identify a

Table 5. Comparison of ages of patients with campylobacteriosis and GBS, New Zealand, July 1995–December 2008*

Calculation	A. Patients with campylobacteriosis	B. Patients hospitalized for campylobacteriosis		C. Patients hospitalized for GBS		D. Patients hospitalized for GBS and campylobacteriosis†	
	Age, y	Age, y	p value for age compared with A§	Age, y	p value for age compared with B§	Age, y	p value for age compared with C‡
Mean	33.7	43.7	<0.0001	48.8	<0.0001	50.3	0.7063
Median	31	41	<0.0001	52.5	<0.0001	54	0.7280

*GBS, Guillain-Barré syndrome.
†Includes subsequent and concurrent hospitalizations (campylobacteriosis and GBS diagnoses at time of hospital discharge).
‡Means compared with Student *t*-test medians compared with median 2-sample test.

reference patient population with no additional GBS risk factors, we considered that the total population provided the most appropriate reference rate.

The association between campylobacteriosis and GBS in New Zealand needs further investigation. It will be useful to continue to follow the trends identified here to assess the stability of the decrease in GBS, which will eventually give greater precision to the estimated contribution of campylobacteriosis. Ongoing monitoring of GBS should be included in the comprehensive surveillance of infectious diseases (*30*). The hypothesis that patients not hospitalized for campylobacteriosis have a lower risk for GBS should be tested by investigation of incidence of GBS among these patients.

Our findings suggest the value of further research to identify other potentially preventable infectious causes of GBS. Table 3 shows a markedly elevated risk for GBS after hospitalization for infectious diseases in general. Investigating these associations in detail may identify other potentially preventable causes of GBS.

Findings of this study have relevant implications for food safety programs. Although GBS is rare, the toll it takes on the individual patient is often high (*1*). Even with treatment, 9%–17% of patients die or remain disabled (*31*), and repeat hospitalizations are common, representing ≈60% of total hospitalizations (online Technical Appendix Table 1). Almost half of all patients report ongoing difficulties 3–6 years after GBS onset (*32*). Consequently, ongoing health care costs for each GBS patient are considerable. In New Zealand during 1988–2008, the GBS case-fatality proportion was 3.0%, and a recent article (*33*) estimated that 204 (13%) of 1,568 disability-adjusted life years for campylobacteriosis in New Zealand were caused by GBS.

This study shows that food safety programs that successfully lower rates of campylobacteriosis might have the additional benefit of preventing GBS. This finding adds to the health and economic arguments for such control measures. The justification for such interventions is particularly strong where a substantial proportion of human disease can be linked to a widely consumed food source, such as contaminated poultry products, as it is in New Zealand (*7*).

Acknowledgments

We thank 2 anonymous reviewers for considerably improving this article.

The New Zealand Ministry of Health provided the hospitalization and mortality data, and the Institute of Environmental Science and Research provided the notification data.

Dr Baker is an associate professor at the University of Otago, Wellington. He is actively investigating the potential for public health surveillance to guide more effective interventions in a range of settings. His research includes a strong focus on infectious diseases and their determinants, particularly the effects of housing conditions and social and ethnic inequalities.

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Pathogenesis of Avian Bornavirus in Experimentally Infected Cockatiels

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Avian bornavirus (ABV) is the presumed causative agent of proventricular dilatation disease (PDD), a major fatal disease in psittacines. However, the influencing factors and pathogenesis of PDD are not known and natural ABV infection exhibits remarkable variability. We investigated the course of infection in 18 cockatiels that were intracerebrally and intravenously inoculated with ABV. A persistent ABV infection developed in all 18 cockatiels, but, as in natural infection, clinical disease patterns varied. Over 33 weeks, we simultaneously studied seroconversion, presence of viral RNA and antigens, infectious virus, histopathologic alterations, and clinical signs of infection in the ABV-infected birds. Our study results further confirm the etiologic role of ABV in the development of PDD, and they provide basis for further investigations of the pathogenetic mechanisms and disease-inducing factors for the development of PDD.

Proventricular dilatation disease (PDD) is a significant cause of disease-related fatalities among birds, primarily psittacines (*1*–*3*). PDD has been observed in >50 psittacine species. Large parrots, including many endangered species, are the most frequently and most severely affected birds (*4*). PDD constitutes a threat to all parrot flocks and aviaries worldwide and endangers the protection and conservation of captive and wild psittacine species.

PPD is caused by a nonpurulent inflammation of the autonomic nervous system of the upper gastrointestinal tract, the peripheral and central nervous tissue, and the cardiac conduction system (*5*,*6*). Gastrointestinal and neurologic signs can appear alone or in combination (*4*,*7*,*8*). The clinical signs are nonspecific, and PDD can be definitively diagnosed only by pathohistologic detection of lymphoplasmacytic infiltrates of ganglia in the upper gastrointestinal tract. However, a negative finding cannot exclude the presence of PDD (*4*,*6*,*8*,*9*).

In 2008, 2 independent groups of research scientists described a new virus, avian bornavirus (ABV), which was amplified from samples from PDD-affected birds. Since then, 6 different ABV genotypes have been detected in psittacines birds. Additional genotypes have been detected in a canary (*Serinus canaria*), wild Canada geese (*Branta canadensis*), and trumpeter swans (*Cygnus buccinator*) (*3*,*10*–*12*). Recent studies substantiate the crucial role of ABV as the etiologic agent for PDD (*10*,*11*). Several scientific groups found ABV in 60%–100% of PDD-affected birds studied (*10*,*11*,*13*–*15*). Surveillance studies in aviaries showed that not all birds were affected after exposure to PDD-diseased and ABV-positive birds, and clinical signs and infection status varied considerably in birds that were affected. In addition, some ABV-positive birds showed no clinical signs (*16*–*20*). These facts indicate that host factors as well as features of the infectious agent, ABV, play a key role for disease induction (*18*).

Initial studies of experimental infections in birds fulfilled Henle-Koch postulates by using small numbers of animals. Gancz et al. (*21*) inoculated 3 cockatiels by multiple routes with brain homogenate containing ABV-4. Sixty-six days postinoculation (dpi), PDD-associated signs, including characteristic histopathologic lesions, developed in 2 of the 3 birds, and test results were positive for ABV-4. The implications of these findings were obscured by the fact that the brain homogenate also included sequences with partial analogy to viruses of the family *Astroviridae* and family *Retroviridae*. After inoculating 4-day-old mallards (*Anas platyrhynchos*; n = 15) with ABV-4, Gray and colleagues (*22*) detected

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DOI: http://dx.doi.org/10.3201/eid1802.111525

ABV RNA in the feces and antibodies against ABV in serum, but they did not detect any clinical signs or PDD-associated lesions. Later, Gray et al. (*23*) inoculated 2 adult Patagonian conures (*Cyanoliseus patagonis*) with ABV-4. The conures were known to be chronic carriers of psittacine herpesvirus, but they appeared to be healthy. Antibodies against ABV were detected 33 dpi, and shedding of viral RNA was detected 62 dpi. Clinical PDD signs developed in both birds, after which 1 bird died and the other was euthanized. Histopathologic analysis showed typical PDD lesions. It was not determined whether the herpesvirus infection was a potentiating factor (*21*–*23*).

Reliable studies that include sufficient numbers of animals to address the host variability over an adequate investigation period are needed to clarify the pathogenetic effects of ABV infection on the development of PDD. More precisely, this implies the need for investigating the course of clinical signs, seroconversion, histopathologic lesions, and virus shedding and distribution in the tissues of affected birds. To further the understanding of ABV and the associated disease, we infected 18 cockatiels with ABV by using 2 different inoculation routes and monitored them for 33 weeks. Our findings show that a persistent ABV infection was induced by in all 18 birds and that it was possible to reliably reproduce all of the known natural ABV disease patterns.

Material and Methods

Inoculum and Sequencing

The virus for inoculation was originally isolated from the brain of a scarlet macaw (*Ara macao*) that died from PDD; the inoculation was passaged 6 times in the quail cell line CEC-32 (*24*). The persistently ABV-infected CEC-32 cells were suspended in medium with 2% fetal bovine serum, sonicated, clarified by centrifugation at 3,000 × *g* for 10 min, and assayed to determine infectivity. For inoculations, we used an infectivity titer of 10^4 50% infectious dose/mL.

By using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, we isolated total RNA for sequencing from 200 μL of virus-containing supernatant. Total RNA was reverse transcribed by using random hexamer primers. PCR for parts of the large viral polymerase and the nucleocapsid protein genes of avian bornavirus was performed as described (*11*). We analyzed PCR products by using gel electrophoresis and purified the products for sequencing by using the QIAquick PCR Purification Kit (QIAGEN). Sequencing was carried out by LGC Genomics (Berlin), and BlastN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to align generated sequence information.

Experimental Infection of Cockatiels

For inoculation purposes, we divided the cockatiels into 2 groups (groups ic and iv), each consisting of 9 animals. We intracerebrally (IC) inoculated birds in group ic and intravenously (IV) inoculated birds in group iv with 0.1 mL of the inoculum described above. All birds were under isoflurane anesthesia when inoculated. One bird remained untreated and served as a sentinel bird in group ic. Another group of 9 birds from the same flock served as controls; they remained untreated and were kept separate from the inoculated birds during the investigation period.

Study Design (Sampling, Clinical Investigations, and Necropsies)

Over a period of 230 days, we surveyed the birds daily to determine their health status. We obtained swab samples from the crop and cloaca to test for the presence of ABV RNA by using real-time reverse transcription PCR as described (*10*). Cycle thresholds (C_t) >36.0 were considered negative (*10*). Swab samples were obtained every other day until all birds of the respective group had ABV-positive test results, then specimens were obtained weekly. In parallel, once a week we obtained 0.3-mL blood samples for indirect immunofluorescence assay (IIFA) detection of antibodies against ABV, as described (*25*). For humane reasons, we euthanized birds with clinical signs typical of PDD (emaciation, undigested seed in the feces, neurologic signs) and reduced general condition. All remaining birds, including control birds, were euthanized 115 or 116 dpi or at the end of the trial (229 or 230 dpi).

We obtained samples of brain, eye, spinal cord, ischiadic nerve, adrenal gland, heart, liver, kidney, spleen, pancreas, crop, proventriculus, gizzard, intestine, pectoral muscle, and skin with feathers from all birds that died or were euthanized. For histopathologic analysis immunohistochemical testing, and other immunohistologic procedures, samples were fixed in 5% buffered formalin, embedded in paraffin wax, and used for preparation of 5-μm sections stained with hematoxylin and eosin. Samples from similar organs were frozen fresh for subsequent real-time PCR to detect ABV RNA. In addition, infectious virus was isolated by using samples from brain and retina as described (*25*). To exclude the presence of any other infection, we examined samples of blood, liver, and lung for bacteria; we used mycological staining to examine samples of proventriculus for infection with yeast; and we examined samples from all intestinal parts for parasites.

Indirect Immunofluorescence Assay

Antibodies against ABV were detected by use of an IIFA on persistently Borna disease virus (BDV)–infected Madin-Darby canine kidney cells. For the assay, we used a 1:50 dilution of fluorescein isothiocyanate–conjugated goat

anti-bird IgG (Bethyl Laboratories, Inc., Montgomery, TX, USA), as described (*25*).

Immunohistochemical Testing

We performed immunohistochemical testing according to the avidin-biotin complex method, as described (*25*,*26*). This method uses a polyclonal rabbit antibody directed against the phosphoprotein and the X protein of BDV.

Virus Isolation

For virus isolation, we performed infectivity assays as described by Narayan et al. (*27*) and Herzog et al. (*25*). Organ samples were homogenized, and 10-fold dilutions were prepared in GIBCO Glasgow Minimum Essential Medium BHK-21 1× (Invitrogen, Paisley, UK) with 10% fetal bovine serum. The mixture was then mixed with equal volumes of freshly dispersed cells of the quail cell line CEC-32 and then incubated on Lab-Tek Chamber Slides (Nunc, Roskilde, Denmark) for 6 days at 37°C. Virus replication was demonstrated by indirect immunofluorescence by using polyclonal serum specimens, which cross-reacted reliably with ABV antigen, from rats experimentally infected with BDV (*25*).

Statistical Analysis

We used the Wilcoxon-Mann-Whitney-test to ascertain differences between the IC- and IV-inoculated groups. U <11 (critical value with $\alpha = 0.005$) was considered highly significant for calculated test statistics.

Results

Sequence Analysis

We used BlastN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare newly generated sequences of parts of the nucleocapsid protein and large viral polymerase genes with sequences available from GenBank. Both genes showed highest accordance (99%) with ABV genotype 4 (EU781959.1 and FJ002323.1).

Clinical Observations

During the 230-day investigation period, 5 of the 18 inoculated birds (3 from group iv and 2 from group ic) showed clinical signs typical of PDD: birds iv1 and iv3 had gastrointestinal signs, birds ic1 and iv5 showed neurologic signs, and bird ic2 had gastrointestinal and neurologic signs. In bird ic2, the following signs developed 33, 37, and 41 dpi, respectively: general symptoms, e.g., apathy; undigested seeds in the feces; and epileptic-like seizures. In birds iv3 and iv2, gastrointestinal signs first became obvious 116 and 126 dpi, respectively. In birds iv5 and ic1, which were only affected neurologically, signs were first noticed 159 and 199 dpi, respectively.

In addition, birds ic6 and iv2 died suddenly and unexpectedly, without obvious signs, 66 and 120 dpi, respectively. Three apparently healthy birds from each group (birds iv3, iv4, iv7, ic3, ic4, and ic8) were euthanized 115 or 116 dpi. At the end of the investigation period, 229 or 230 dpi, the remaining birds (iv1, iv5, iv8, iv9, ic5, ic7, and ic9) and the sentinel bird (se1), which all appeared clinically healthy, were euthanized (Table 1). The control birds were in a good health status during the entire investigation period and were euthanized with the other birds after 230 days.

Gross Findings and Histopathologic Lesions

Necropsy revealed a dilated proventriculus in 7 of the 18 inoculated birds (ic1, ic2, ic3, iv1, iv2, iv3, and iv4), 4 of which had shown signs typical of PDD (Table 2). Dilatation of the proventriculus was most severe in bird ic2, which had both gastrointestinal and neurologic signs. No macroscopic alterations were detected in the remaining 11 inoculated birds, the sentinel bird, or the 9 control birds.

Histopathologic examination revealed that all infected birds and the sentinel bird had mononuclear infiltrates characteristic of PDD in a wide range of organs and of considerable severity. Most of the immune cell infiltrates were in the central nervous system and the gastrointestinal tract, but they were also present in heart; liver; kidney; pancreas; skin with feathers; and, in 1 case, the spleen. In some of the animals, the infiltrates had a follicle-like appearance. Infiltrates were not detected in the pectoral muscle of any birds. No other infections were detected during bacteriologic, mycologic, and parasitologic examinations. The control group did not have histopathologic alterations in organs and did not have any other infection.

Detection of ABV RNA and Antibody against ABV

We detected ABV RNA in swab specimens from all inoculated birds and the sentinel bird. We obtained the first positive test results for group ic 19–29 dpi, whereas we first amplified ABV RNA from group iv samples 25–72 dpi (Figure 1). By using the Wilcoxon-Mann-Whitney test, we determined that ABV RNA was detected significantly earlier in group ic than in group iv (calculated value for test statistic U = 5.5, which is <11 [critical value with $n_1 = 9$ and $n_2 = 9$] and therefore considered to be highly significant, with $\alpha = 0.005$). We obtained the first positive ABV RNA test results for the sentinel bird 76 dpi (Figure 1). The C_t for all birds constantly decreased during the trial, from 35.6 at first ABV RNA detection to 19.8 at last sampling (geometric mean values).

Antibodies against ABV were first detected in group ic 7–63 dpi, and group iv birds seroconverted 29–57 dpi (Figure 2). Statistical analysis did not show any noticeable difference between the ic and iv group (calculated value for

Table 1. Premortem findings in cockatiels experimentally infected with ABV*

Bird	Age at inoculation, d/sex	Antibodies against ABV		RNA‡, first detected, dpi	Gastrointestinal signs		Neurologic signs		Died, dpi	Euthanized, dpi
		First detected, dpi	Titer†		First detected, dpi	Titer†	First detected, dpi	Titer†		
ic1	137/F	43	160	27	–	–	199	5,120	–	206
ic2	121/F	29	20	21	37	320	41	320	–	60
ic3	137/F	35	320	25	–	–	–	–	–	115
ic4	137/F	7	20	19	–	–	–	–	–	115
ic5	121/F	57	320	29	–	–	–	–	–	230
ic6	137/M	43	1,280	25	–	–	–	–	66	–
ic7	137/M	63	160	29	–	–	–	–	–	230
ic8	137/F	43	640	27	–	–	–	–	–	115
ic9	44/M	7	10	25	–	–	–	–	–	230
iv1	137/F	57	320	71	126	5,120	–	–	–	229
iv2	137/F	35	160	35	–		–	–	120	–
iv3	137/M	57	640	43	116	10,240	–	–	–	116
iv4	137/M	29	40	63	–	–	–	–	–	116
iv5	121/F	35	640	72	–	–	159	5,120	–	229
iv6	121/F	43	640	33	–	–	–	–	–	229
iv7	137/M	35	40	35	–	–	–	–	–	116
iv8	137/M	35	40	43	–	–	–	–	–	229
iv9	44/F	29	40	25	–	–	–	–	–	229
se1	137/M	NA	–	76	–	–	–	–	–	230

*Investigation period was 230 d. ABV, avian bornavirus; dpi, days postinoculation; ic, intracerebrally inoculated; –, not detected or not applicable; iv, intravenously inoculated; se, sentinel.
†Antibodies detected by use of indirect immunofluorescence assay; titers <10.0 are considered negative.
‡Avian bornavirus RNA detected by real-time reverse transcription PCR as described by Honkavuori et al. (*10*); cycle thresholds >36.0 are negative.

test statistic U = 39.5, which is >21 [critical value with n_1 = 9 and n_2 = 9] and thus considered not significant, with α = 0.05). Antibody titers in groups ic and iv steadily increased to ≤20,480 during the investigation period (Figure 3). We did not detect ABV RNA or antibodies against ABV in control group birds during the investigation period.

Table 2. Postmortem findings in cockatiels experimentally infected with ABV*

Bird	Died, dpi	Euthanized, dpi	Antibody titer†	Dilatation of proventriculus‡	Antigen detection§		Infectious virus¶
					p14	p24	
ic1			10,240	2	+	+	+
ic2	–	206	5,120	3	+	+	+
ic3	–	60	20,480	1	+	+	+
ic4	–	115	20,480	0	+	+	+
ic5	–	115	20,480	0	+	+	+
ic6	–	230	5,120	0	+	+	+
ic7	66	–	1,0240	0	+	+	+
ic8	–	230	1,0240	0	+	+	+
ic9	–	115	5,120	0	+	+	+
iv1	–	230	10,240	2	+	+	+
iv2	–	229	5,120	2	+	+	+
iv3	120	–	10,240	2	+	–	+
iv4	–	116	5,120	1	+	+	+
iv5	–	116	20,480	0	+	+	+
iv6	–	229	10,240	0	+	+	+
iv7	–	229	10,240	0	+	+	+
iv8	–	116	5,120	0	+	+	+
iv9	–	229	20,480	0	+	+	+
se1	–	229	<10	0	–	–	–

*Proventricular dilatation disease was confirmed in all birds by histopathologic confirmation of lymphoplasmacytic infiltrates of/near ganglia in the central nervous system and/or upper gastrointestinal tract. ABV, avian bornavirus; dpi, days postinoculation; ic, intracerebrally inoculated; +, positive; –, negative; iv, intravenously inoculated; se, sentinel.
†Antibodies against avian bornavirus detected by use of indirect immunofluorescence assay; titers <10.0 are considered negative.
‡0, no dilatation; 1, mild dilatation; 2, moderate dilatation; 3, severe dilatation.
§Detection of the X-protein (p14) and the phosphoprotein (p24) of Borna disease virus by immunohistochemical testing.
¶Re-isolation of infectious ABV in quail cell line CEC-32.

Detection of ABV RNA in Organs

We detected ABV RNA in all organs that were examined 115 or 116 dpi and 229 or 230 dpi. A high amount of ABV RNA (geometric mean C_t 12.0–17.0) was found in the central nervous system, gastrointestinal tract, and in skin with feathers. Moderate amounts of ABV RNA (geometric mean C_t 17.0–23.0) were found in heart, kidney, spleen, and pancreas. The lowest amounts of ABV RNA (geometric mean C_t 23.0–29.0) were found in pectoral muscle and liver. In the sentinel bird, only skin with feathers had positive test results (C_t 28.77), but brain, crop, gizzard, and intestine had high C_t (>36.0); all other organs remained negative for ABV RNA (Figure 4). We did not detect ABV RNA in the organs of control birds.

Immunohistochemical Testing

The detection of viral phosphoprotein and X-protein was mostly restricted to the central nervous system and the heart. In some cases, ABV antigen was also found in some parts of the gastrointestinal tract. We detected X-protein in all group ic birds but not in the sentinel bird. We could not detect phosphoprotein p24 in bird iv3 or in the sentinel bird. ABV antigen was not found in any of the organs of the control birds.

Virus Isolation

We isolated infectious ABV from all inoculated birds. No infectious virus was detected from the organs of the sentinel bird or birds in the control group.

Discussion

PPD was first described in the late 1970s, but its etiology remained unknown until 2008 when ABV and its correlation to PDD were discovered. Numerous studies using naturally infected animals followed this discovery and substantiated the close association between ABV infection and PDD. This association has been further confirmed by several experimental trials (*21–23*,*28*) that used various bird species. However, detailed information about the occurrence of clinical signs, seroconversion, histologic lesions, viral RNA, and infectious virus using a statistically adequate group and sample size was still lacking. We reliably and successfully demonstrated these details in our study. We reproduced the natural pattern of ABV disease variation and the typical signs. We also achieved an infection rate of 100%, with the development of a persistent ABV infection in all animals, as indicated by the constant presence of ABV RNA, ABV antigen, infectious virus, and serum antibodies. These results are in agreement with other experimental data (*21*) and with infection in birds with natural PDD (*18–20*,*28*).

In our experiments, the discrepancies in infection status and in the development of clinical signs in infected but clinically healthy birds could be a result of several host factors (e.g., age, immune status), virulence, and adaption of the inoculated ABV suspension to the cell culture. These variations have already been described for mammalian BDV infections (*29–32*). The cockatiels most likely had considerable variability in their genetic makeup, in contrast, for example, with inbred strains of laboratory mice. Such genetic variability could have a substantial effect on disease susceptibility. Bird ic2 did show a severe progression of clinical signs, and clinical signs developed in 4 other cockatiels. This variability in the course of ABV infection and development of PDD is similar to the variability observed in natural cases of ABV infection (*33*).

ABV RNA and antibodies to ABV were detected in all inoculated birds, and titers of antibody against ABV steadily increased during the investigation period; however,

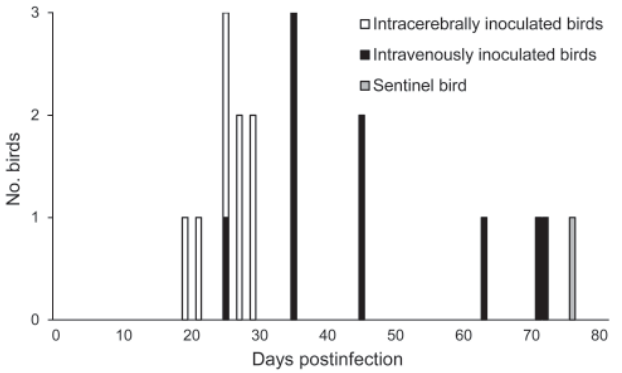

Figure 1. Timing of the first detection of avian bornavirus (ABV) RNA in cockatiels that had been intracerebrally or intravenously inoculated with ABV. ABV RNA was amplified significantly earlier in samples from intracerebrally inoculated birds compared with intravenously inoculated birds (α = 0.005 by using the Wilcoxon-Mann-Whitney test). A noninoculated sentinel bird, which was housed with the intracerebrally inoculated group of cockatiels, was the last bird to shed ABV RNA.

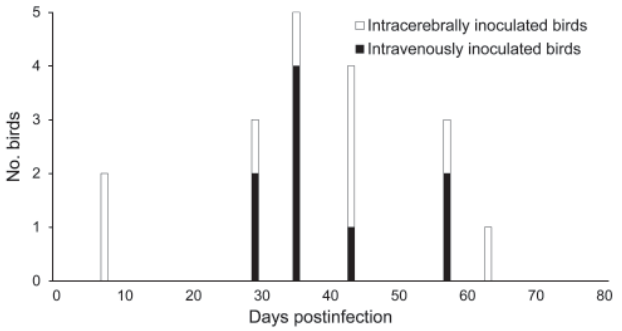

Figure 2. Timing of the first detection of antibodies against avian bornavirus (ABV) in cockatiels that had been intracerebrally or intravenously inoculated with ABV. The time of ABV antibody detection did not differ substantially between the 2 inoculation groups.

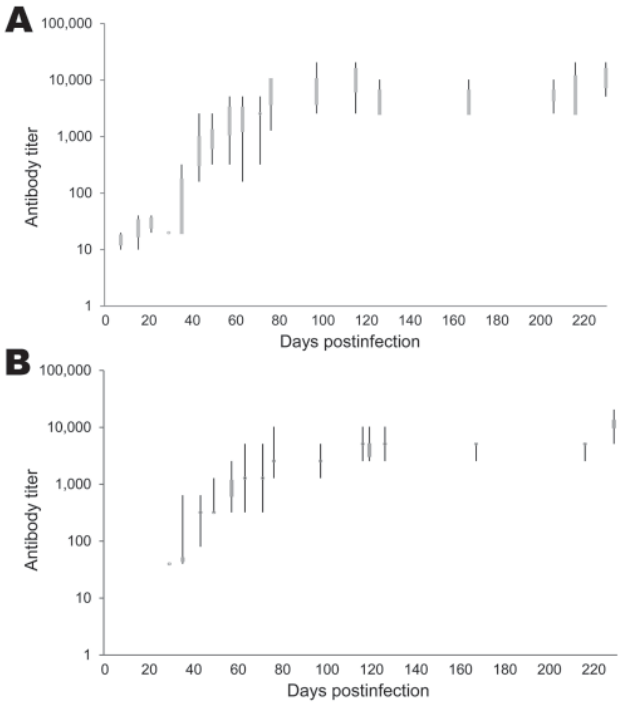

Figure 3. Avian bornavirus antibody in cockatiels inoculated intracerebrally (A) and intravenously (B) with avian bornavirus. In both groups, an exponential rise in antibody titers was detected within the first 12 weeks after inoculation and was followed by a plateau of high antibody titers (≤20,480).

these antibodies did not influence the outcome of clinical disease. Payne et al. (*28*) also reported on ABV-infected cockatiels in which antibodies to ABV did not influence the outcome of clinical disease. Narayan et al. (*27*) similarly reported that in mammals with Borna disease, antibodies against ABV do not exhibit protective properties and seem not to play a role in immunopathogenesis. Thus, detection of antibodies to ABV does not indicate antiviral immunity; on the contrary, they indicate a resolved or ongoing ABV infection with possible risk for the development of PDD. The relevance of these findings should be considered in the diagnosis of possible ABV infections.

We detected ABV RNA considerably earlier in group ic than in group iv. This finding agrees with results of studies in which rats were experimentally infected with BDV and in which the IC route was shown to be the most efficient route for reproducing the disease; however, it was never possible in those studies to infect rats with BDV by iv inoculation (*27*,*34*). Therefore, the successful infection of cockatiels with ABV by IV inoculation represents a notable difference between ABV and BDV infection. This difference is highlighted by similarities in the postinoculation timing of seroconversion and the appearance of clinical signs in bird groups iv and ic. Gastrointestinal as well as neurologic signs could be noted in some birds of both groups. Moreover, we detected infectious virus and ABV RNA in all organs of all infected birds, whereas BDV exhibits strict neurotropism in immune-competent mammals, and spread of the virus to peripheral organs is only possible in immune-incompetent mammals (*30*,*35*). Whether this is also the case for ABV infection remains unclear. However, in our studies, ABV RNA was constantly present at the highest levels in the central nervous system and gastrointestinal tract, indicating that ABV also has an affinity for central and peripheral nervous tissue, as previously described (*13*,*18*,*20*,*21*,*36*). Additional investigations are warranted to resolve this issue.

Antibodies to ABV did not develop in the sentinel bird (se1) during the investigation period; however, increasing amounts of ABV RNA were detected in swab samples from the bird beginnning 76 dpi. After Se1 was euthanized, ABV RNA was amplified from the bird's skin with feathers but not from other organs. The presence of ABV RNA in skin with feathers might be a result of contamination by virus that was constantly shed from other birds in the same cage. In contrast, se1 did show lymphoplasmacytic infiltrates typical for PDD in brain, heart, intestine, or liver. These

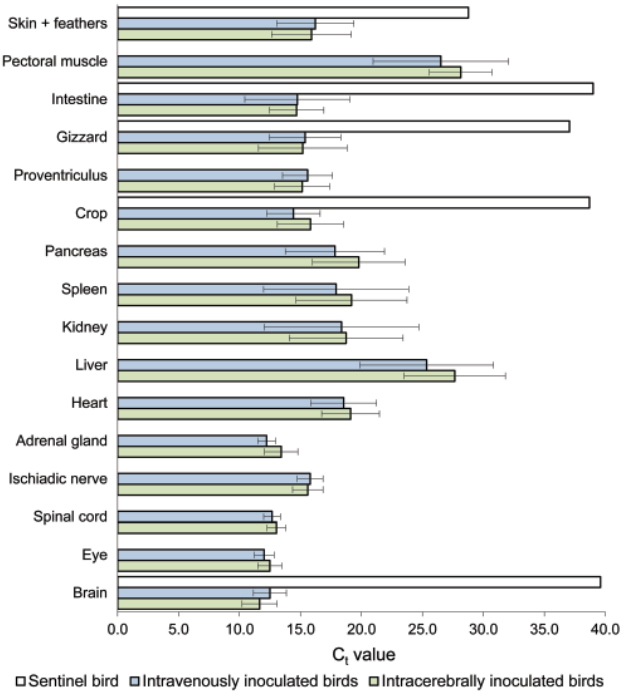

Figure 4. Detection of avian bornavirus (ABV) RNA in different tissues from cockatiels that had been intracerebrally or intravenously inoculated with ABV and from a noninoculated sentinel bird. The geometric mean cycle thresholds (C_t) are shown with their respective standard deviations. C_t >36.0 is considered negative. Low C_t, implying high amounts of ABV RNA, was detected in neuronal and gastrointestinal tissue.

findings indicate that bird se1 reacted differently than the experimentally infected birds, and it may have been more efficient than the experimental cohort in combatting the infection. This might be a result of infection by a different route, such as the oral or intranasal route. Additional investigations are needed to characterize the effects of different infection routes on the outcome of ABV infection and the development of PDD. It is already known from BDV infection of rats that the route of infection determines the severity of disease and that an early up-regulation of BDV-specific CD4 T cells can efficiently protect against infection by the virus (*37*).

In summary, the experimental infection of cockatiels in this study provides reliable evidence that ABV can induce a persistent infection by various routes and lead to disease patterns similar to those in natural infection. Moreover, the etiologic role of ABV for the development of PDD was further confirmed in an adequately sized cohort of cockatiels. Our detailed investigation of clinical signs, seroconversion, histopathologic lesions, and various viral parameters allowed us to document essential data on the course and clinical outcome of ABV infections and on the similarities and differences between ABV and BDV infections. This will serve as a basis for further investigations on the underlying pathogenesis and the main contributing virus and host factors in ABV infection. It remains to be determined whether immunopathologic mechanisms that are based on a T cell–mediated immune reaction, as known for BDV infection, play a role in ABV infection and the development of PDD. Findings in the present study add to our understanding of the pathogenesis of ABV infection and will facilitate interpretation of clinical findings. Antibodies to ABV do not indicate immunity; instead, they point toward a resolved or ongoing ABV infection and a possible risk for the development of PDD.

Acknowledgments

We thank Kristina Maier for help with sampling and surveying of the birds and Philipp Olias for his advice.

The Loro Parque Fundacion provided financial support for the study (project identification: PP-65-2009-1).

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Effect of Surveillance Method on Reported Characteristics of Lyme Disease, Connecticut, 1996–2007

Starr-Hope Ertel, Randall S. Nelson, and Matthew L. Cartter

To determine the effect of changing public health surveillance methods on the reported epidemiology of Lyme disease, we analyzed Connecticut data for 1996–2007. Data were stratified by 4 surveillance methods and compared. A total of 87,174 reports were received that included 79,896 potential cases. Variations based on surveillance methods were seen. Cases reported through physician-based surveillance were significantly more likely to be classified as confirmed; such case-patients were significantly more likely to have symptoms of erythema migrans only and to have illness onset during summer months. Case-patients reported through laboratory-based surveillance were significantly more likely to have late manifestations only and to be older. Use of multiple surveillance methods provided a more complete clinical and demographic description of cases but lacked efficiency. When interpreting data, changes in surveillance method must be considered.

Lyme disease, a multisystem disease caused by the spirochete bacterium *Borrelia burgdorferi*, is the most commonly reported vector-borne disease in Connecticut and in the United States (*1*). During 1996–2007, Connecticut contributed 33,457 (15%) cases to the national surveillance case count, with a mean of 83.4 cases per 100,000 population reported annually, and consistently led the nation in reported annual incidence rate during the study period (*2*). Diagnosis of Lyme disease is based on clinical findings, serologic evidence of infection, and history of exposure to *Ixodes scapularis* ticks. Early stages of illness are most readily diagnosed by identification of erythema migrans. Later-stage illness can involve the musculoskeletal, neurologic, or cardiovascular systems. Positive serologic results are necessary for identifying and classifying patients with later manifestations.

During 1996–2007, the Connecticut Department of Public Health (CDPH) received Lyme disease reports through 4 surveillance methods: passive physician, active physician, enhanced laboratory, and mandatory laboratory. Physician-based surveillance (passive and active) was conducted during the entire study period and relied on health care providers to report new diagnoses of Lyme disease. Active surveillance comprised a voluntary network of health care providers who reported cases 1× per month. Enhanced laboratory surveillance, conducted during 1996–1997, required participating Connecticut laboratories to send supplemental case report forms with each positive *B. burgdorferi* result to the ordering physician. In January 1998, to study the effectiveness of a newly released Lyme disease vaccine, mandatory laboratory surveillance was implemented that required all laboratories to report positive and equivocal results to CDPH. Follow-up, conducted by CDPH staff, involved sending a letter and supplemental report form to the ordering physician. To assist the physician, demographic and patient-identifying information from the laboratory report was incorporated into the form. Mandatory laboratory surveillance ended after 2002 when the Lyme disease vaccine was removed from the market. In 2007, mandatory reporting of positive Lyme disease results was reinstated for laboratories with electronic reporting capability. Two large commercial laboratories provided electronic reports. Follow-up was reestablished by using the previous method, i.e., CDPH staff sent a letter and supplemental report form to the ordering physician.

Public health surveillance methods for infectious diseases change over time, depending on program priorities and resources, advancements in diagnostic testing, modifications to surveillance case definitions, and changing

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DOI: http://dx.doi.org/10.3201/eid1802.101219

reporting modalities (e.g., electronic laboratory reporting). Lyme disease surveillance data provide a measure of the relative geographic distribution of this disease and its effect on public health in Connecticut and have been used to assess the effectiveness of control and prevention activities (*3*–*5*). These data also form part of the risk communication messages provided to the general public, advocacy groups, media, political leaders, health care providers, and public health professionals. We examined how surveillance method affected the classification of reported clinical and demographic characteristics of case-patients and the incidence of Lyme disease in Connecticut, during 1996–2007.

Materials and Methods

Surveillance Case Definition

Lyme disease reports were categorized by using the national surveillance case definition issued in 1996 (*6*). A case was defined as 1) physician report of erythema migrans of ≥5 cm in diameter or 2) at least 1 objective late manifestation (i.e., musculoskeletal, neurologic, or cardiovascular) with laboratory confirmation of infection with *B. burgdorferi* by enzyme immunoassay, immunofluorescent assay, or Western immunoblot. CDPH classified reports that did not meet the case definition as not a case. Because clinical information is required for case classification, when supplemental follow-up reports were not returned, they were considered lost to follow-up. The distinct report forms used for each surveillance method contained the following data elements: case-patient demographic characteristics (sex, age, race, ethnicity), clinical findings (erythema migrans or late manifestations), seasonality, and case status. Seasons were defined as winter (December–February), spring (March–May), summer (June–August), and fall (September–November).

Data Collection

The statewide Lyme disease surveillance system was maintained by an average of 1.5 full-time employees. Reports were entered into the National Electronic Telecommunications System for Surveillance (NETSS), a public health surveillance information system that used Epi Info 6.0 (*7*). In 2007, NETSS and Microsoft Excel (Microsoft, Redmond, WA, USA) were used. Supplementary variables necessary for follow-up and maintenance of reports were added to the standard NETSS variables by CDPH staff and included health care provider name and contact information, license number, and origin of report.

A potential case could have been reported through >1 surveillance method. For consistency in classification, the origin of case reports was entered in the following hierarchy: active surveillance, passive surveillance, enhanced laboratory surveillance, and mandatory laboratory surveillance. Data were cleaned and duplications were removed at the end of each year.

Data Analyzed

The following statistics were calculated across surveillance methods: annual mean number of reports and cases, annual incidence rates, proportion of reports by case status, demographic characteristics of case-patients, seasonality of cases, and clinical and laboratory findings. Incidence per 100,000 population was determined by using decennial census data covering the year of data collection (1990 or 2000). Statistical tests were performed by using Epi Info 6.0. We used χ^2 test with the Yates continuity correction. A p value ≤ 0.05 was considered significant. The positive predictive value (PPV) of reports of potential cases was calculated for each type of surveillance method by determining the ratio of cases to reports.

Results

Overall Analysis

During 1996–2007, CDPH staff processed 87,174 Lyme disease reports, of which 7,278 (8.3%) were duplicate entries and were removed from the database. A total of 79,896 individual reports were analyzed. Of these, 43,767 (54.8%) were reported through mandatory laboratory surveillance, 19,350 (24.2%) through passive physician surveillance, 13,040 (16.3%) through active physician surveillance, and 3,739 (4.7%) through enhanced laboratory surveillance. Overall, 33,457 (41.9%) reports were classified as cases, and 26,318 (32.9%) as not cases; 20,121 (25.2%) were lost to follow-up (Table 1). Except for calculation of PPV, reports classified as lost to follow-up were excluded from further analyses.

During 1996, Connecticut had 5,473 reports of Lyme disease. The number of reports increased with the successive implementation of enhanced and mandatory laboratory surveillance reporting, peaking at 12,947 in 2002 (Figure 1). In 1998, the first year of mandatory laboratory surveillance, the overall number of Lyme disease reports

Table 1. Number of Lyme disease reports, by status and surveillance method, Connecticut, 1996–2007*

Status	PS	AS	ELS	MLS	Total
Case	12,185	8,666	1,949	10,657	33,457
Not a case	4,962	4,316	1,783	15,257	26,318
Lost to follow-up	2,203	58	7	17,853	20,121
Total†	19,350	13,040	3,739	43,767	79,896

*PS, passive physician surveillance 1996–2007; AS, active physician surveillance 1996–2007; ELS, enhanced laboratory surveillance 1996–1997; MLS, mandatory laboratory surveillance 1998–2002 and 2007.
†Positive predictive values: PS, 63.0; AS, 66.5; ELS, 52.1; MLS, 24.3; total, 41.9.

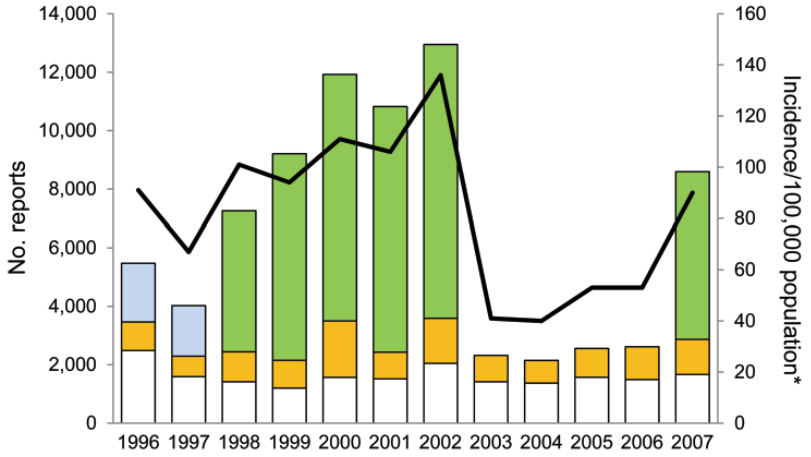

Figure 1. Number of Lyme disease surveillance reports received and incidence per 100,000 population, Connecticut, 1996–2007. White bar sections, passive surveillance; gold bar sections, active surveillance; blue bar sections, enhanced laboratory surveillance; green bar sections, mandatory laboratory surveillance; line, incidence, determined by using decennial census data encompassing the year data were reported.

increased by 80.3%, cases increased by 49.4%, and physician-based reporting increased by 26.9% over the previous year; incidence was 104.5 cases per 100,000 population. In 2003, the first year after laboratory reporting ended, the overall number of reports decreased by 82.1%, cases decreased by 69.7%, and physician-based reporting decreased by 37.8% over the previous year; incidence was 41.2 cases per 100,000 population. During 2003–2006, the period with no laboratory surveillance, the number of total reports dropped substantially to an annual mean of 2,411, a 78.5% decrease from the 1999–2002 annual mean. In 2007, laboratory surveillance was reinstated for laboratories with the capability to electronically report results. The total number of reports increased by 228.3%, the number of cases increased by 71.0% over the previous year (Figure 1), and the incidence nearly doubled to 89.8 cases per 100,000 population. An average of 16.0% more cases were reported through physician-based surveillance during years with mandatory laboratory reporting.

The PPV varied across surveillance methods and was highest for physician-based surveillance methods (Table 1). Less than 25% of reports received through mandatory laboratory surveillance were classified as cases. Cases reported through this method accounted for nearly one third (31.9%) of all cases during the study period.

Demographic Characteristics

The median age of case-patients was 38 years (range 34–43 years). Case-patients <20 years of age were more likely to be reported through physician-based surveillance ($p<0.001$); laboratory-based surveillance was more likely to report case-patients >40 years of age ($p<0.001$) (Figure 2). Overall, whites accounted for 82.0% of cases, similar to the state's racial distribution, and the distribution did not differ significantly by surveillance method. Ethnicity data were available for approximately one third (32.6%) of case-patients; only 1.2% were reported as Hispanic. Laboratory-based surveillance reported an average of 32 Hispanic case-patients annually, compared with 20 reported through physician-based surveillance (Table 2). On average, 9.6% more male than female case-patients were reported by each surveillance method.

Clinical Features

Of the 33,457 cases, 66.2% were characterized by erythema migrans only, 27.6% by ≥1 late manifestation and

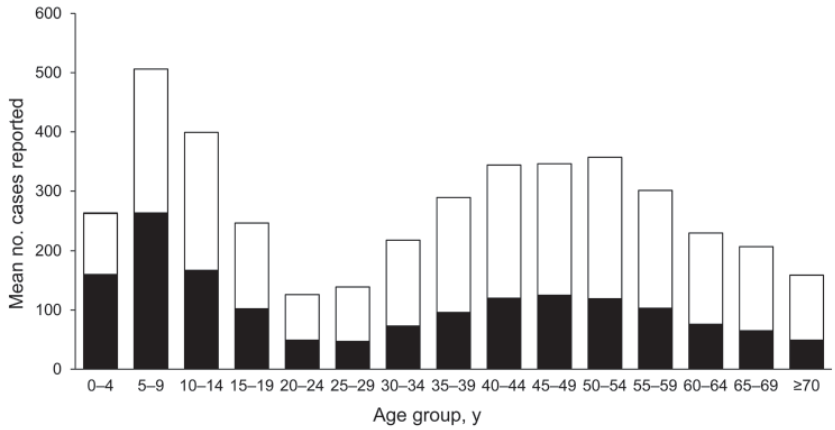

Figure 2. Mean annual number of Lyme disease cases, by age group and surveillance method, Connecticut, 1996–2007. Black bar sections, physician-based surveillance; white bar sections, laboratory-based surveillance.

Table 2. Demographic characteristics of Lyme disease case-patients, by surveillance method, Connecticut, 1996–2007*

Characteristic†	No. (%) case-patients				
	PS, n = 12,185	AS, n = 8,666	ELS, n = 1,949	MLS, n = 10,657	Total, n = 33,457
Sex					
M	6,707 (55.0)	4,690 (54.1)	1,128 (57.9)	5,761 (54.1)	18,286 (54.7)
F	5,453 (44.8)	3,969 (45.8)	814 (41.8)	4,856 (45.6)	15,092 (45.1)
Unknown	25 (0.2)	7 (0.1)	7 (0.4)	40 (0.4)	79 (0.2)
Race					
White	10,402 (85.4)	6,772 (78.1)	1,440 (73.9)	8,811 (82.7)	27,425 (82.0)
Black	83 (0.7)	47 (0.5)	14 (0.7)	119 (1.1)	263 (0.8)
Asian/Pacific Islander	83 (0.7)	34 (0.4)	10 (0.5)	63 (0.6)	190 (0.6)
American Indian/Alaska Native	18 (0.1)	7 (0.1)	1 (0.1)	6 (0.1)	32 (0.1)
Other	83 (0.7)	17 (0.2)	1 (0.1)	32 (0.3)	133 (0.4)
Unknown	1,516 (12.4)	1,789 (20.6)	483 (24.8)	1,626 (15.3)	5,414 (16.2)
Ethnicity					
Hispanic	202 (1.7)	33 (0.4)	3 (0.2)	156 (1.5)	394 (1.2)
Non-Hispanic	7,051 (57.9)	884 (10.2)	16 (0.8)	2,550 (23.9)	10,501 (31.4)
Unknown	4,932 (40.5)	7,749 (89.4)	1,930 (99.0)	7,951 (74.6)	22,562 (67.4)

*PS, passive surveillance; AS, active surveillance; ELS, enhanced laboratory surveillance; MLS, mandatory laboratory surveillance.
†Median ages: PS, 34 y; AS, 36 y; ELS, 43 y; MLS, 42 y; total, 38 y.

positive laboratory findings, and 6.2% by both (Tables 3, 4). Overall, symptoms of erythema migrans only were more likely to be reported through physician-based surveillance than through laboratory-based surveillance (75.8% vs. 50.3%; p<0.001). Conversely, late manifestations were more likely to be reported through laboratory-based surveillance than through physician-based surveillance (43.2% vs. 18.1%; p<0.001). Of all case-patients reported through laboratory-based surveillance, 30.5% had Lyme arthritis, compared with 13.4% of those reported through physician-based surveillance. Of cases characterized by late manifestations only, arthritis was most frequently reported (72.1%). Of late manifestation cases for which arthritis was reported, 74.0% were based on physician surveillance and 70.8% on laboratory surveillance (p<0.001). Laboratory-based surveillance was more likely to report case-patients with second- or third-degree atrioventricular block (p = 0.051).

Seasonality

In 72.9% of cases, illness onset occurred during the summer (76.3% physician-based vs. 66.8% laboratory-based cases) (Table 5). Erythema migrans occurred in 84.2% of cases with onset during the summer. Erythema migrans was significantly more likely to be reported during the summer through physician-based surveillance than through laboratory-based surveillance (71.3% vs. 28.7%; p<0.001). Late manifestations were 2× more likely to be reported through laboratory-based surveillance during the summer months (17.5% vs. 8.2%; p<0.001).

Discussion

In Connecticut, data obtained through 4 surveillance methods during 1996–2007 demonstrated that the epidemiology of Lyme disease is subject to variation by surveillance method. The number of reports, proportion of reports classified as cases, incidence, and demographic and clinical characteristics of case-patients differed between physician-based and laboratory-based surveillance. Although some of the annual fluctuation in reports and cases might be attributable to an actual increase or decrease in disease, the substantial changes seen indicate that the principal factor most likely resulted from changes in surveillance method over time. As these surveillance artifacts show, changes in surveillance methods can cause changes in trends. Therefore, the nature of the surveillance method and the effect of changes in the method are necessary to consider when interpreting the data.

Lyme disease surveillance methods ultimately rely on physicians to report the necessary clinical information to classify cases. Because health care providers in outpatient settings often underreport commonly seen illnesses (*8*,*9*), in Connecticut, follow-up for mandatory laboratory surveillance might help serve as a reminder system for physicians to report cases. This fact could explain the 16.0% increase in the average annual number of cases

Table 3. Clinical manifestations of Lyme disease, by surveillance method, Connecticut, 1996–2007*

Clinical manifestation	No. (%) cases				
	PS, n = 12,185	AS, n = 8,666	ELS, n = 1,949	MLS, n = 10,657	Total, n = 33,457
Erythema migrans only	9,489 (77.9)	6,324 (73.0)	1,032 (53.0)	5,305 (49.8)	22,150 (66.2)
Late manifestations only	2,059 (16.9)	1,725 (19.9)	763 (39.1)	4,678 (43.9)	9,225 (27.6)
Both	637 (5.2)	617 (7.1)	154 (7.9)	674 (6.3)	2,082 (6.2)

*PS, passive surveillance; AS, active surveillance; ELS, enhanced laboratory surveillance; MLS, mandatory laboratory surveillance.

Table 4. Late manifestations of Lyme disease, by surveillance method, Connecticut, 1996–2007*

Manifestation†	No. cases (%)				
	PS, n = 2,059	AS, n = 1,725	ELS, n = 763	MLS, n = 4,678	Total, n = 9,225
Lyme arthritis	1,448 (70.3)	1,353 (78.4)	532 (69.7)	3,318 (70.9)	6,651 (72.1)
Bell palsy	454 (22.1)	259 (15.0)	143 (18.7)	814 (17.4)	1,670 (18.1)
Radiculoneuropathy	117 (5.7)	131 (7.6)	84 (11.0)	501 (10.7)	833 (9.0)
Lymphocytic meningitis	61 (3.0)	35 (2.0)	19 (2.5)	108 (2.3)	223 (2.4)
Encephalitis/encephalomylitis	38 (1.5)	18 (1.0)	26 (3.4)	116 (2.5)	198 (2.1)
Second- or third-degree heart block	40 (1.9)	26 (1.5)	10 (1.3)	58 (1.2)	134 (1.4)

*PS, passive surveillance; AS, active surveillance; ELS, enhanced laboratory surveillance; MLS, mandatory laboratory surveillance.
†May have included >1 late manifestation per case. Percentages do not add to 100%.

reported through physician-based surveillance during years when laboratory surveillance was mandatory.

Our data showed that physician-based surveillance, combined with laboratory-based surveillance, resulted in more comprehensive clinical and demographic information and higher incidence of illness than each method alone. Of all reported cases, nearly one third (31.9%) originated through laboratory-based surveillance. However, use of laboratory-based surveillance is inefficient: only 24.3% were classified as cases. Case-patients reported through laboratory-based surveillance also differed significantly in age groups, reporting older case-patients; clinical information, reporting more late stage illness; and seasonal data, reporting more cases during the fall and winter months. Our combined surveillance methods contributed to, and broadened, the overall epidemiologic description of Lyme disease in Connecticut.

This study has several limitations. First, laboratory-based surveillance was difficult to evaluate independently of physician-based surveillance. Because health care professionals could potentially report by using each of the surveillance methods, we used a hierarchy to help reduce bias toward 1 surveillance method over another. Second, because active surveillance providers volunteered to participate, these physicians were more likely to be those most interested in Lyme disease surveillance. Therefore, physicians who volunteered to participate in active surveillance might have been more likely to report cases in a strictly passive surveillance system.

To satisfy the sometimes conflicting goals of surveillance methods and allocation of public health resources, collection of case data needs to be streamlined. Two potential alternatives may be the following: modeling by using sampling schemes or greater use of electronic information systems, which is planned in Connecticut. Electronic laboratory reporting, automation of follow-up requests, and Web-based provider reporting will conserve resources, and provide incident data information demanded of public health agencies.

When determining which methods of Lyme disease surveillance to use, the purpose of that surveillance and available resources need to be considered. In Lyme disease–endemic states where the epidemiologic purpose might primarily be to monitor geographic, clinical, and demographic trends, intensive statewide surveillance is not essential. Rather, surveillance needs to be conducted

Table 5. Lyme disease cases, by clinical manifestation, season, and surveillance method, Connecticut, 1996–2007

Clinical manifestation/season	No. (%) cases				
	PS, n = 10,535*	AS, n = 8,281*	ELS, n = 1,767*	MLS, n = 8,685*	Total, n = 29,268
All					
Winter	278 (2.6)	244 (2.9)	76 (4.3)	548 (6.3)	1,146 (3.9)
Spring	1,029 (9.8)	923 (11.1)	154 (8.7)	1,118 (12.9)	3,224 (11.0)
Summer	8,121 (77.1)	6,234 (75.3)	1,276 (72.2)	5,707 (65.7)	21,338 (72.9)
Fall	1,107 (10.5)	880 (10.6)	261 (14.8)	1,312 (15.1)	3,560 (12.2)
Erythema migrans					
Winter	84 (0.8)	56 (0.7)	7 (0.4)	73 (0.8)	220 (0.8)
Spring	741 (7.0)	624 (7.5)	34 (1.9)	425 (4.9)	1,824 (6.2)
Summer	7,315 (69.4)	5,500 (66.4)	993 (56.2)	4,164 (47.9)	17,972 (61.4)
Fall	739 (7.0)	515 (6.2)	78 (4.4)	501 (5.8)	1,833 (6.3)
Total	8,879 (84.3)	6,695 (80.8)	1,112 (62.9)	5,163 (59.4)	21,849 (74.7)
Late manifestations					
Winter	194 (11.7)	188 (11.9)	69 (10.5)	475 (13.5)	926 (3.2)
Spring	288 (17.4)	299 (18.8)	120 (18.3)	693 (19.7)	1,400 (4.8)
Summer	806 (48.7)	734 (46.3)	283 (43.2)	1,543 (43.8)	3,366 (11.5)
Fall	368 (22.2)	365 (23.0)	183 (27.9)	811 (23.0)	1,727 (5.9)
Total	1,656 (15.7)	1,586 (19.2)	655 (37.1)	3,522 (40.6)	7,419 (25.3)

*PS, passive surveillance; AS, active surveillance; ELS, enhanced laboratory surveillance; MLS, mandatory laboratory surveillance.

consistently over time. Intensive surveillance efforts may even be counterproductive when not sustainable because of limited resources or when resources are diverted from other public health activities. Replacing traditional case-reporting surveillance methods with less labor-intensive data collection methods, such as regular population-based surveys, may be suitable for following trends and estimating disease (*10*).

Acknowledgments

We thank Brenda Esponda, Carmen Marquez, Paul Gacek, and Heather Altier for their data entry assistance. We also thank participating active surveillance physicians and laboratories that participated in enhanced laboratory surveillance.

The Lyme Disease Surveillance System was funded in part by federal funds from the Centers for Disease Control and Prevention (cooperative agreement U50/CCU106598).

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Characterization of Nipah Virus from Outbreaks in Bangladesh, 2008–2010

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Nipah virus (NiV) is a highly pathogenic paramyxovirus that causes fatal encephalitis in humans. The initial outbreak of NiV infection occurred in Malaysia and Singapore in 1998–1999; relatively small, sporadic outbreaks among humans have occurred in Bangladesh since 2001. We characterized the complete genomic sequences of identical NiV isolates from 2 patients in 2008 and partial genomic sequences of throat swab samples from 3 patients in 2010, all from Bangladesh. All sequences from patients in Bangladesh comprised a distinct genetic group. However, the detection of 3 genetically distinct sequences from patients in the districts of Faridpur and Gopalganj indicated multiple co-circulating lineages in a localized region over a short time (January–March 2010). Sequence comparisons between the open reading frames of all available NiV genes led us to propose a standardized protocol for genotyping NiV; this protcol provides a simple and accurate way to classify current and future NiV sequences.

Nipah virus (NiV) is a deadly paramyxovirus that was first described during 1998–1999 in Malaysia and Singapore, when a large epidemic of fatal encephalitis occurred in humans (283 cases, 109 deaths) (*1*). In this initial outbreak, most human cases were epidemiologically linked with activities involving close contact with sick pigs; the outbreak ended after >1 million pigs were culled and movement of pigs was stopped (*2*). Although NiV infection has not been detected in Malaysia or Singapore since 1999, NiV has caused recurring (almost annual) outbreaks of fatal encephalitis in Bangladesh and sporadic outbreaks in India since 2001 (*3*–*6*). The outbreaks in Bangladesh have demonstrated human-to-human and foodborne transmission of NiV (*7*–*9*). Although the outbreaks in Bangladesh have been smaller, the case-fatality rates have been consistently higher (≈75%) than those from the initial outbreak in Malaysia and Singapore (≈40%) (*8*,*10*). The clinical case definition used in Bangladesh differs from that used during the Malaysia outbreak and focuses on fatal or severe neurologic signs and symptoms. Sequence analysis of virus isolates and clinical samples obtained from persons affected by the outbreaks in Bangladesh and India indicated greater nucleotide heterogeneity than those from Malaysia (*3*,*4*,*11*).

Within 2 weeks in Bangladesh during February 2008, 2 clusters of human NiV infection resulted in 10 cases with 9 deaths (90% case-fatality rate). The locations of the clusters (Rajbari and Manikgonj districts) were ≈44 km apart, separated by the intersection of the Padma and Jamuna Rivers. The outbreak was linked to ingestion of raw date palm sap (*12*). From December 2009 through March 2010, an outbreak of NiV infection in Fardipur and Gopalganj districts was responsible for 17 cases and 15 deaths (88% case-fatality rate) (*6*).

In this study, we confirmed the suspected clinical cases of NiV infection from both outbreaks by using IgM and IgG ELISAs, real-time and conventional reverse transcription PCR (RT-PCR), and virus isolation. We characterized the complete genomic sequences of 2 identical NiV isolates from 2008 and 3 partial genomic sequences of isolates from 2010. Our results indicate the presence of multiple co-circulating lineages of NiV in a localized region over a short time in 2010. Phylogenetic and sequence analysis of

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DOI: http://dx.doi.org/10.3201/eid1802.111492

all currently available full-length NiV gene open reading frames (ORFs) led us to propose a standardized protocol for genotyping NiV.

Methods

Sample Collection and Case Definition

We collected blood, cerebrospinal fluid (CSF), urine, and throat swab samples from patients with suspected cases. The serum and CSF samples were separated into aliquots locally, and all specimens were transported to the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) in cold packs or in liquid nitrogen for subsequent storage at −70°C. Serum and CSF samples were initially tested for IgM against NiV at ICDDR,B and then sent to the Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) for confirmatory testing. Samples were confirmed as NiV positive if IgM against NiV was found in serum or CSF; if NiV RNA was amplified; or if NiV was isolated from CSF, urine, or throat swab samples (*6*,*12*).

Serologic Testing

Serum samples were tested at ICDDR,B for IgM against NiV by ELISA as described (*1*,*3*,*13*). At CDC, samples were irradiated with gamma rays before confirmatory testing for IgM and IgG against NiV as described (*3*).

Detection of NiV by Real-Time, Conventional RT-PCR, and Virus Isolation

Virus isolation was attempted on Vero E6 cells as described (*1*). Human urine, CSF, and oropharyngeal swab samples were inactivated in guanidine isothiocynate (GITC) at a dilution of 1 part sample to 5 parts GITC. RNA was extracted by the acid GITC–phenol–chloroform method (*14*). Real-time RT-PCR (rRT-PCR) was performed by using the following primers that amplified a 112-nt fragment spanning from positions 538 to 650 in the NiV N gene: forward primer NVBNF2B 5′-CTGG TCTCTGCAGTTATCACCATCGA-3′, reverse primer NVBN593R 5′-ACGTACTTAGCCCATCTTCTAGTTT CA-3′, and probe NVBN542P 5′-CAGCTCCCGACAC TGCCGAGGAT-3′, with the FAM dye incorporated at the 5′ terminus and a BHQ1 quencher molecule at the 3′ terminus. The rRT-PCR cycling conditions were as follows: 48°C for 30 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s followed by 1 min at 60°C. Synthetic NiV N gene RNA was produced by in vitro transcription that used pTM1-N plasmid (*15*) with Megascript kit (Ambion, Austin, TX, USA) according to manufacturer's instructions. An Applied Biosystems 7900HT machine was used for rRT-PCRs, and the PCR Core Kit along with MultiScribe Reverse Transcriptase were used for the rRT-PCR master mix (all from Applied Biosystems, Foster City, CA, USA). Conventional RT-PCR was performed with the SuperScript One-Step RT-PCR kit with Platinum Taq (Invitrogen, Carlsbad, CA, USA) as described (*11*). Two-step RT-PCR was performed for selected samples by using SuperScript III First-Strand Synthesis System (Invitrogen) to generate cDNA and Platinum Taq DNA Polymerase High Fidelity (Invitrogen) for the PCR. Briefly, 8 μL of extracted RNA was used in a 20-μL RT reaction with a primer complementary to the 3′ leader NVB3END (5′-ACCAAACAAGGGAAAATATGGATACGTT-3′) and the 5′ trailer NIP5END (5′-ACCGAACAAGGG TAAAGAAGAATCG-3′) sequences of the NiV genome from the 2004 Bangladesh outbreak (GenBank accession no. AY988601). Subsequently, 2 μL of the cDNA reaction was used in 50-μL PCRs to amplify the N, P, M, F, G, and L ORFs with corresponding primer sets that anneal to the noncoding regions for the respective genes: N ORF NVBN5NCF1 (5′-GGTCTTGGTATTGGATCCTC-3′) and NVBN3NCR1 (5′-GTTTAATCTAAGTTAAGATTG-3′); P ORF NVBPPCRFW (5′-AGCAGTTATCAGCTGGG AGTTCAACTTAC-3′) and NVBPPCRREV (5′-ATGC GTGAATGAACTACAATACGAATCGAC-3′); M ORF NVBMPCRFW (5′-TCCAATAACTGGTCAATTGAG GACAGAAATCCTG-3′) and NVBMPCRREV (5′-CATA ATAGTTGTCTAATTATTAACCGAATATTCAC-3′); F ORF NVBPCRFFW (5′-CAAGCATTATTACTATCT GATCAACAAAAGGATTGG-3′) and NVBPCRFREV (5′-GAATATCAACTGTTCATTCATGGTTGAGTAC-3′); G ORF NVBPCRGFW (5′-CAGGTCCATAACT CATTGGATATTAAACTGTGTCC-3′) and NVBPCR GREV (5′-CAAGATTTAGCTCTACTATATCAAATG GAGTTTCAGTCAAG-3′); and L ORF (amplified in 2 fragments) NVBPCRL1FW (5′-CAGGTCCTTGATTGTG CTAATTTTCTTGAG-3′) and FRAG4REV (5′-GAT CTTATCAGGCCTTTAGTTGTATCTAATAGACC-3′), FRAG5FW (5′-TGAGGACCTTGAACTAGCTAGCTTC CT-3′) and NVBLREV (5′-AATTGTCGGTCGGTTC TGGACTTGGAAGATCAAATCAGATAATGGAT ATG-3′). PCR products were analyzed by agarose gel electrophoresis with GelRed staining (Biotium, Hayward, CA, USA), gel purified, and sequenced as described (*3*,*11*). Rapid amplification of cDNA ends was performed by using the 5′ RACE Kit 2.0 (Invitrogen). Phylogenetic and molecular analyses of the sequences were performed by using MEGA5 (*16*).

Results

Of the 10 cases from the 2008 outbreak in Bangladesh, 5 were confirmed positive for NiV infection by at least 1 laboratory test at CDC. Of those 5 positive cases, 4 were positive for IgM; 2 for IgG; 3 by rRT-PCR; and 2 by conventional RT-PCR; 2 throat swab samples yielded

live NiV, 1 from Manikgonj (NiV/BD/HU/2008/MA [BD, Bangladesh; HU, human]; accession no. JN808857) and 1 from Rajbari (NiV/BD/HU/2008/RA; accession no. JN808863) (Table 1). Despite the isolates having come from patients from 2 districts, sequence analysis of the entire genome of the 2 isolates indicated that they were identical. Phylogenetic analysis of ORFs from each NiV gene indicated that this strain was similar to, but distinct from, the 2007 isolate from India (NiV/IN/HU/2007/FG [IN, India]; accession no. FJ513078) (Figure 1, panel A; Figure 2, panels A–E). To rule out the possibility of laboratory contamination, we performed 2-step conventional RT-PCR by using RNA from duplicate samples of the original throat swab samples from which the 2 viruses were isolated. We amplified the entire N gene ORF from each sample and confirmed that the sequences were identical. Although there were 4 isolated cases of NiV infection in Bangladesh in 2009 as confirmed by IgM or IgG ELISA, or both, we were not able to obtain NiV sequences from those case-patients (Table 1).

Of the 17 cases from the 2010 outbreak in Bangladesh, 12 were confirmed positive. All 12 were positive for IgM, 2 for IgG, 5 by rRT-PCR, and 3 by conventional 2-step RT-PCR (Table 1). Although we detected NiV RNA by rRT-PCR from urine, CSF, and throat swab samples, we were unable to isolate virus from any of those sources. Of the 3 samples from which we were able to amplify NiV sequences, 1 was from a 10-year-old girl from the initial cluster (NiV/BD/HU/2010/FA2; accession no. JN808859) and the other 2 were from patients with isolated cases. The patients with isolated cases were a medical intern (NiV/BD/HU/2010/FA1; accession no. JN808864) who was working in the pediatric department at Faridpur Medical College Hospital and a 7-year-old girl (NiV/BD/HU/2010/GO; accession no. JN808860) who was examined by the same medical intern; both died. The illness developed in the intern only 6 days after the 7-year-old girl died; this incubation period was atypically short for NiV infection, indicating the possibility of separate infections (*6*). Sequence analysis of the N ORFs amplified from throat swab samples confirmed that the intern and the girl were infected with distinct lineages of NiV (Figure 1, panel A). Our attempts to recover NiV sequences from prior contacts of the medical intern who were IgM positive for NiV infection were unsuccessful. Phylogenetic analysis indicated that the sequence from the 7-year-old girl was similar to, but distinct from, the 2007 isolate from India, whereas the sequence from the intern was closer to that of the 2004 isolate from Bangladesh (NiV/BD/HU/2004/RA1; accession no. AY988601). The N sequence obtained from the 10-year-old girl from the initial cluster was shown to be slightly more similar to the 2007 isolate from India (Figure 1, panel A). We were only able to amplify the complete N ORF from the throat swab samples from the 7-year-old and 10-year-old girls because our rRT-PCR indicated the presence of ≈10^3 to 10^4 copies of NiV N RNA (cycle threshold ≈26–30). The rRT-PCR conducted on the throat swab sample from the medical intern indicated the presence of ≈10^6 copies of NiV N RNA (cycle threshold ≈20), which corroborated

Table 1. Results from patients with confirmed Nipah virus infection, Bangladesh, 2008–2010*

			Serologic result		RT-PCR result		
Patient no.	Year isolated	Case type	IgM	IgG	Conventional	Real-time	Virus isolation
1	2008	Cluster	+	+	–	–	–
2	2008	Cluster	+	–	–	+	–
3	2008	Cluster	–	–	+	+	+
4	2008	Cluster	+	–	+	+	+
5	2008	Cluster	+	+	–	–	–
6	2009	Isolated	+	+	NA	NA	NA
7	2009	Isolated	+	–	NA	NA	NA
8	2009	Isolated	+	–	NA	NA	NA
9	2009	Isolated	+	–	NA	NA	NA
10	2010	Cluster	+	–	NA	NA	NA
11	2010	Cluster	+	–	+	+	–
12	2010	Cluster	+	–	–	+	–
13	2010	Cluster	+	–	NA	NA	NA
14	2010	Cluster	+	+	NA	NA	NA
15	2010	Isolated	+	–	–	+	–
16	2010	Isolated	+	+	+	+	–
17	2010	Isolated	+	–	+	+	–
18	2010	Isolated	+	–	NA	NA	NA
19	2010	Isolated	+	–	NA	NA	NA
20	2010	Isolated	+	–	NA	NA	NA
21	2010	Isolated	+	–	NA	NA	NA

*RT-PCR, reverse transcription PCR; +, positive; –, negative; NA, sample not available.

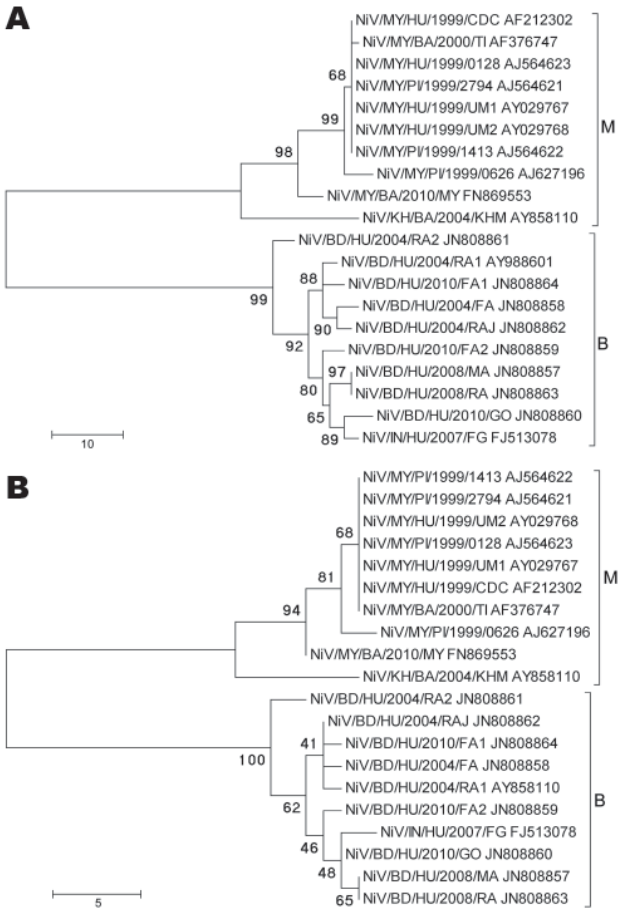

Figure 1. Phylogenetic analyses of sequences from the complete Nipah virus N ORF (A) and the 729-nt proposed N ORF genotyping window (B). Tree created with maximum parsimony, close-neighbor-interchange algorithm, 1,000 bootstrap replicates (*16*). Branch lengths are in units of number of changes over the whole sequence. Available GenBank accession numbers are shown for corresponding sequences. Proposed genotype groupings are indicated by brackets (M, B). ORF, open reading frame; MY, Malaysia; KH, Cambodia; BD, Bangladesh; IN, India; HU, human; PI, pig; BA, bat. Scale bars indicate number of sequence changes corresponding to illustrated branch length.

our ability to amplify nearly the entire genome except for the 3′ leader and 5′ trailer (data not shown) from this sample.

Since the initial molecular characterization of NiV from Bangladesh in 2004 (*11*), there has been a shortage of full-length NiV ORF sequences from Bangladesh. However, the sequence data obtained from the 2008 and 2010 Bangladesh outbreaks in this study, along with the recent characterization of the 2007 isolate from India (*4*), support the previous observation of relative heterogeneity among NiV nucleotide sequences from humans affected by outbreaks in Bangladesh compared with sequences from Malaysia (*11*). Phylogenetic analysis indicated that these new sequences from Bangladesh and India group substantially closer to the sequences from Bangladesh in 2004, which led us to propose a system to describe the distinct lineages of NiV (Figure 1; Figure 2, panels A–E). We propose to designate the current sequences obtained from Malaysia (MY) and Cambodia (KH) as genotype M and the sequences obtained from Bangladesh and India as genotype B. By using a 729-nt window in the N terminal region of the N gene ORF (N ORF nt 123–852, NiV genome positions 236–964), we were able to determine 25 distinct nucleotides that universally differentiated the genotypes (Figure 1, panel B). The topology of the phylogenetic tree and the positions of the branches generated from this smaller nucleotide window were similar to those of the tree generated with the full-length N ORF sequences and have reasonably high bootstrap values at the root branch junctions, albeit with lower bootstrap values at the distal branch junctions (Figure 1, panels A, B). In support of this scheme, we observed similar topologies and branching patterns in phylogenetic trees generated for the complete P, M, F, G, and L ORFs, all with strong bootstrap values (Figure 2, panels A–E).

Pairwise sequence comparisons conducted across each individual NiV gene ORF indicated a nucleotide variation range of 6.32%–9.15% between genotype M and B viruses and an amino acid variation range of 1.42%–9.87% (Table 2; online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1492-Techapp.pdf). The ranges of nucleotide and amino acid variation of sequences within genotype M were 0.19%–2.21% and 0.18–3.67%, respectively, and within genotype B were 0.28%–1.06% and 0.28% –0.56%, respectively. The apparently higher levels of variation found among ORFs within genotype M is mostly caused by the comparatively divergent sequences obtained from *Pteropus vampyrus* (NiV/MY/BA/2010/MY; accession no. FN869553) and *P. lylei* (NiV/KH/BA/2004/KHM; accession nos. AY858110, AY858111) bats. Not only is the proposed genotyping scheme supported by consistent phylogenetic tree topologies, but pairwise nucleotide comparisons of the 729-nt region yield similar percentages of variability as seen in the full-length N ORF comparisons. This finding indicates that this sequence window is a relatively accurate indicator of overall nucleotide variability within and across genotypes M and B (online Technical Appendix Figure 1, panels A, C).

A comprehensive amino acid alignment of currently available complete N protein ORFs indicated that the 4 residues that distinguish between genotype M and B viruses are almost all located in the COOH-terminus (Table 3). Of these residues, only 1 (position 387) is located within the putative minimum contiguous sequence required for capsid assembly (*17*), and none were located in the 29

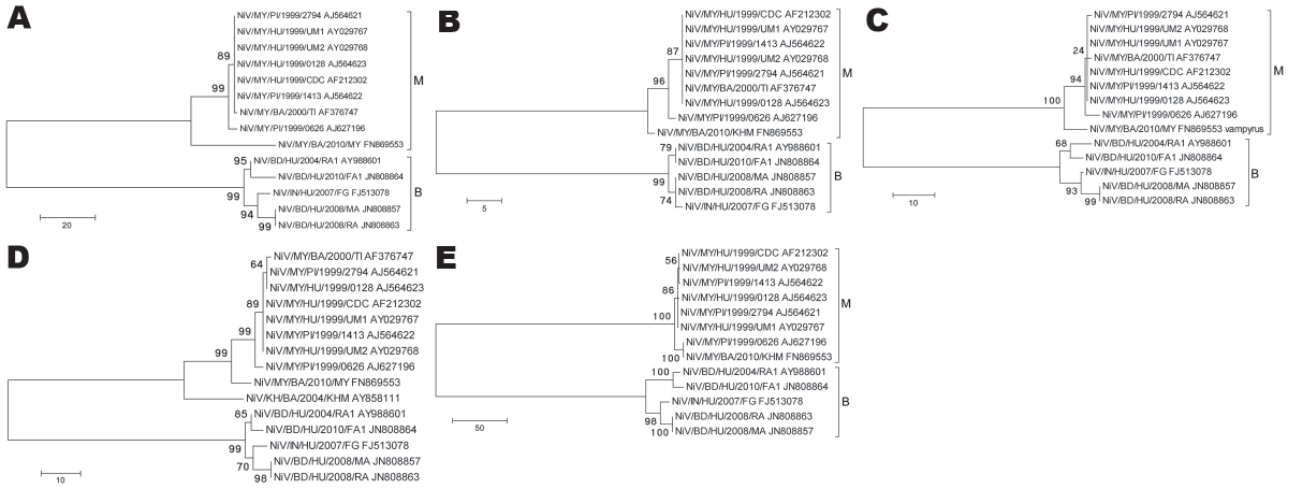

Figure 2. Phylogenetic analyses of sequences from the complete Nipah virus P ORF (A), M ORF (B), F ORF (C), G ORF (D), and L ORF (E). Tree created with maximum parsimony, close-neighbor-interchange algorithm, 1,000 bootstrap replicates (*16*). Branch lengths are in units of number of changes over the whole sequence. Available GenBank accession numbers are shown for corresponding sequences. Proposed genotype groupings are indicated by brackets (M, B). ORF, open reading frame; MY, Malaysia; KH, Cambodia; BD, Bangladesh; IN, India; HU, human; PI, pig; BA, bat. Scale bars indicate number of sequence changes corresponding to illustrated branch length.

COOH-terminal and 10 NH-terminal residues required for interaction with the P protein (*18*,*19*). Of note, there are 4 residues (V429, E432, D457, and T521) in the COOH-terminal region common to all genotype B sequences that are shared with 2 of the comparatively divergent genotype M sequences. In light of the overall nucleotide and amino acid sequence comparisons, however, the divergent genotype M sequences from the bats still differ substantially from genotype B sequences (Figure 1, panel A; Figure 2, panels A–E; online Technical Appendix Figure 1, panels A, B).

Amino acid alignments of the P protein indicated numerous differences between genotype M and B sequences in the first 400 residues, which comprise the shared N terminal region between the P, V, and W proteins. Of the differences in this region, there were neither changes that would be predicted to alter the STAT-1 binding ability of the P, V, and W proteins nor changes that could adversely affect RNA replication (*20*–*22*). There were only 4 changes in the COOH-terminal region of P, which is required for direct N–P interactions, 2 of which were nonconservative changes (N590→S, E635→G) (*18*). The P sequence derived from *P. vampyrus* bats had an intriguing sequence of amino acids from residues 408–440, in which there was substantial sequence divergence from genotypes M and B at the nucleotide and amino acid levels (*23*). These particular nucleotide changes in the P sequence also introduced several amino acid changes in the unique COOH-terminal regions of the V (11 changes) and W (9 changes) ORFs, which distinguish them from any genotype M and B sequences.

We observed the M protein to be highly conserved across genotypes M and B, and we found just 2 aa residues exclusive to genotype B that are not located in any region of the protein with a known function, such as budding (*24*,*25*), nuclear localization, or ubiquitination (*26*). In the F protein, the predicted cleavage site, F1 amino-terminal domain, transmembrane domain, and predicted N-linked glycosylation sites are all conserved across both genotypes. Although the percentage of amino acid variation in the G protein is higher than that in all other NiV proteins (except the P protein), it is not surprising that the residues implicated in Ephrin B2 and B3 binding are conserved across the genotypes (*27*,*28*). The amino acid differences between genotypes M and B sequences are predominantly found

Table 2. Percentage nucleotide and amino acid variability among available complete Nipah virus open reading frame sequences

Gene	Open reading frame length, nt/aa	% nt variation			% aa variation		
		Overall	Genotype M	Genotype B	Overall	Genotype M	Genotype B
N	1,599/532	0.0–6.32	0.0–2.19	0.0–1.06	0.0–2.26	0.0–1.69	0.0–0.56
P	2,130/709	0.0–9.15	0.0–2.21	0.0–0.99	0.0–9.87	0.0–3.67	0.0–0.99
M	1,059/352	0.0–6.70	0.0–0.57	0.0–0.28	0.0–1.42	0.0–0.85	0.0–0.28
F	1,641/546	0.0–6.76	0.0–0.85	0.0–0.98	0.0–1.65	0.0–0.75	0.0–0.55
G	1,809/602	0.0–7.35	0.0–1.93	0.0–0.55	0.0–4.65	0.0–1.83	0.0–0.33
L	6,735/2244	0.0–6.68	0.01–0.19	0.0–0.82	0.0–1.92	0.0–0.18	0.0–0.45

Table 3. Amino acid differences among available complete Nipah virus N gene open reading frame sequences*

Sequence and accession no.	Amino acid position																						
	G	30	139	188	211	318	345	380	381	387	414	429	432	436	457	502	505	506	508	511	518	521
NiV/MY/HU/1999/CDC, AF212302	M	T	S	E	Q	I	M	N	R	D	K	I	G	I	N	I	R	T	G	E	L	A
NiV/MY/PI/1999/1413, AJ564622	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MY/PI/1999/2794, AJ564621	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MY/PI/1999/0626, AJ627196	M	.	R	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MY/HU/1999/0128, AJ564623	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MY/HU/1999/UM1, AY029767	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MY/HU/1999/UM2, AY029768	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MY/BA/2000/TI, AF376747	M	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MY/BA/2010/MY, FN869553	M	.	.	.	.	.	.	.	.	.	.	V	E	.	D	.	.	.	.	.	.	.
NiV/KH/BA/2004/KHM, AY858110	M	.	.	.	.	.	.	.	.	.	.	V	E	.	D	T	.	.	.	G	P	T
NiV/BD/HU/2004/1, AY988601	B	.	.	D	.	.	.	.	.	N	.	V	E	.	D	.	K	D	R	.	.	T
NiV/BD/HU/2004/FA, JN808858	B	.	.	.	.	.	.	.	.	N	N	V	E	M	D	.	K	D	R	.	.	T
NiV/BD/HU/2004/RA2, JN808861	B	.	.	.	.	.	.	.	.	N	.	V	E	.	D	.	K	D	R	.	.	T
NiV/BD/HU/2004/RAJ, JN808862	B	.	.	.	.	.	.	I	.	N	.	V	E	M	D	.	K	D	R	.	.	T
NiV/BD/HU/2008/MA, JN808857	B	.	.	.	.	.	.	.	.	N	.	V	E	.	D	.	K	D	R	.	.	T
NiV/BD/HU/2008/RA, JN808863	B	.	.	.	.	.	.	.	.	N	.	V	E	.	D	.	K	D	R	.	.	T
NiV/BD/HU/2010/FA1, JN808864	B	.	.	.	.	.	.	.	K	N	.	V	E	.	D	.	K	D	R	.	.	T
NiV/BD/HU/2010/GO, JN808860	B	.	.	.	.	V	.	.	.	N	.	V	E	.	D	.	K	D	R	.	.	T
NiV/BD/HU/2010/FA2, JN808859	B	.	.	.	.	.	.	.	.	N	.	V	E	.	D	.	K	D	R	.	.	T
NiV/IN/HU/2007/FG, FJ513078	B	.	.	.	R	.	.	.	.	N	.	V	E	.	D	.	K	D	R	.	.	T

*Dots indicate sequence identity with AF212302. NiV, Nipah virus; MY, Malaysia; HU, human; G, genotype classification; genotype M, sequences from Malaysia and Cambodia; genotype B, sequences from Bangladesh and India; T, threonine, S, serine; E, glutamate; Q, glutamine; I, isoleucine; M, methionine; N, asparagine; R, arginine; D, aspartate; K, lysine; G, glycine; V; valine; P, proline; PI, pig; BA, bat; KH, Cambodia; BD, Bangladesh; IN, India.

at residues that are distant from the receptor binding site. Two differences were found in the intracellular domain, 3 differences in the stalk region (positions 72–182), 9 differences in a span of ≈100 aa (positions 236–344) along the side of the globular head domain, 4 differences closer to the top of the globular head domain (positions 385–424), and only 2 differences (positions 498 and 502) that were close to the tryptophan residue at position 504, which is part of the receptor-binding pocket. As in other NiV proteins, several amino acids were shared between genotype B sequences and 2 genotype M sequences derived from the bat isolates. The significance of these changes has yet to be explored.

The level of amino acid conservation throughout the L proteins was high; the purported GDNE catalytic site and the K-X_{21}-GEGSG ATP binding site were conserved across genotypes M and B. Most distinct differences between genotypes M and B sequences (26 of 32) were located outside the 6 linear domains typically found in nonsegmented negative strand virus polymerases (*29*,*30*). The cis-acting control sequences in NiV are usually well conserved. The tri-nucleotide intergenic sequences amplified from the throat swab sample from the medical intern in 2010 had GAA for all 6 intergenic regions, which was identical to the 2007 isolate from India. For the 2008 isolates, the intergenic sequence between the N and P

genes was AAA, and the rest of the intergenic sequences were GAA. The biological implications of finding adenosine in the first position of NiV intergenic sequences is unknown. The 3′ leader and 5′ trailer sequences of the 2008 NiV isolates were identical to those found in the 2004 Bangladesh and 2007 India isolates.

Discussion

From the initial outbreak of NiV in Malaysia until now, there has not been a standard method by which to classify NiVs. With the accumulation of sequences from subsequent human outbreaks in Bangladesh and India, along with an increasing number of bat-derived sequences, we propose a standardized genotyping method for NiV. The goal behind a genotyping scheme is to classify viruses by using a smaller sequence window that has levels of sequence variability that correspond to variability between complete genomes and that would give the same phylogenetic tree topology with high bootstrap values. Genotyping schemes for other paramyxoviruses, such as measles virus and mumps virus, have been delineated (*31*,*32*).

Before this study, there has been a growing body of partial-sequence data obtained from a 357-nt region coding for the COOH-terminus of N (NiV genome positions 1197–1553) (*23*,*33*). Although obtaining sequence information from this window has the advantage of tracking more variability at the nucleotide and the amino acid levels, it could potentially overestimate the level of variability between sequences within and across genotypes. Pairwise nucleotide sequence comparisons performed by using the 357-nt window estimate the overall sequence variation at ≈8%, whereas the sequence variation of the complete N ORF is ≈6% (Table 2; online Technical Appendix Figure 1, panels A, D). In particular, the 357-nt window overestimates the variability within genotype M of the *P. vampyrus* bat sequence at ≈2%, whereas variability of the sequence within genotype M is <1% when the complete N ORF is taken into account. The 729-nt window in the N terminal region proposed in this study would serve as a more conservative scheme for genotyping because it has little amino acid variation but has nucleotide variability of ≈5.5% according to pairwise comparisons between the 2 proposed genotypes, which only slightly underestimates the variability among sequences for the complete N ORF (online Technical Appendix Figure 1, panels A, C). As with other genotyping schemes that facilitate the classification of viruses, our proposed scheme is amenable to corrective measures as warranted by evidence from sequences obtained from future outbreaks and bat surveillance studies.

In summary, we conducted a comprehensive molecular phylogenetic analysis of currently available complete NiV gene ORFs at the nucleotide and amino acid levels, including newly obtained sequence data from NiV outbreaks in Bangladesh in 2008 and 2010. Analyses of the combined sequence data obtained from Bangladesh and India in the past decade led us to propose a genotyping scheme based on a 729-nt window of the NiV N ORF. This genotyping scheme provides a simple and accurate way to classify current and future NiV sequences.

M.K.L. was supported by an American Society for Microbiology postdoctoral fellowship. Financial support for this research came from CDC core funding, ICDDR,B, and US National Institutes of Health, grant no. 07-015-0712-52200 (Bangladesh-NIH/EID).

Dr Lo is a microbiologist with the Viral Special Pathogens Branch at CDC. His research interests include the molecular pathogenesis and epidemiology of Nipah virus.

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Unsuspected Dengue and Acute Febrile Illness in Rural and Semi-Urban Southern Sri Lanka

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Dengue virus (DENV), a globally emerging cause of undifferentiated fever, has been documented in the heavily urbanized western coast of Sri Lanka since the 1960s. New areas of Sri Lanka are now being affected, and the reported number and severity of cases have increased. To study emerging DENV in southern Sri Lanka, we obtained epidemiologic and clinical data and acute- and convalescent-phase serum samples from patients ≥2 years old with febrile illness. We tested paired serum samples for DENV IgG and IgM and serotyped virus by using isolation and reverse transcription PCR. We identified acute DENV infection (serotypes 2, 3, and 4) in 54 (6.3%) of 859 patients. Only 14% of patients had clinically suspected dengue; however, 54% had serologically confirmed acute or past DENV infection. DENV is a major and largely unrecognized cause of fever in southern Sri Lanka, especially in young adults.

Dengue virus (DENV), with 4 antigenically distinct serotypes (DEN1–4), is the most common cause of arboviral disease; ≈50 million cases of dengue occur annually in >100 countries (*1*). Manifestations of dengue infection range from asymptomatic or mild febrile illness to circulatory failure and death from dengue hemorrhagic fever (DHF). Urbanization and geographic expansion of the primary vector for DENV, *Aedes aegypti*, have fueled the current global dengue pandemic (*2*).

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DOI: http://dx.doi.org/10.3201/eid1802.110962

DENV has been documented in the Indian subcontinent since the 1960s, and there are recent reports of DENV in new areas and of severe disease (*2*,*3*). Epidemic dengue was first recognized in Colombo, the capital of Sri Lanka, in 1965–1966 (*4*). Outpatient, clinic-based surveillance at Colombo's Lady Ridgeway Children's Hospital during 1980–1984 found dengue accounted for 16% of acute febrile illness, among which 66% were secondary (recurrent) dengue cases. A 1980–1985 school-based study found a baseline DENV seroprevalence of 50% in Colombo and a 6-month dengue incidence of 15.6%, of which 37% were secondary cases (*4*). In the early 1980s, severe dengue was rare in Sri Lanka: <10 reported cases were DHF (*4*). However, since 1989, many cases of DHF have been reported from the heavily urbanized western coastal belt of Sri Lanka, which includes Colombo (*5*), and cases have recently been reported elsewhere in the country.

DENV began emerging in southern Sri Lanka recently. To define the epidemiology of dengue in this previously unstudied region, we prospectively enrolled patients seeking care for acute febrile illness at a local hospital. Study participants lived in Galle, a seaport city (population 100,000), and the surrounding coastal plain and heavily vegetated foothills at the southernmost tip of the island nation. During the study months, the temperature ranged from highs of 27.5°C to 32°C and lows of 24°C to 26°C, and rainfall was variable (mean 301 mm, range 36–657 mm).

Materials and Methods

Febrile Cohort

During March–October 2007, we recruited patients in the emergency department, acute care clinics, and

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adult and pediatric wards of Teaching Hospital Karapitiya in Galle, the largest (1,300-bed) hospital in southern Sri Lanka. Consecutive febrile (≥38°C tympanic) patients ≥2 years old without trauma or hospitalization within 7 days were eligible for study participation. Study doctors verified eligibility and willingness to return for convalescent-phase follow-up and obtained written consent from patients or from parents (for patients <18 years old) and assent from those ≥12–17 years old. The institutional review boards of the University of Ruhuna, Johns Hopkins University, and Duke University Medical Center approved the study.

Study personnel recorded epidemiologic and clinical data on a standardized form. Study doctors obtained blood for on-site, clinician-requested testing and off-site, research-related testing. Patients returned for clinical and serologic follow-up 2–4 weeks later or were visited in their home if unable to return and their address was known. Blood and serum samples were stored promptly at −80°C and shipped on dry ice to the University of North Carolina School of Medicine (laboratory of A. de S.), where paired serum samples were tested by ELISA and acute-phase serum samples were cultured and tested by PCR.

Serologic Testing for Dengue

IgM ELISA

We performed dengue IgM capture ELISA as described (*6*), except we used anti-flavivirus monoclonal antibody (mAb) 4G2 followed by enzyme-conjugated goat anti-mouse IgG to detect captured DENV antigen. In brief, 96-well plates were coated (overnight, 4°C) with 100 μL/well (1 ng/μL) of goat anti-human IgM (Sigma, St. Louis, MO, USA) at a concentration of 0.1 mol/L in carbonate buffer (pH 9.6). Plates were washed 3× in Tris-buffered saline with 0.2% Tween 20 (TBST) and blocked with 200 μL/well of 1× Tris-buffered saline with 0.05% Tween 20 and 3% nonfat dry milk. Paired serum samples were tested on the same plate. Diluted serum (1:50) was loaded in duplicate and incubated (37°C, 1 h) to capture IgM antibody. Unbound antibody was washed, and wells were successively incubated with DENV antigen (mix of serotypes DEN1–4), mouse anti-flavivirus 4G2 mAb, and human-absorbed alkaline phosphatase (AP)–conjugated goat anti-mouse IgG antibody (Sigma). Optical density (OD) was measured at 405 nm after final incubation with AP substrate.

IgG ELISA

Dengue IgG ELISA was performed as described (*7*). Plates were coated overnight (4°C) with 100 μL/well of mouse anti-flavivirus 4G2 mAb at a concentration of 0.1 mol/L in carbonate buffer (pH 9.6) and then washed 3× in TBST. Plates were then blocked with standard diluents and successively incubated (37°C, 1 h) with DEN1–4 antigen, diluted serum (1:100) in duplicate wells, and AP-conjugated goat anti-human IgG (Fc portion), with 3 washings (TBST) between incubations. Plates were read at 405 nm after a final incubation with AP substrate (15 min, room temperature, in the dark).

Serologic Interpretation

We defined acute dengue as IgG seroconversion (acute-phase OD <0.20 and convalescent-phase OD ≥0.20) or as a substantial increase in antibody titer (convalescent-phase IgG OD ≥0.30 or IgM OD ≥0.20 than acute-phase). Acute primary (first episode) and acute secondary (recurrent) dengue were distinguished by the absence or presence of IgG (OD <0.35 and ≥0.35, respectively) in acute-phase serum samples. The presence of IgG without a substantial increase in titer defined past dengue infection. Seroprevalence was defined as the presence of IgG (OD >0.20) in acute-phase serum samples; other samples were seronegative. For each ELISA, we used 2 negative control human serum samples, each tested in duplicate. Positive cut-off values were determined during assay validation and were based on the mean OD +2 SD for negative control serum specimens.

Isolation of Dengue

Individual 15-μL aliquots of undiluted acute-phase serum (from all patients with confirmed acute dengue as well as negative and positive controls) were each mixed with 185 μL of Eagle minimal essential medium supplemented with 2% fetal bovine serum, and then each was added to C6/36 cells (27°C, 5% CO_2). After 7 and 10 days, cells were fixed and a direct immunofluorescent antibody assay was performed by using anti-dengue mAb (2H2-Alexa488) (*8*). Positive samples were centrifuged (1,500 × *g*, 5 min). Supernatants containing virus were supplemented with 20% fetal bovine serum, divided into aliquots, and preserved at −80°C.

Dengue Virus Neutralization

We tested the convalescent-phase serum samples from 35 patients with serologically defined acute dengue. Dengue neutralizing antibodies were detected by using a flow cytometry–based assay as described (*9*). In brief, serially diluted immune serum samples were incubated with each of the 4 dengue serotypes (37°C, 1 h). Next, the virus and serum mixture was added to a human monocyte cell line (U937DC-SIGN) that was engineered to express the dengue receptor DC-SIGN and incubated with 5% CO_2 (37°C, 24 h). The cells were washed, fixed, and stained with Alexa 488–conjugated anti-dengue mAb 2H2. The percentage of infected cells was measured by flow cytometry. GraphPad Prism 4 for Windows (www.graphpad.com/welcome.htm)

and nonlinear regression analysis were used to calculate 50% neutralization values.

PCR for DENV

To yield cDNA, we used RNA eluted serum samples (QIAmp Viral RNA Mini Kit; QIAGEN, Valencia, CA, USA) from 25–140 μL of acute-phase serum samples, reverse transcriptase (RT), and DENV downstream consensus primer D2 or random primers. To confirm and serotype DENV, we used consensus primers D1 and D2 to amplify cDNA by standard PCR; positive first-round PCR products were diluted 1:100 and used as template in second-round PCR with consensus primer D1 and nested serotype DEN1–4 type-specific primers (*10*). Negative controls were included for the RT and PCR steps. The negative control for the RT step consisted of a sample to which all reagents, except RNA, were added. The negative control step for PCR consisted of a sample to which all reagents, except cDNA, were added.

Statistical Analysis

Proportions were compared by the χ^2 test or Fisher exact test and continuous variables by Student *t* test or the rank sum test, if not normally distributed. Analyses were performed by using Stata IC 11.0 (StataCorp LP, College Station, TX, USA).

Results

Febrile Cohort

Paired serum samples to identify acute and past dengue were available for 859 (79.6%) of 1,079 patients enrolled. The likelihood of follow-up did not differ by age (p = 0.30), sex (p = 0.22), or level of education (p = 0.74). Most (90.2%) patients reported rural residence, and follow-up was more likely among rural (80.6%) than urban (69.5%) dwellers (p = 0.008). The reported duration of fever and illness was similar in patients who did and did not return for follow-up.

Among the 859 patients with paired serum samples, 61.2% were male, and the median age was 30.7 years (interquartile range [IQR] 19–48 years), which did not differ by sex (p = 0.97). The median reported duration of fever and of illness before seeking medical care was 3 days (IQR 2–5 and 2–7 days, respectively). Many patients (36.3%) reported having taken an antimicrobial drug for the illness. The median time between acute-phase and convalescent-phase follow-up was 21 days (IQR 15–32 days).

Acute Dengue

Acute dengue occurred during each month of the study and accounted for 3.0% (May) to 11.1% (October) of acute febrile illnesses. Fifty-four patients (6.3%) had acute dengue (27 primary and 27 secondary infections). Of patients ≥18 and <18 years old, 48 (7.0%) and 6 (3.5%), respectively, had acute dengue (p = 0.09). The age distribution of patients with and without acute dengue is shown in Figure 1. The median age of patients with acute dengue was 27.6 years (IQR 22–45 days), and a similar proportion were male versus female (p = 0.26).

The clinical features of patients with and without acute dengue are detailed in Table 1. Headache was the most frequent (75.9%) symptom, and lethargy and muscle and joint pain were also reported by >50% of patients with dengue; however, these symptoms were just as frequent in patients without dengue. Patients with dengue were less likely than those without it to report cough and sore throat and to have lymphadenopathy, but they were more likely to have conjunctivitis. Although gastrointestinal symptoms and signs were uncommon overall, diarrhea, jaundice, hepatomegaly, and abdominal tenderness occurred more frequently in dengue patients. Patients with acute dengue had statistically significantly lower leukocyte and platelet counts than patients without dengue. No dengue patients had petechiae.

Only 3 acute dengue patients (men 23–27 years old; 2 with secondary and 1 with primary infection) had platelet

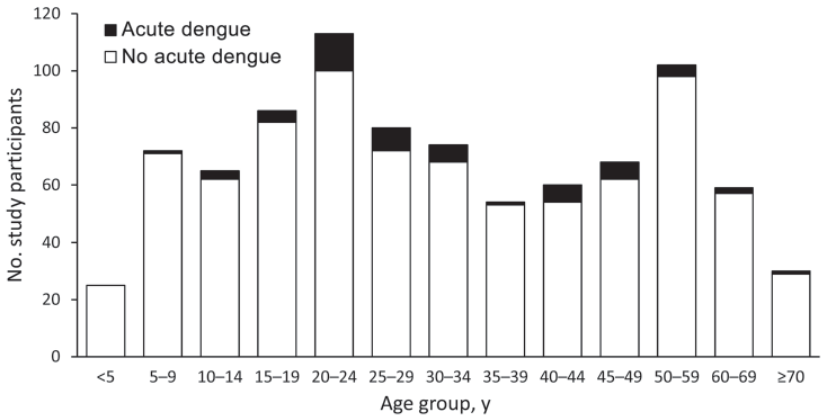

Figure 1. Age distribution of patients with and without acute dengue, southern Sri Lanka, 2007.

Table 1. Clinical characteristics of febrile patients with and without acute dengue, southern Sri Lanka, 2007*

Characteristic	Acute dengue, n = 54	Not acute dengue, n = 805	p value
Symptoms			
Headache	75.9	78.1	0.70
Sore throat	13.0	29.3	0.01
Cough	35.2	58.5	0.001
Dyspnea	15.1	16.7	0.76
Joint pain	50.9	44.3	0.35
Muscle pain	57.7	48.7	0.21
Lethargy	68.6	66.6	0.76
Abdominal pain	25.0	19.0	0.29
Emesis	43.4	36.9	0.35
Diarrhea	22.2	10.7	0.01
Dysuria	16.7	14.4	0.65
Oliguria	14.8	9.1	0.17
Signs			
Mean temperature, °C, ± SD	38.5 ± 0.70	38.4 ± 0.61	0.66
Mean heart rate, beats/min, ± SD	85 ± 17	84 ± 16	0.57
Mean systolic blood pressure, mm Hg, ± SD	114 ± 16	111 ± 16	0.32
Mean diastolic blood pressure, mm Hg, ± SD	72 ± 16	73 ± 11	0.58
Conjunctivitis	27.8	14.0	0.006
Pharyngeal erythema or exudates	5.6	14.0	0.08
Lymphadenopathy	3.7	24	0.001
Jaundice	7.6	1.4	0.001
Lung crackles	5.7	14.2	0.08
Tender abdomen on palpation	18.5	9.4	0.03
Hepatomegaly	11.3	5.0	0.05
Swollen joint	1.9	0.5	0.27
Rash	5.6	2.3	0.14
Laboratory parameters			
Leukocytes/μL, median (IQR)	5,300 (3,600–12,000)	8,000 (5,800–11,100)	0.009
ANC/μL, median (IQR)	3,600 (2,304–8,560)	5,396 (3,534–7,980)	0.03
ALC/μL, median (IQR)	1,602 (1,044–2,400)	2,085 (1,540–2,829)	0.002
Hemoglobin, g/dL, mean ± SD	12.9 ± 1.6	12.6 ± 1.7	0.25
Platelets, × 1,000/μL, mean (IQR)	190 (156–242)	232 (190–293)	0.0002

*Values are percentages unless otherwise stated. Leukocytes, white blood cell count; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; IQR, interquartile range.

counts consistent with DHF (<100,000/μL) (*11*). At admission, 2 of the 3 men reported myalgia and arthralgia; all 3 had headache, leukopenia (leukocytes 2,400–3,600 cells/μL), severe thrombocytopenia (platelets 19,000–47,000/μL), and compensated shock (blood pressure 100–110/70–80 mm Hg, with heart rate 88–100 beats/min). Additional laboratory testing only included determination of transaminase levels, which were elevated in the 1 patient in whom they were evaluated. All 3 patients were well at follow-up.

Patients with acute dengue were more likely than those with other causes of fever to be hospitalized (92.6% vs. 71.3%; p = 0.001), but their duration of stay was similar (median 5 vs. 4 days [IQR 3–7 and 3–6 days, respectively]; p = 0.23). A presumptive clinical diagnosis was available for 791 patients, including 50 with acute dengue. Few adults and children were suspected to have acute dengue on the basis of clinical features (3.3% vs. 1.2%, respectively; p = 0.16), and only 7 of the 23 patients (all adults) with clinically suspected dengue had laboratory-confirmed infection (positive predictive value 30.4%, 95% CI 27.2–33.6). Clinical diagnosis also had poor sensitivity (14.0%; 95% CI 11.6–16.4) and high specificity (97.8%; 95% CI 96.8–98.9). Compared with other patients, those with suspected dengue were more likely to be admitted to the hospital (70.4% vs. 95.7%; p = 0.008) and less likely to be treated with antimicrobial drugs (72.6% vs. 20.0%; p = 0.02). However, 50.0% of those with confirmed acute dengue reported taking antimicrobial drugs before seeking medical care.

Clinical illness was similar in patients with primary and secondary dengue. Median platelet counts were 186,000/μL (IQR 153,000–234,000/μL) and 198,000/μL (IQR 160,000–270,000/μL) for patients with primary and secondary dengue, respectively (p = 0.60). Diarrhea was more common in patients with secondary than with primary dengue (33.3% vs. 11.1%; p = 0.05), as were jaundice (14.8% vs. 0.0%; p = 0.04) and hepatomegaly (22.2 vs. 0.0%; p = 0.01). The duration of illness before

patients sought care was similar for those with primary and secondary dengue. Patients with primary dengue were as likely as those with secondary dengue to be admitted to the hospital (both 92.6%; p = 1.0) and to remain for a similar duration (median 5 days for both [IQR 3–6 and 3–8 days, respectively]; p = 0.89).

Acute or Past Dengue

Fifty-four percent (464/859) of the study cohort had either acute or past dengue (see Table 2 for patient characteristics). The overall seroprevalence was 50.9% (437/859 patients) because the 27 patients with acute primary infections were seronegative at enrollment. The proportion of patients who were seropositive at enrollment increased in each older age group, from 9% in those <5 years old to 72% in those 40–44 years old (Figure 2). Overall, seropositivity was more likely in male than in female patients (55.9% vs. 42.9%; p<0.0001), and in urban than in rural dwellers (75.3% vs. 52.0%; p<0.0001).

Confirmation of Acute Dengue and Serotyping of Virus

We isolated DENV from 12 (22.2%) of the 54 acute dengue patients. The likelihood of isolating DENV was higher when the patient had fewer reported days of fever (median 3 vs. 4 days; p = 0.04), and DENV was isolated almost exclusively from patients with primary versus secondary dengue (91.7% vs. 8.3%; p = 0.001).

Dengue PCR results were positive in 19 (35.2%) patients; positive results were higher among patients with positive versus negative culture results (75.0% vs. 23.8%; p = 0.001). Fever duration was similar in patients with dengue-positive and -negative PCR results (median 4 days; p = 0.22). A positive PCR result was not statistically more likely in patients with primary versus those with secondary dengue (57.9% vs. 42.1%; p = 0.39). PCR testing confirmed serotype DEN2 (2 primary cases), D3 (8 primary, 7 secondary), and D4 (1 primary, 1 secondary); no cases of serotype DEN1 were identified. DENV neutralization testing confirmed the specific presence of dengue antibodies in 33 (94.3%) of 35 IgG-positive serum samples (95% CI 80.8–99.3).

Discussion

We sought to define the epidemiology of dengue in the southern tip of Sri Lanka. We prospectively enrolled patients with a reproducible criterion (documented fever) and rigorously distinguished acute from past infections by using paired serum samples. The seroprevalence of dengue in our cohort increased with age; however, half of those 20–25 years old were seronegative. In contrast, 50% of children in Colombo are positive for dengue IgG antibody by 5 years of age, and >70% have been found to be seropositive by 12 years of age (*4*,*12*). The high proportion of susceptible adults in Galle suggests the recent emergence of DENV in this area (*4*,*5*).

Table 2. Demographic characteristics of febrile patients with or without evidence of dengue virus infection, southern Sri Lanka, 2007*

Characteristic	Acute dengue, n = 54†	Past dengue, n = 410†	Not dengue, n = 395†	p value
Median age, years (IQR)	27.6 (22.4–44.5)	41.3 (27.0–53.4)	21.7 (13.3–35.3)	0.0001
Male	68.5	66.3	54.9	0.002
Residence				0.001
Rural	86.8	88.2	95.4	
Urban	13.2	11.8	4.6	
Type of work				<0.001
Home	11.5	25.9	27.8	
Laborer	19.2	32.4	18.3	
Farmer, e.g., rice paddy	5.8	3.0	2.3	
Merchant	3.9	4.0	2.1	
Student	13.5	7.3	37.8	
Other	46.2	27.4	11.8	
Swim/bathe/wade in freshwater				0.63
None	74.1	73.1	76.4	
River	20.4	13.5	12.2	
Paddy field	3.7	10.8	9.1	
Pond/lake	0	1.0	1.0	
Other	1.9	1.7	1.3	
Water source				0.003
Tap	42.6	35.1	25.5	
Boiled	3.7	8.8	10.2	
Well	51.9	54.9	64.4	
Other	1.9	1.2	0	

*Except for median age, data are percentages. IQR, interquartile range.
†Values are percentages unless otherwise stated.

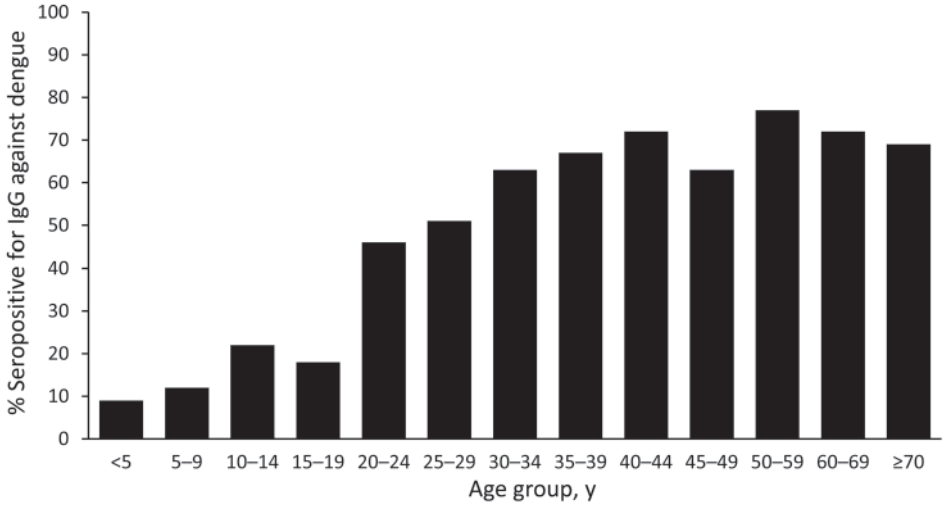

Figure 2. Presence of IgG against dengue in febrile patients, by age, southern Sri Lanka, 2007.

Dengue is considered an urban and peri-urban disease, and the current pandemic is partly attributed to increasingly populous cities (*2*). We documented cases of acute dengue during March–October in rural and semi-urban areas. In rural Cambodia, dengue is a major cause of hospitalization and death among children, with delays in recognition and care-seeking contributing to its impact in rural areas (*13*,*14*). Discarded water storage jars and concrete water tanks are commonly used breeding sites for the vector (*15*), and mosquito populations may be higher in rural than urban areas (*16*). Dengue has also been documented in rural Vietnam (*17*). Control efforts in urban Sri Lanka have included covering cement water tanks, which are established breeding sites for *Aedes* spp. *(18)*; however, data to direct control efforts in Southern Sri Lanka are lacking.

We describe the clinical manifestations of endemic dengue in an unselected febrile cohort in southern Sri Lanka. Dengue accounted for 6.3% (54/859) of acute febrile illnesses in this cohort; serotypes DEN2–4 caused illness, with DEN3 being the most frequent. In the cohort (predominantly adults who sought care early in illness), manifestations of acute dengue were similar to those of other causes of fever, except for more diarrhea, jaundice, and abdominal tenderness and less cough and sore throat. Ramos et al. (*19*) similarly identified the presence of diarrhea and absence of respiratory symptoms as early features of confirmed acute dengue infection in adults; however, 53.5% of the patients in that study were excluded from analyses because only single serum samples were tested.

We identified a disease spectrum different from that of classic severe dengue reported in hospital-based studies during epidemics in urban centers (Colombo and Kandy) in Sri Lanka (*20*–*22*); the spectrum we report is similar to that described in studies of acute febrile illness elsewhere in Asia. In a report from Singapore, headache, retro-orbital pain, arthralgia, anorexia, nausea, and vomiting were symptoms statistically associated with dengue, but, as in our study, the symptoms were common in their entire febrile cohort (*23*). In the same study, signs more suggestive of dengue were infrequent: red eyes (33.6% of patients), rash (11.2%), and bleeding (7.5%). The only signs we found statistically associated with acute dengue were jaundice, abdominal tenderness, and hepatomegaly. Others have found abnormal test results for liver function to be common among patients with secondary dengue (*24*). That we observed jaundice only in secondary cases lends credence to the hypothesis that host response to the virus may be a major factor in the pathogenesis of dengue (*24*).

Our finding that only 14% of patients with dengue were identified clinically is consistent with the recent emergence of dengue in southern Sri Lanka. In addition, the finding emphasizes the difficulties with clinical diagnosis, particularly in unselected patients with recent onset of fever (median 3 days) in the absence of a recognized epidemic. Clinical acumen is difficult to develop when confirmatory testing is not available, even in a subset of patients. This fact highlights the need for rapid, accurate point-of-care diagnosis (*25*), which could also limit the frequent use of unnecessary antimicrobial drugs.

A limitation of many studies is that diagnosis of acute dengue was made on the basis of a single serum sample. In studies of severe dengue in urban Sri Lanka (*20*,*22*) and in a more recent case-control study of dengue (*26*), acute dengue was defined by a positive result from a PanBio dual IgM/IgG rapid strip test (PanBio Pty Ltd., Brisbane, Australia) (*27*), which was performed on a single serum sample obtained on day 7 of illness. This strategy may be adequate during an epidemic in a patient with classic severe dengue; however, in a setting of high dengue seroprevalence and lower pretest probability (undifferentiated fever, no

recognized epidemic), a single positive serologic test result may more often denote past dengue, and the true cause of illness might remain unrecognized and untreated.

Unique strengths of our study include rigorous confirmation of acute infection by World Health Organization criteria (*28*), a large sample size with 80% follow-up (critical to reference-standard diagnosis and assessment of outcomes), and prospective clinical correlation. To confirm dengue, the World Health Organization requires detection of virus by isolation or PCR or a 4-fold rise in antibody in paired serum samples. Cases with supportive serologic test results (single high antibody titer) are considered probable cases because antibody may not be present early (poor sensitivity) or may represent past infection (poor specificity) (*28*). In our study, we would have identified only secondary cases if a probable case definition had been used because IgG is initially absent in primary dengue. We assessed paired serum samples by using an ELISA comparable to the more difficult and time-consuming hemagglutinin inhibition test used for determining dengue seroprevalence in populations (*7*) and the ELISA used for dengue surveillance in Colombo, Sri Lanka (*12*). A recent systematic review scrutinized clinical studies designed to aid clinicians in resource-poor settings by identifying features predictive of acute dengue (*29*). Most studies had myriad methodologic flaws, including inadequate diagnosis, which made prediction impossible. We prospectively studied unselected patients by using rigorous diagnostic criteria to minimize recall, selection, and diagnostic verification bias.

A limitation of serologic testing is that serologic cross-reactions occur between dengue and other flaviviruses, but we believe this is unlikely to bias our results. West Nile virus has not been described in the region, yellow fever is not present in Asia, and Japanese encephalitis is of low endemicity, with no recent outbreaks reported in the area. Furthermore, we used dengue virus neutralization testing to confirm the specific presence of dengue in a subset of IgG-positive serum samples in addition to performing PCR and viral isolation on the acute-phase serum samples of patients with serologically confirmed acute dengue. The median duration of fever in those in whom virus was isolated is consistent with the reported persistence of viremia for 5–6 days after onset of symptoms (*1*,*30*). Because most patients were adults, we could not compare the clinical features of symptomatic dengue in adults versus those in children.

We have not delineated the full clinical spectrum of dengue, which would require a prospective population-based study. However, we believe that the population studied is representative of patients in the region with clinically noteworthy symptomatic dengue: Teaching Hospital Karapitiya has a large catchment area and is 1 of 2 public teaching hospitals in the Southern Province, and there are no large private hospitals in the area. Patients with fulminant dengue may die before hospital evaluation, but most, including indigent patients from outlying areas, seek care because of free access.

In summary, we found that dengue is responsible for 6.3% of undifferentiated febrile illnesses in southern Sri Lanka. We identified relatively mild disease, despite identifying mostly serotype DEN3 infection, which is difficult to distinguish from other acute febrile illnesses, and we found acute infections were mostly in young adults. We hypothesize that the burden and spectrum of acute dengue is under-recognized in other regions because of limited study and limited tools for rapid diagnosis. Clinical recognition is poor for less severe dengue, particularly, perhaps, when endemicity is low or transmission is sporadic. Assays to measure antibody in single serum samples may be misleading, especially early in illness, and PCR and viral isolation are often unavailable and may be insensitive. Other causes of acute febrile illness require antimicrobial drug therapies, but dengue only requires supportive care; thus, improved low-cost diagnostic tools are urgently needed to guide clinical management.

Acknowledgments

We thank the members of the microbiology laboratory at the Medical Faculty, University of Ruhuna, and the clinical staff at Teaching Hospital Karapitiya for their assistance; P.L. Ariyananda for support of the study; Cynthia Binanay for project management; and Siddharth Srivastava for performing the dengue neutralization tests. We also thank our clinical research team, especially Vathsala Abeygunawardane.

Patient enrollment was supported by the Hubert-Yeargan Center for Global Health and the Duke University Medical Center Chancellor's Tsunami Relief Fund. M.E.R. was supported by a Johns Hopkins Center for Global Health Junior Faculty Grant, a Clinician Scientist Career Development Award from Johns Hopkins School of Medicine, and the National Institute of Allergy and Infectious Diseases, National Institutes of Health (K23AIO83931). A. de S. was supported by a National Institutes of Health Fogarty International Research Award (R03 TW007319).

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Association of Human Bocavirus 1 Infection with Respiratory Disease in Childhood Follow-up Study, Finland

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Human bocavirus 1 (HBoV1) DNA is frequently detected in the upper airways of young children with respiratory symptoms. Because of its persistence and frequent co-detection with other viruses, however, its etiologic role has remained controversial. During 2009–2011, using HBoV1 IgM, IgG, and IgG-avidity enzyme immunoassays and quantitative PCR, we examined 1,952 serum samples collected consecutively at 3- to 6-month intervals from 109 constitutionally healthy children from infancy to early adolescence. Primary HBoV1 infection, as indicated by seroconversion, appeared in 102 (94%) of 109 children at a mean age of 2.3 years; the remaining 7 children were IgG antibody positive from birth. Subsequent secondary infections or IgG antibody increases were evident in 38 children and IgG reversions in 10. Comparison of the seroconversion interval with the next sampling interval for clinical events indicated that HBoV1 primary infection, but not secondary immune response, was significantly associated with acute otitis media and respiratory illness.

Human bocavirus 1 (HBoV1), a new member of the *Bocavirus* genus of the family *Parvoviridae*, was discovered in 2005 by large-scale sequencing in nasopharyngeal samples from children (*1*). HBoV1 DNA has since been frequently detected by PCR in the upper airways of young children who have upper or lower respiratory tract illness (URTI, LRTI) and, less frequently, in their feces (*2*,*3*). Furthermore, 3 other bocaviruses, HBoV2, 3, and 4, were recently detected in human feces (*4*–*6*), and HBoV2 has been associated with acute gastroenteritis (*5*).

HBoV1 in the upper airways also occurs persistently or recurrently in asymptomatic children (*7*–*11*). Because of these characteristics and frequent co-detection with other viruses, the role of HBoV1 in respiratory illness has been questioned. Circumventing the PCR-related problems of prolonged or recurrent positivity and disclosing the association of HBoV1 infection with disease require a more reliable diagnosis that uses serum for PCR and antibody detection (*12*–*16*). By using serology, one can distinguish between primary and secondary HBoV1 infections. We recently detected secondary HBoV1 immunoactivations in immunocompetent adults (*17*), but no data exist on the clinical effects of such events or on their frequency in children. Furthermore, to our knowledge, no prospective studies with reliable diagnostics have been conducted to determine the clinical associations of primary HBoV1 infection.

We determined HBoV1 primary infection in relation to clinical symptoms among constitutionally healthy children who were serologically followed from infancy up to age 13 years. In addition, we investigated the kinetics of HBoV1 viremia and IgG and IgM antibody responses, IgG avidity maturation, and the occurrence and clinical effects of secondary infections or immunoactivations.

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DOI: http://dx.doi.org/10.3201/eid1802.111293

Materials and Methods

Patients and Samples

We conducted this study during 2009–2011. Participants were from the ongoing population-based Diabetes Prediction and Prevention (DIPP) study, a prospective survey of the preclinical events preceding type 1 diabetes among genetically susceptible children in Finland (*18*,*19*). These children, who carry specific human leukocyte antigen (HLA)–DQ genotypes conferring increased susceptibility to type 1 diabetes, were observed from birth for the appearance of diabetes-associated antibodies and viral infections. By the end of 2002, a total of 68,953 newborn children (27,030 in Turku) had been tested for their HLA-conferred risk for type 1 diabetes. From this group, 10,743 (4,391 in Turku) were invited to join the DIPP study, and 8,014 (2,942) of these participated.

The 109 DIPP children in this study were randomly chosen (computer algorithm that gives equal relative amounts of all HLA types studied) from children born during 1995–2002 in Turku, fulfilling the following criteria: 1) to ensure that all their samples were not contaminated or otherwise compromised (e.g., multiple thaws), these children's samples had never been used in any previous studies; 2) participating children had been followed up according to the sampling schedule as promptly as possible; and 3) the children had to be of normal health and did not have type 1 diabetes or any diabetes-related antibodies by the end of 2002. Of these 109 constitutionally healthy children, 56 were girls. We analyzed the children's 1,952 serum samples (mean of 18 samples per child, median 17, range 12–27), obtained from the average age of 3 months (median 0.31 years, range 0.20–0.91 years) to an average of 8 years (median 8.5 years, range 4–13 years), as well as umbilical cord blood samples from 9 selected children. The 109 children were examined at a mean interval of 110 days (median 96 days, range 55–484 days) until age 2 years and subsequently at a mean interval of 197 days (median 182 days, range 92–849 days) until October 2008 (unless they were discontinued earlier). At each examination, a serum sample was drawn, divided into aliquots, and stored at –70°C. All serum samples were tested for IgG and IgM antibodies against HBoV1. HBoV1 IgG avidity and HBoV1 quantitative PCR (qPCR) were conducted on the 3 specimens flanking each serodiagnosis (primary and secondary IgG antibody increases). At each child's scheduled visit, the parents completed a questionnaire and were interviewed by a study nurse about any clinical symptoms or illnesses since the previous visit. Acute otitis media (AOM), sinusitis, tonsillitis and LRTI were diagnosed by a physician. All prescribed antimicrobial drugs were also recorded. Thirty (28%) children had physician-diagnosed allergic diseases, e.g., asthma; diabetes-associated autoantibodies developed in 7 (6%) children, but none of these children progressed to clinical diabetes.

The ethics committee of the Hospital District of Southwest Finland approved the study protocol. The legal guardians of the study participants provided written informed consent.

Enzyme Immunoassays

HBoV1 IgG and μ-capture IgM enzyme immunoassays (EIAs) were conducted as described (*13*) with biotinylated virus–like viral protein 2 particles as the antigen. The diagnostic sensitivity was 97% and specificity 99.5% for these EIAs done in combination (*13*). Our diagnostic criteria for HBoV1 primary infection were seroconversion or PCR positivity in serum occurring for the first time; and for an HBoV1 secondary immunoactivation, a ≥4-fold titer increase in IgG antibodies in 2 adjacent serum samples after seropositivity. We used a protein-denaturing EIA to analyze HBoV1 IgG avidity (*17*). All samples from each child were studied in parallel.

Real-time qPCR

The DNA in 20 μL of serum was extracted by phenol-chloroform, precipitated by sodium acetate and ethanol, and then eluted in 20 μL of 10 mmol/L Tris-Cl buffer (pH 8.0); 5 μL was assayed by using PCR. The HBoV1 nucleoprotein 1 (NP1) gene-based qPCR was performed as described (*12*,*20*) with a Stratagene Mx3005P instrument (Agilent Technologies, Santa Clara, CA, USA). The quantification standard was a plasmid (pSt2; GenBank accession no. DQ000496) containing the HBoV1 NP1 gene comprising serial dilutions covering 7 logs. Water served as negative controls and pSt2 as the positive control. The 3 serum samples from each child, including and flanking the seroconversion or secondary increase(s), were studied in parallel.

Statistics of Clinical Correlates

Any infection-related illnesses—URTI (with fever or ≥2 respiratory symptoms), LRTI, fever without respiratory tract infection, tonsillitis, AOM, conjunctivitis, sinusitis, gastroenteritis (with vomiting or diarrhea), exanthema with fever, and other infection-related illnesses—during the HBoV1 primary infection or during the secondary immune response were compared with illnesses during the previous sample interval and the subsequent interval in each child. One child, for whom complete clinical information was lacking, was excluded. Liddell exact test served for statistical analyses, and p values <0.05 were considered significant.

Additionally, we compared the stability of or decrease in IgG antibodies after conversion in each child during the entire study, with the presence of allergic diseases, diabetes-

associated antibodies, symptoms at HBoV1 primary infection, and with the child's increased susceptibility to infections in general, as defined by the pediatric infectious disease specialists of Turku University Hospital on the basis of clinical features in primary immunodeficiency diseases (*21*), by the number of infection episodes (>10 AOM, >2 acute sinusitis, >1 pneumonia or >1 acute pyelonephritis), tonsillectomy or insertion of tympanostomy tubes because of recurrent infections. We used the Fisher exact test and SAS version 9.2 (SAS Institute, Cary, NC, USA) for these statistical analyses.

Results

HBoV1 Immune Response

A total of 1,961 consecutive serum samples from 109 constitutionally healthy children, including the 9 cord blood samples, were studied for HBoV1 IgG and IgM antibodies. All 109 children were seropositive for HBoV1 by age 6 years (Figure 1). Seven children remained seropositive from birth, and 102 children showed seroconversion at the mean age of 2.3 years (median 2.1, range 0.3–6.0) (Tables 1–3). Ages did not change substantially when calculated according to the mid-point of the seroconversion interval (mean 2.1 years, median 1.9 years, range 0.16–5.7 years). At primary infection of the 102 seroconverters, 53 also showed other markers of HBoV1 infection (Table 2): viremia in 24, IgM antibodies in 28, and low avidity of IgG in 34. In subsequent follow-up, IgG avidity matured in all but 2 children.

Only 2 children showed IgM antibodies in 2 consecutive samples, with intervals of 83 and 174 days. The corresponding intervals for the other children were 74–311 days, median 164. Only 4 children showed transient low-level IgM antibody reactivity (mean absorbance 0.335, median 0.285) in 1 serum sample long after seroconversion. One of these 4 IgM-ambiguous samples also was falsely IgM antibody positive for B19 parvovirus, which suggested nonspecificity. None of them contained HBoV1 DNA, but all contained high-avidity HBoV1 IgG antibodies, indicating preexisting immunity.

In 73 (67%) of the 109 children, IgG antibody levels after seroconversion remained stable or decreased only slightly during follow-up (Tables 1, 3; Figure 2). They decreased substantially over time in 26 (24%) children, 10 of whom turned seronegative during follow-up. In all, 38 (35%) children had ≥1 (44 in total) diagnostic secondary HBoV1 immunoactivations (≥4-fold increase in IgG antibody titer in 2 consecutive samples; Figure 1), including 7 reconversions (Figure 2). None of the 44 serum pairs showing secondary responses contained HBoV1 DNA or IgM antibodies (the latter with 1 exception; the nonspecific B19 IgM-reactive serum), and 42 contained IgG of high avidity. In addition, some of the children showed nondiagnostic fluctuations in the IgG antibody level.

All 7 children who lacked a primary seroconversion had been IgG positive from birth (Figure 2). Their umbilical cord samples showed high IgG avidity and lacked IgM and viral DNA. During follow-up, each of these 7 children experienced ≥1 (2 children had 3) secondary IgG increases within their first 5 years of life.

For 88 children, the first samples were taken during the first 6 months (median 3.7 months of life); 35 (40%) had maternal IgG antibodies (Table 1). One child in this age group showed an HBoV1 primary infection (at 3.7 months): presence of IgM antibodies, IgG conversion, and viremia. An episode of AOM preceded the viremic sample. Another child had a borderline IgM antibody result in her first available sample but exhibited waning (maternal) IgG

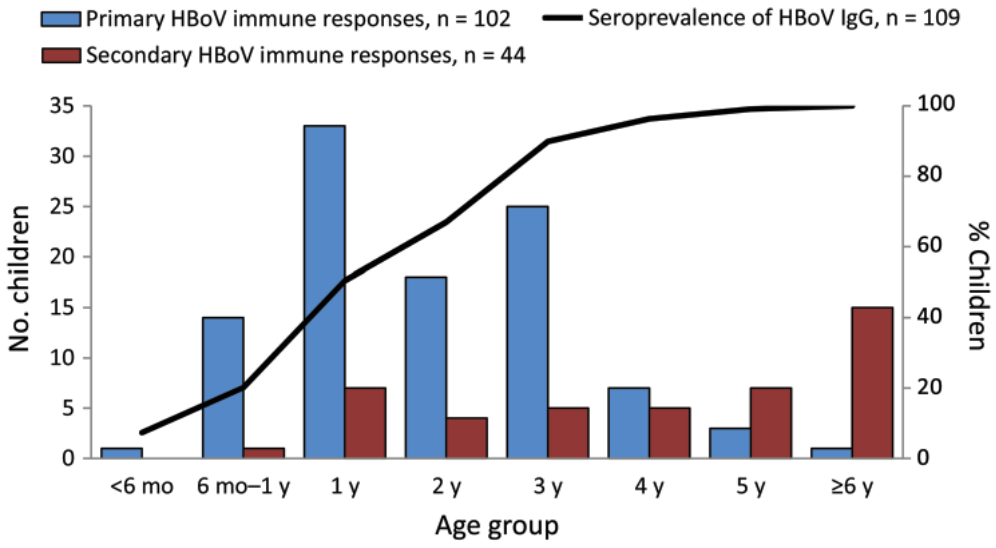

Figure 1. Age distribution of children with primary and secondary human bocavirus (HBoV) immune responses and seroprevalence of HBoV1 IgG, Finland.

Table 1. Human bocavirus IgG results from 109 constitutionally healthy children, Finland

Result*	No. (%)	Age, y		
		Mean	Median	Range
Seroconversion	102 (94)	2.30	2.08	0.31–6.00
Secondary response†	38 (35)	4.79	4.77	0.73–9.79
Reconversion	7 (6)	6.29	7.07	1.59–8.15

*Maternal antibodies, i.e., low-level (vanishing) IgG, were detected in 35/88 children from whom serum was taken ≤6 mo. of age. Seven children were IgG positive from birth, and their maternal antibodies were not seen to disappear before induction of their own immunity. For 73, the IgG level remained high; for 26, the IgG level decreased with time; and 10 underwent IgG reversion.
†≥4-fold increase in, or reconversion of, high avidity IgG (2 children had 2 and 2 other children had 3 secondary immune responses).

antibodies. However, umbilical cord blood samples from both of these children were negative for IgM antibodies and DNA, and IgG antibodies were of high avidity, ruling against congenital infection.

Real-time qPCR

At primary infection, viral DNA was detectable in the serum of 24 of the 102 children with a mean of 1.21×10^5 copies/mL (Table 2). However, none of the serum samples with secondary immune responses was PCR positive. No child had HBoV1 DNA in 2 consecutive samples (postviremic sampling interval 74–207 days, mean 128 days, median 96 days).

Clinical Correlates

Infection-related symptoms were reported in 90% of the children during the HBoV1 primary infection and in 70% during the subsequent sampling interval (Table 4) ($p = 0.0003$). When comparing individual symptoms, 2 conditions, URTI (60%) and AOM (47%), occurred significantly more frequently during the HBoV1 primary infection than during the subsequent interval (36% and 31%; $p = 0.0002$ and $p = 0.026$). LRTI was also more frequent during the HBoV1 primary infection (5%) than during the next interval (0%) but was too rare for the difference to reach significance; however, when combined with URTI, the difference was highly significant ($p<0.0001$). The secondary immune responses, including reconversions, showed no association with symptoms (Table 4). The results were similar, regardless of whether the symptoms of HBoV1 infection were compared with those of the previous or the subsequent interval (Table 4). Neither the previous nor the subsequent sampling interval differed significantly in length from the seroconversion interval. The mean differences in length compared with the seroconversion interval were the following: –16.6 days for the previous and +0.5 days for the subsequent interval.

Stability and decline in HBoV1 IgG absorbance level in long-term follow-up were not associated either with diabetes-related autoantibody positivity, allergic disease, or symptoms during HBoV1 primary infection. They were also not associated with excess susceptibility to infections in general.

Discussion

By comprehensive serologic and molecular testing and follow-up, we observed that by 6 years of age, all children were infected with HBoV1. Reports state that HBoV1 infects predominantly children, and at a young age (*2*,*3*), but almost all studies have been symptom and PCR based and cross-sectional, whereas our study was serum based and longitudinal and spanned the entire period from infancy through 13 years of age.

We determined the seroepidemiology and clinical correlates of HBoV1 infections and the kinetics of HBoV1 infection markers in sequential serum samples from constitutionally healthy children. We showed that HBoV1 primary infections, but not secondary immunoactivations, were significantly associated with respiratory illness and with AOM.

A definitive IgG seroconversion was evident in 102 of the 109 children, half of whom showed further markers of HBoV1 primary infection: viremia, IgM antibodies, or low avidity of IgG. The frequency of viremia or IgM antibody positivity in children with HBoV1 primary infection was considerably lower here than in our earlier study in which 45 of 48 wheezing children with serologically verified HBoV1 primary infection were viremic, and all but 1 who seroconverted had IgM antibodies (*13*). Unlike our current population-based study, our earlier study comprised symptomatic children who gave samples at short intervals during their acute disease. Our PCR results are concordant with the earlier results that showed the brevity of HBoV1 viremia. IgM antibodies persisted slightly longer than did the viremia and were detectable in one fourth of the first IgG-positive serum samples. For human parvovirus B19 (B19V), another pathogenic human parvovirus, the kinetics are the reverse, with viremia usually outlasting IgM antibodies and persisting at a low level for months or years (*22*). In longevity of diagnostic findings, the 3 assays

Table 2. Human bocavirus 1 findings of 102 constitutionally healthy children at seroconversion, Finland*

No. (%) children	Virologic finding	Mean	Median	Range
28 (27)	IgM antibodies, abs	0.69	0.66	0.17–1.54
24 (24)	qPCR positive, copies/mL	1.21×10^5	4.13×10^4	1.26×10^0 to $–9.09 \times 10^5$
34 (33)	Low IgG avidity, %	8.6	9.4	1.5–14.4

*Abs, absorbance value; qPCR, quantitative PCR in serum.

Table 3. Serologic and quantitative PCR results of consecutive serum samples from a representative child, showing all acute HBoV markers, Finland*

Sample no.	Age at sample collection, y	Sampling interval, d	IgG absorbance	IgG interpretation†	IgM absorbance	IgM interpretation‡	IgG avidity, %§	qPCR, copies/mL
1	0.33	120	0.061	Neg	0.015	Neg		
2	0.99	235	0.010	Neg	0.014	Neg		
3	1.25	96	0.029	Neg	0.025	Neg		
4	1.52	96	0.032	Neg	0.023	Neg		
5	1.77	90	0.032	Neg	0.021	Neg		
6	2.02	89	0.017	Neg	0.022	Neg		
7	2.52	180	0.019	Neg	0.028	Neg		
8	2.97	165	0.014	Neg	0.028	Neg	Neg	Neg
9	**3.44**	**167**	**1.536**	**Pos**	**0.730**	**Pos**	**2.8**	**7.67×10^4**
10	3.93	178	2.883	Pos	0.023	Neg	60.8	Neg
11	4.46	190	3.412	Pos	0.035	Neg		
12	4.91	163	3.111	Pos	0.022	Neg		
13	5.45	193	3.754	Pos	0.033	Neg		
14	5.96	185	3.228	Pos	0.020	Neg		
15	7.07	398	3.183	Pos	0.030	Neg		
16	7.52	163	3.102	Pos	0.026	Neg		
17	8.07	198	3.450	Pos	0.027	Neg		
18	8.53	165	3.149	Pos	0.016	Neg		
19	9.07	195	3.252	Pos	0.024	Neg	49.2	

*This child experienced an acute HBoV infection at 3 years of age (sample no. 9), evidenced by: IgG conversion, IgM, low IgG avidity and viremia (**boldface**). HBoV, human bocavirus; qPCR, quantitative PCR in serum; neg, negative; pos, positive; blank cells, not done.
†The cutoff absorbances for negative and positive IgG results were 0.154 (mean + 3 SD) and 0.188 (mean + 4 SD), respectively (*13*).
‡The cutoff absorbances for negative and positive IgM results were 0.136 (mean + 3 SD) and 0.167 (mean + 4 SD), respectively (*13*).
§The low- and high-avidity cutoff values: 15% and 25%, respectively (*17*).

for HBoV1 ranked in this order: IgG-avidity EIA, IgM EIA, serum PCR.

Regarding the high HBoV1 IgG antibody seroprevalence, which has exceeded 90% in adults (*13*,*23*–*26*), we recently observed HBoV1 IgG secondary responses in a large proportion of immunocompetent adults (*17*). In the current study, two thirds of children maintained steady IgG antibody levels for years after seroconversion. In one fourth, however, antibody levels declined substantially with time, and in some cases fell below the detection limit. In most of the children with such a reversion, the HBoV1 IgG antibodies later reconverted. Altogether, 38 (35%) children exhibited diagnostic secondary HBoV1 IgG antibody responses: in 2 children 2× and in another 2 children 3×. The secondary immunoactivations were generally of high avidity and lacked IgM antibodies and were always nonviremic. If these events represent HBoV1 secondary infections, they must be local rather than systemic infections, or they produce short-lived or low-titer viremia that escaped PCR detection. That they were nonviremic would agree with the possibility of B cell boosting by related viruses. The most plausible candidates for such closely related immunogens are the recently discovered HBoV species HBoV2–4 (*4*–*6*), or even more intriguingly, some currently unknown viruses. Past-immunity IgG antibodies against HBoV1–4 cross-react; however, both IgM and IgG antibodies of the acute phase are HBoV1 specific (*27*).

Seven children remained HBoV IgG antibody positive from birth through follow-up, without any observable seroconversion or other acute HBoV-infection markers. The absence of HBoV IgM antibodies and DNA from their umbilical cord blood samples argues against congenital infection. This is in line with a recently noted absence of HBoV from amniotic fluid or fetal tissues (*28*,*29*). The lengths of the sampling intervals may have concealed the disappearance of maternal antibodies and the appearance of markers for HBoV acute infection in the infant. Alternatively, instead of a full replicative infection, the virus could induce a vaccination-like immunity because of preexisting maternal antibodies. Within the first months of life, maternal HBoV IgG antibodies have been observable at prevalences of 26%–78%, depending on the child's age (*13*,*23*,*24*,*30*), similar to the 40% at a mean age of 4 months in our study. Because only 1 child had detectable HBoV primary infection during the first 6 months of life, the maternal antibodies as a rule seem to be protective—or the infants were less exposed.

The first HBoV prevalence studies showed low detection frequencies of HBoV DNA in the respiratory tracts of asymptomatic children and a high co-infection rate (*31*–*33*). However, subsequent studies indicated a prolonged and frequent presence of HBoV DNA also in asymptomatic children (*7*–*11*). Because of these features and the frequent co-detection of other viruses, the role of HBoV as a respiratory pathogen has been questioned. In

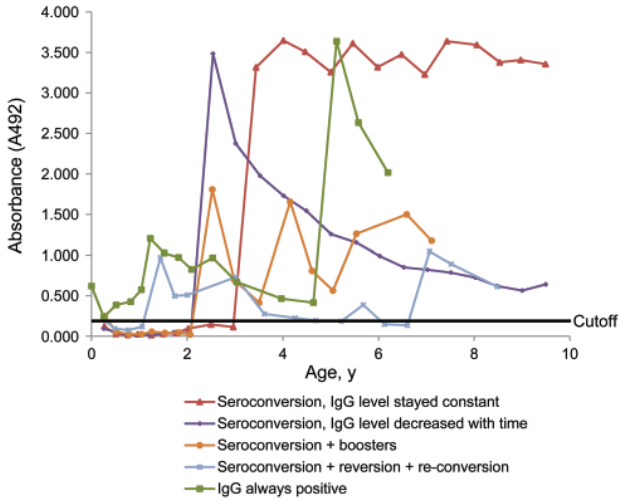

Figure 2. IgG responses in follow-up serum samples from 5 representative children in a study of human bocavirus 1 infection, Finland.

a recent prospective study of children in day care, neither HBoV DNA presence nor its load in nasal swabs was associated with the presence or severity of respiratory illness (*11*). On the other hand, low-load HBoV PCR positivity in the upper respiratory tract does not reliably indicate acute HBoV infection, and therefore an accurate diagnosis of acute HBoV infection requires a serum specimen (*12–16*).

We collected consecutive serum samples from constitutionally healthy children and recorded the appearance of HBoV antibodies. The parents were interviewed during each sample-collection visit about the child's symptoms within that interval. We compared symptoms during the seroconversion interval with those during the subsequent interval for each child, and, taking into account the slight age difference and variations in occurrences of other respiratory viruses, also to symptoms during the previous sampling interval. With both approaches, HBoV primary infection was unambiguously associated with URTI and with combined URTI and LRTI, strongly suggesting that HBoV does cause respiratory illness. Furthermore, our result linking AOM with HBoV primary infection, in concert with 2 previous studies' detection of HBoV DNA in middle ear fluid (*34*,*35*), indicates a close association for HBoV also with AOM and middle ear effusion. That these children were genetically susceptible to type 1 diabetes (*18*) most likely does not affect this interpretation of our data.

Even though the strength of our study is the close monitoring of children over a long period (1996–2006), the sampling intervals were too long for any detailed analysis of the seasonal distribution of the infections. However, even distribution throughout the year was evident, which reduced the possibility that HBoV1 infections would accumulate within peak season(s) of other particularly pathogenic viruses, which, at least in theory, could have resulted in a false disease association. Although we did

Table 4. Infection-related signs and symptoms during human bocavirus 1 primary seroconversions and secondary responses compared with the previous and subsequent sampling interval, Finland*

Sign or symptom	Primary immune response, n = 101					Secondary immune response, n = 43				
	Interval,†	Previous interval‡		Next interval§		Interval,¶	Previous interval#		Next interval**	
	no. (%)	No. (%)	p value	No. (%)	p value	no. (%)	No. (%)	p value	No. (%)	p value
URTI	61 (60.4)	34 (33.7)	**0.0002**	36 (35.6)	**0.0002**	24 (55.8)	21 (48.8)	0.53	23 (53.5)	1
LRTI	5 (4.9)	2 (2.0)	0.45	0	–	1 (2.3)	3 (7.0)	0.62	2 (4.6)	1
URTI or LRTI	62 (61.4)	36 (35.6)	**0.0003**	36 (35.6)	**<0.0001**	24 (55.8)	22 (51.2)	0.83	23 (53.5)	1
Fever without RTI	14 (13.9)	9 (8.9)	0.30	10 (9.9)	0.54	7 (16.3)	3 (7.0)	0.22	3 (7.0)	0.34
Acute otitis media	47 (46.5)	33 (32.7)	**0.024**	31 (30.7)	**0.026**	12 (27.9)	8 (18.6)	0.45	5 (11.6)	0.06
Acute tonsillitis	0	1 (1.0)	–	1 (1.0)	–	1 (2.3)	1 (2.3)	1	0	–
Acute conjunctivitis	5 (4.9)	3 (3.0)	0.72	8 (7.9)	0.51	1 (2.3)	5 (11.6)	0.12	2 (4.6)	1
Acute sinusitis	2 (2.0)	0	–	1 (1.0)	1	2 (4.6)	3 (7.0)	1	2 (4.6)	–
Gastroenteritis	23 (22.8)	19 (18.8)	0.62	16 (15.8)	0.30	9 (20.9)	8 (18.6)	1	10 (23.3)	1
Exanthema, fever	8 (7.9)	4 (4.0)	0.34	0	–	0	1 (2.3)	–	0	–
Other	8 (7.9)	3 (3.0)	0.23	6 (5.9)	0.79	4 (9.3)	2 (4.6)	0.62	3 (7.0)	1
Totals	91 (90.1)	74 (73.3)	**0.003**	71 (70.3)	**0.0003**	35 (81.4)	30 (69.8)	0.27	32 (74.4)	0.58

*URTI, upper respiratory tract illness; LRTI, lower respiratory tract illness; RTI, respiratory tract infection; –, could not be calculated. **Boldface** indicates statistical significance by Liddell exact test (<0.05).
†Length of intervals, d: mean 155, median 166, range 75–361; mean age 2.3 y, median age 2.1 y.
‡Length of intervals, d: mean 139, median 130, range 73–358.
§Length of intervals, d: mean 155, median 169, range 61–615.
¶Length of intervals, d: mean 188, median 176, range 78–537, mean age 4.8 y, median 4.8 y.
#Length of intervals, d: mean 181, median 182, range 61–415.
**Length of intervals, d: mean 183, median 176, range 88–408. One child was omitted because of lack of clinical information for 1 interval.

not screen for other respiratory pathogens, the scheduled sampling according to the ages of the children instead of seasons further reduced the possibility of seasonal bias.

In our longitudinal study, all children acquired HBoV infection by 6 years of age. Although most of them subsequently maintained stable HBoV IgG antibody levels, in one fourth, levels substantially declined, and some children lost their antibodies completely, with subsequent reconversion. Secondary infections or anamnestic immune responses commonly occurred. Among the children with HBoV primary infection, >60% had respiratory symptoms. Whereas HBoV primary infections were strongly associated with respiratory illness, the secondary immunoactivations were not. Our results indicate that HBoV1 is a true and common respiratory pathogen.

Acknowledgments

We thank the dedicated personnel of this study; the study children and their families for their essential contributions; and Carol Norris for language revision. We also thank Tobias Allander for providing the plasmid (pSt2; GenBank accession no. DQ000496) containing the HBoV1 NP1 gene.

This work was supported by grants from the Juvenile Diabetes Research Foundation International (grants 4-1998-274, 4-1999-731, 4-2001-435); the European Union (grant BMH4-CT98-3314); the Academy of Finland (grants 68292, 1122539); the Novo Nordisk Foundation; the Finnish Funding Agency for Technology and Innovation (Tekes); the Special Research Funds for University Hospitals in Finland; the Finnish Office for Health Technology Assessment; the Diabetes Research Foundation in Finland; the Sigrid Juselius Foundation; the Emil Aaltonen Foundation; the Signe and Ane Gyllenberg Foundation; the Foundation for Pediatric Research; the Päivikki and Sakari Sohlberg Foundation; the Helsinki University Central Hospital Research & Education and Research & Development Funds; the Research Funds of the University of Helsinki; and the Medical Society of Finland.

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Lack of Decline in Childhood Malaria, Malawi, 2001–2010

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In some areas of Africa, health facility data have indicated declines in malaria that might have resulted from increasingly effective control programs. Most such reports have been from countries where malaria transmission is highly seasonal or of modest intensity. In Malawi, perennial malaria transmission is intense, and malaria control measures have been scaled up during the past decade. We examined health facility data for children seen as outpatients and parasitemia-positive children hospitalized with cerebral malaria in a large national hospital. The proportion of *Plasmodium falciparum*–positive slides among febrile children at the hospital declined early in the decade, but no further reductions were observed after 2005. The number of admissions for cerebral malaria did not differ significantly by year. Continued surveillance for malaria is needed to evaluate the effects of the increased malaria control efforts.

Malaria is a leading cause of illness and death among children in countries in which it is endemic (*1*). An increasing number of countries in sub-Saharan Africa are rapidly scaling up malaria control interventions as broad programmatic measures designed to achieve Millennium Development Goal 4 (*2*). To reduce malaria effectively, countries should reach at least 70% coverage of the 4 main malaria control tools: long-lasting insecticide-treated bed nets, indoor residual spraying, intermittent presumptive treatment for pregnant women, and prompt treatment with artemisinin-based combination therapy for symptomatic uncomplicated malaria for which parasitemia was confirmed (*3*). As malaria control interventions increase, several reports based on analyses of long-term surveillance data (*4*–*8*) have emerged from countries with long-standing programs that showed a substantial drop in malaria-associated hospitalizations.

Malawi, in south-central Africa, has year-round malaria transmission that peaks during the long rainy season (late November–April) (*9*) and accounts for 30%–40% of all outpatient visits (*2*,*10*,*11*). Since 2005, and with the support of the President's Malaria Initiative and the Global Fund, Malawi has started to widely scale up malaria control interventions. In 2000, bed net use was 6% nationally; by 2004, a total of 36% of children <5 years of age reportedly had slept under an insecticide-impregnated bed net during the previous night (*12*). Since 2007, ≈3 million extra nets have been distributed free of charge through health facilities, reaching ≈60% coverage (*13*). Coverage of indoor residual spraying, however, remained low in 2010, even in urban areas (e.g., 3% in Blantyre city) where it was limited mainly to the private sector (*13*). Artemisinin-based combination therapies were adopted as the recommended method of treatment in November 2007. Despite increasing efforts, according to the most recent World Malaria Report, no evidence exists of decreased malaria since 2000 in Malawi (*14*).

Even with the implementation of an improved Health Management Information System in 2002, obtaining complete facility-based routine data remains a challenge

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DOI: http://dx.doi.org/10.3201/eid1802.111108

in Malawi (*15*). Time trends in health facility records may show evidence of fewer malaria cases and may provide a useful complementary approach to monitor disease changes in settings where routine surveillance systems are incomplete.

Malaria has been studied extensively since the late 1990s at the Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi (*16*–*19*). In 2001, an improved Pediatric Accident and Emergency Unit (PAEU) was opened, and routine malaria testing of all febrile children in the unit was introduced. A high-dependency research ward in the Department of Pediatrics has been fully functional since 1987 during January–June each year, with the main aim of improving care and undertaking research on severe malaria during the peak malaria season. This arrangement allows monitoring of malaria at a health facility level. We report trends in outpatient visits by malaria parasite–positive children and in admissions for cerebral malaria among children during the past 10 years in the main referral public hospital of Blantyre, Malawi.

Methods

Study Site and Population

We examined health facility data for children in the QECH PAEU and for children admitted with cerebral malaria to the high-dependency research ward at QECH during January 2001–December 2010. To provide information about background rates of asymptomatic parasitemia in nonfebrile children, we monitored trends in the prevalence of parasitemia among children admitted for elective surgery to Beit CURE International Hospital (BCIH), an orthopedic hospital in Blantyre, where all patients are routinely tested at admission for parasites.

QECH is a 1,250-bed, government-funded central hospital that provides primary to tertiary care and admits an average of 50,000 patients per year. QECH serves a population of ≈1 million in Blantyre, surrounding townships, and outlying villages. Most hospitalizations are for community-acquired illnesses, and patients admitted are largely either self-referred or referred from a primary health center within Blantyre. Malaria diagnosis in both the PAEU and research ward was performed by the same personnel, who received regular refresher training throughout the study period. BCIH is a 66-bed teaching hospital that specializes in treating the orthopedic needs of children and adults. It is a referral hospital and therefore covers a population from both urban and rural areas in the same region. Health care is provided free to children at both facilities.

Data Abstraction

From every child in the PAEU who had a febrile illness, blood was obtained for a thick blood film malaria parasite examination and for measurement of hematocrit. These criteria did not change during the study period. Total monthly numbers of outpatient visits to QECH by children, malaria slides taken, and results of thick-film microscopy were available from the PAEU laboratory records. To obtain estimates of parasite density and blood hematocrit levels, we abstracted individual-level records from January 2001 through December 2010. Because of the large volume of data recorded since 2001, we abstracted data from only 2 days (Monday and Thursday) of the first and last weeks of each month.

The criteria for admission into the research ward with cerebral malaria were consistent with World Health Organization guidelines (*20*) and remained unchanged over time. Diagnostic requirements for cerebral malaria were coma (Blantyre coma score <3) persisting for at least 4 hours after admission, *Plasmodium falciparum* parasitemia, negative cerebrospinal fluid, and no other cause of coma identified on examination or investigation. These children were a subset of children admitted to QECH and represent virtually all children admitted with cerebral malaria each year during the rainy seasons. All the diagnoses were reviewed daily by an experienced clinician.

Records from BCIH were available only from 2003 onward. The proportion of children admitted to this hospital for elective surgery who had parasitemia served as a proxy measure of the prevalence of parasitemia in the referred community (*5*) because children attending a hospital offering free orthopedic care are unlikely to be biased with regard to malaria risk.

Statistical Methods

We used Stata version 10.1 (StataCorp LP, College Station, TX, USA) for descriptive and statistical analyses. Monthly slide positivity rates (SPRs) were calculated as the proportion of positive slides of all slides in a given month. Monthly figures for the prevalence of severe anemia (packed cell volume [PCV] ≤15), moderate-to-severe anemia (PCV 15%–24%), and mild (PCV 24%–33%) anemia also were obtained for the same period. Approximate parasite densities were based on numbers of asexual-stage *P. falciparum* parasites per oil-immersion field on the thick blood film (*21*,*22*): + = 1–10 parasites/100 fields; ++ = 11–100 parasites/100 fields; +++ = 1–10 parasites/field; and ++++ = >10 parasites/field . For our analysis, sick children attending the outpatient clinic who had >1 parasite per field (last 2 categories) were considered to be more likely than others to have a fever that was actually attributable to malaria; changes in the proportion of +/++ and +++/++++ parasite levels over time were therefore assessed by logistic regression. Trends in the malaria SPR and anemia prevalence from the PAEU

at QECH, as well as malaria SPR trends from BCIH, were assessed by using logistic regression weighting by the total number of slides taken (for SPRs) or by the total number of children tested (for anemia prevalence). Because of the large number of statistical comparisons performed, differences were considered significant only for p values <0.001, although results with a significance level of 0.05 also were reported. Annual trends of cerebral malaria were tested by Poisson regression for 2001–2010 (dependent variable: cerebral malaria counts; independent variable: 2001–2010, reference year 2001).

Results

Slide Positivity Rates among Children in the PAEU

During January 2001–December 2010, monthly data on the total numbers of outpatient visits, malaria slides, and positive slides were available from the PAEU. During the decade, 686,118 outpatient visits were recorded. Of these, a malaria slide was obtained at 242,953 (35.4%) visits, and 61,320 (25.2%) of these were parasite positive for *P. falciparum*. The total number of outpatient visits in successive years increased gradually, but numbers of slides taken and numbers of positive slides remained approximately constant over time, except for an increase during 2001–2003 (Figure 1). The patterns of outpatient visits for malaria were highly seasonal, with peaks of total positive slides after the main rainy season (December–May) (Figure 1). SPRs peaked in 2001 and 2002 and fell significantly by approximately one third in 2003 and 2004 from 2001 (p<0.001). In 2005 and 2006, SPRs significantly decreased from 2001 by an additional one quarter (p<0.001), but then returned to the 2003 and 2004 levels in 2007–2010 (p<0.001). Although annual SPRs during 2005–2010 were significantly lower than in 2001, annual SPRs did not decline further within the last 6-year period (2005–2010) (Table).

Parasite Density and Anemia Prevalence among Children in the PAEU

To analyze parasite densities and hematocrits of children in the PAEU, we abstracted 22,397 records (22,267 with hematocrit information) from the PAEU laboratory books. Books were available for the entire period, except for 2 quarters for which books were missing (October–December 2004 and January–March 2010). No clear differences were observed in parasite density levels over time (Figure 2, panel A). Overall, 15.7% of slides (95% CI 15.2–16.1) had >1 parasite/field (i.e., PAEU visits more likely to be attributable to malaria). This proportion was significantly lower in 2003 (odds ratio [OR] 0.54, 95% CI 0.45–0.64) and 2005 (OR 0.58, 95% CI 0.48–0.69) than in 2001, but no significant differences were seen in the remaining years, and no further reductions were observed after 2005.

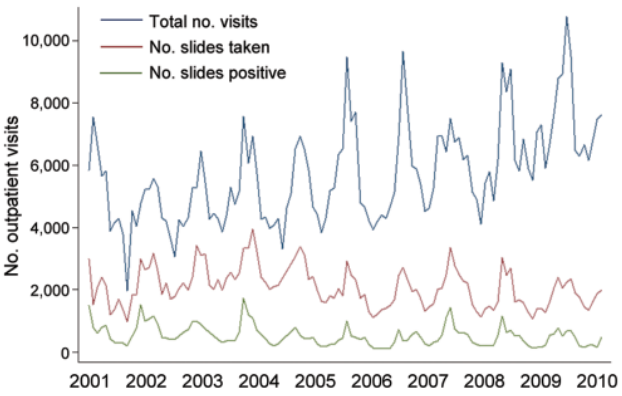

Figure 1. Temporal trends of total monthly outpatient visits, malaria slides taken, and parasitemia-positive slides recorded in the Pediatric Accident and Emergency Unit at Queen Elizabeth Central Hospital, Blantyre, Malawi, 2001–2010.

The overall prevalences of mild, moderate, and severe anemia were 49.7 (95% CI 49.1–50.4), 12.9 (95% CI 12.5–13.4), and 3.2 (95% CI 2.9–3.4), respectively. Each anemia category peaked in 2001 and significantly fell by 23%, 26%, and 39%, respectively, in 2002 (p<0.001). In 2003, the prevalences of all anemia categories significantly decreased by an additional 13%, 29%, and 23%, respectively (p<0.001). No further reductions occurred after 2004 (Figure 2, panel B).

Admissions for Cerebral Malaria

During January–June in 2001–2010, a total of 1,048 children with cerebral malaria were admitted to the research ward at QECH; 433 (41%) also had severe malarial anemia (PCV ≤15%). Data for 2005 were not included because the research ward was not fully functional during that year.

During the 10-year study period, the number of children admitted with cerebral malaria per season ranged from 85 to 150, with the maximum in 2010 (Table). When monthly trends were assessed by Poisson regression and by using 2001 as the reference year, no significant differences were observed by year. The overall linear trend was also tested, but no significant trend was found over time (p = 0.906).

Compared with 2001, the mean age of patients with cerebral malaria did not significantly vary each year up to 2007 but then significantly increased in 2008–2010 (Table; Figure 3). Mean ages during 2008–2010 (49.0 months [95% CI 46.2–51.8 months]) remained significantly higher (p<0.001) than the mean age during 2001–2007 (43.3 months [95% CI 38.2–42.4 months]).

Table. Annual numbers of malaria-related outpatient visits and admissions of children with cerebral malaria at QECH and children hospitalized for elective surgery at BCIH who had a parasitemia-positive slide, Malawi, 2001–2010*

	QECH				BCIH	
	Outpatients		Research ward		Trauma patients	
Year	Malaria slides taken	Parasitemia-positive slides, no. (%)†	Cerebral malaria cases‡	Mean patient age, mo (95% CI)	Malaria slides taken	Parasitemia-positive slides, no. (%)§
2001	21,432	7,392 (34.5)	131	34.2 (30.0–38.4)	NA	NA
2002	27,898	9,251 (33.2)¶	123	42.7 (38.0–47.4)	NA	NA
2003	30,238	7,217 (23.9)#	106	45.0 (39.1–50.9)	320	49 (15.3)
2004	24,675	6,429 (26.1)#	124	43.8 (37.7–49.9)	448	50 (11.2)
2005	26,853	4,591 (17.1)#	NA	NA	551	102 (18.5)
2006	20,850	4,062 (19.5)#	86	38.0 (33.0–43.0)	481	80 (16.6)
2007	23,996	5,969 (24.9)#	105	38.3 (32.8–43.9)	651	97 (14.9)
2008	22,907	5,903 (25.8)#	99	51.8 (46.1–57.5)#	755	98 (13.0)
2009	21,126	5,374 (25.4)#	118	47.8 (42.8–52.8)#	406	74 (18.2)
2010	22,978	5,132 (22.3)#	151	48.8 (44.6–53.0)#	420	43 (10.2)¶
Total	242,953	61,320 (25.2)	1,043	43.5 (41.8–45.2)	4,032	593 (14.7)

*QECH, Queen Elizabeth Central Hospital; BCIH, Beit CURE International Hospital; NA, not available.
†Reference year 2001.
‡Five records had missing information on year.
§Reference year 2003.
¶p<0.05.
#p<0.001.

Malaria Parasitemia in Children Admitted to BCIH

Data from children admitted to BCIH for elective surgery were available for 2003–2010. During this period, a total of 4,033 slides were taken, and an average of 14.7% of patients had parasitemia. Although the SPR varied slightly by year, no significant trend was observed over the 8-year period (Table). Compared with 2003 (reference year), SPRs did not differ significantly for any year except 2010, when SPRs were 27% lower (p<0.05).

Discussion

In several countries, including Malawi's neighbor Zambia, health facility data have indicated significantly declining trends in malaria (*4–8*,*23–25*). Investigators have attributed such declines (at least partially) to the rapid scaling up of malaria control interventions. To our knowledge, this study is one of the few hospital-based retrospective analyses to show no markedly declining trends in malaria during the past decade in a sub-Saharan African country (*26*). The apparently undiminishing malaria at QECH has occurred in the context of increasing efforts to increase malaria control interventions in the country and despite marked reductions in overall deaths among children <5 years of age (*27*). Progress has in fact been substantial in scaling up malaria control interventions in Malawi in recent years, but some parts of the country did not reach high coverage until 2010 (*13*). That evidence of a reduction in malaria in children was not observed in the period during 2001–2010 is therefore not entirely surprising; such a reduction might not become apparent until after 2010. Although use of insecticide-treated bed nets for children <5 years of age has increased quickly in recent years, reaching ≈52% in urban areas and 57% in the southern region (including Blantyre), coverage of other interventions, such as indoor residual spraying, remained low (≈3%) in Blantyre in 2010 (*13*). In areas where dramatic reductions of malaria attributable to rapid scale-up of interventions have been reported (such as in neighboring Zambia), high coverage of interventions with both insecticide-treated bed nets and indoor residual spraying was achieved (*8*,*28–30*). Therefore, because indoor residual spraying coverage is likely to play a critical role in reducing transmission intensity and, ultimately, the incidence and prevalence of malaria, in 2011, Malawi expanded its indoor residual spraying activities to 7 districts (*13*) to maximize the effects of malaria control interventions. Strengthened surveillance systems capable of monitoring short-term changes in disease patterns and evaluating in-country differences in uptake of interventions will become key for assessing these expected effects.

The data presented in this retrospective analysis show little change in the pattern of malaria in children during 2001–2010 in Blantyre. The proportion of malaria-positive slides among febrile children in the PAEU declined in 2003 from the preceding 2 years, but no further reductions were observed after 2005. Approximate parasite densities assessed from thick blood films also remained similar throughout the decade. The prevalence of anemia decreased significantly during the first 3 years of the study period, but no further changes of note occurred after 2004. Trends in parasite density and anemia prevalence in children in the PAEU were based on a systematic sample of records. Although a systematic sampling approach could have led to a potential underestimate or overestimation of monthly figures, these potential differences are likely to have been diluted after monthly estimates were pooled to obtain overall annual figures and are therefore unlikely to have

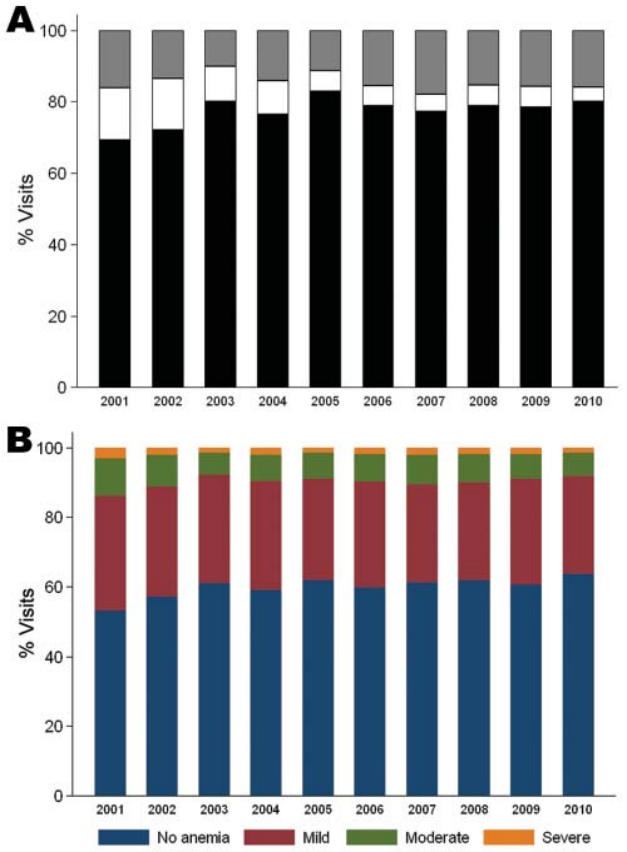

Figure 2. Proportion of parasite density levels (A) and anemia categories (B) over time in the Pediatric Accident and Emergency Unit at Queen Elizabeth Central Hospital, Blantyre, Malawi, 2001–2010.

distorted true trends of parasite density and anemia levels over time.

The unexpectedly high SPRs and anemia levels in 2001 might have resulted from the severe food crisis that affected the entire country during that period. There were reports at the time of increases in the prevalence of malaria and anemia in the Blantyre area (*31*). Because laboratory records from outpatient visits were not available before that period, we were unable to assess trends before 2001.

The number of children with cerebral malaria admitted each season to QECH has fluctuated moderately since 2001 but with no downward trend and with no significant reduction in any year since 2001. Variations in the rainfall pattern are unlikely to have played a major role in the variations in patients admitted with cerebral malaria. The amount of rainfall was fairly constant from year to year, and the months of peak rain coincided with the months of peak cerebral malaria admissions in all years except 2008 (N. Feasey et al., unpub. data).

The mean age of children with cerebral malaria was significantly higher after 2007 than in 2001. A shift of susceptibility to severe malaria toward older children is theoretically possible in areas where malaria control interventions are being rapidly scaled up and has been reported elsewhere (*5*). Continued monitoring of our patient population is needed to confirm whether this trend is maintained. Unlike the situation in other sites, in Blantyre this recent age trend in cerebral malaria patients has not been accompanied by equivalent changes in SPR or anemia prevalence among hospital outpatients.

The use of health facility–based data is subject to several limitations. These limitations include lack of a known community denominator, likely variations in access to or use of the facility, and uncertain quality and standardization of diagnoses. Such data are best interpreted in the context of community-based surveys, which can provide data on the extent of coverage by various interventions and provide indicators of infection and disease prevalences among the population. We used malaria indicators from children admitted for elective surgery to an adjacent pediatric orthopedic hospital (BCIH) as a proxy for indicators likely to prevail in the community surrounding both hospitals. These data showed no substantial reductions in SPR during 2003–2009 but some evidence of a reduction ($p<0.05$) in 2010 from 2003. The SPR obtained from these trauma patients is a good proxy for the malaria prevalence in the community (*5*) because children attending a free trauma hospital are unlikely to be a biased sample with regard to malaria risk. Although the catchment area of BCIH is wider than that of QECH (because it admits children from the southern and central regions), and therefore could include children exposed to higher or lower intensities of malaria transmission than those of Blantyre district, the catchment area and referral patterns have not changed over time. Individual SPR trends are therefore unlikely to have been

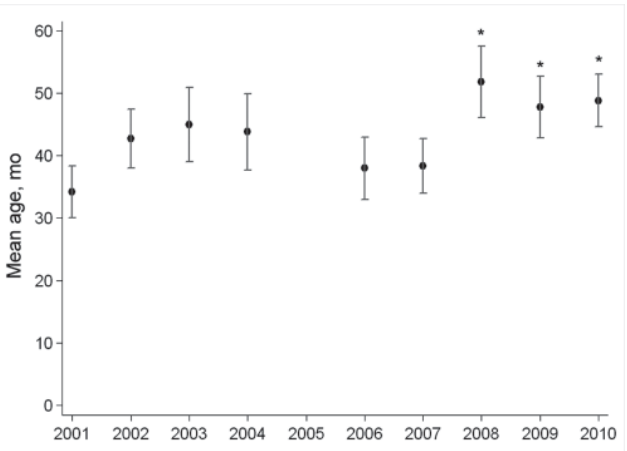

Figure 3. Mean age (95% CI) of children with cerebral malaria admitted to the research ward at Queen Elizabeth Central Hospital, Blantyre, Malawi, during January–June, 2001–2010. Data were not available for 2005. *Denotes a significant difference ($p<0.001$) in the mean age compared with that in the reference year (2001).

biased in this respect. These data, based on a retrospective health-facility analysis that used a significance level of 0.001, suggest that the persisting levels of *P. falciparum* infection among children with fever at the hospital reflect a similar persistence of the infection in the community over the last decade.

Several factors might have led to underestimation of a decline in malaria prevalence, however. The increase in the number of outpatient visits over time probably reflects population growth of the area or changes in health-seeking behavior (from increased activities in health promotion that would have resulted in increased self-reported outpatient visits), and the number of slides taken remained roughly constant over time. This trend could represent a decrease in the proportion of attendees with a malaria-like illness; alternatively, the facility might have reached its capacity to conduct malaria tests, and when that capacity was exceeded, patients were treated for malaria on the basis of a presumptive diagnosis. If the latter occurred, we would have underestimated malaria in the later years of our study period. This potential underestimation is an intrinsic problem of retrospective health facility–based studies that might have resulted in a systematic bias in observed trends. Although a systematic bias could have occurred in the PAEU, we are confident that it did not affect the high-dependency ward because all cerebral malaria patients are admitted to this ward during the rainy season. However, previous studies reporting declining trends of hospital admissions for malaria (*4*,*6*–*8*,*23*–*25*) also were subject to similar limitations and should be interpreted with caution. Lastly, many children seen at QECH who were residents of Blantyre might have acquired their malaria in their journeys to rural areas because use of bed nets by short-term travelers is less common than for those sleeping in their permanent residence (*32*), which partially could have masked a true decline in malaria in Blantyre.

As malaria control efforts increase in Malawi and in similar areas, continued surveillance is needed to describe changes in malaria among a population exposed to lower intensities of malaria transmission. Significant increases in the mean age of outpatient and hospitalized children with positive slides and a rise in the proportion of cerebral malaria among all those admitted for severe malaria are among changes that might occur (*33*). Field-based surveillance and regular malaria indicator surveys are major methods by which to monitor the effectiveness of malaria control. However, to identify changes in disease patterns, mechanisms for the sustainable, prospective collection of surveillance data from health facilities will be increasingly needed to evaluate the effect of malaria control programs.

Acknowledgments

We thank the Statistics and Data Department at the Malawi–Liverpool–Wellcome Trust Clinical Research Program, the data management team of the Blantyre Malaria Project, and John Cashman for their contribution to the data abstraction. We also extend special thanks to the patients at QECH, the medical and nursing staff of the QECH medical and pediatric wards, and the Malawi–Liverpool–Wellcome Research Ward for their contribution to this work.

The surveillance of cerebral malaria at the research ward is supported by the Wellcome Trust Major Overseas program award (award no. 084679/Z/08/Z) and the US National Institutes of Health (R01 AI34969). K.G. was supported by the Center for Host–Parasite Interactions (Canada).

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Non-O157 Shiga Toxin–producing *Escherichia coli* Associated with Venison

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We investigated an outbreak of non-O157 Shiga toxin–producing *Escherichia coli* at a high school in Minnesota, USA, in November 2010. Consuming undercooked venison and not washing hands after handling raw venison were associated with illness. *E. coli* O103:H2 and non-Shiga toxin–producing *E. coli* O145:NM were isolated from ill students and venison.

Non-O157 Shiga toxin–producing *Escherichia coli* (STEC) are emerging pathogens (*1,2*) but are underrecognized because relatively few clinical laboratories routinely use culture-independent testing methods necessary for their identification (*3,4*). Ruminants (e.g., cattle, goats) can be colonized by non-O157 STEC and are reservoirs of these organisms. Non-O157 STEC outbreaks have been associated with contaminated food and recreational water and with direct contact with infected animals or humans (*2,4,5*). However, much is still unknown about sources and risk factors for non-O157 STEC infection.

The Study

On December 1, 2010, the Minnesota Department of Health (MDH) was notified that 2 students from the same high school were hospitalized with bloody diarrhea. As part of a physical education/environmental science class, 7 white-tailed deer (*Odocoileus virginianus*) had been processed on school grounds on November 16, and venison kabobs were grilled and consumed in class on November 23.

MDH and Minnesota Department of Agriculture (MDA) staff interviewed the course instructor and the butcher who processed the deer. The school provided names and contact information for students enrolled in the class. A case–control study was conducted; students were interviewed about illness, food consumption, and venison handling in class. A case-patient was defined as a class enrollee in whom diarrhea (≥3 loose stools in 24 hours) developed after November 16 and lasted ≥3 days. Diarrhea duration was included in the case definition to exclude possible background norovirus infections.

Stool specimens from 6 students were submitted to MDH. Specimens were tested for *E. coli* O157 and *Salmonella*, *Shigella*, *Yersinia*, and *Campylobacter* spp. by culture and for norovirus genogroups I and II by PCR. Non-O157 STEC testing was conducted by using sweep PCR for Shiga toxin genes (*stx1* and *stx2*), *hlyA*, and *eaeA* (*6*) and by culture using immunomagnetic separation.

If Shiga toxin genes were detected by sweep PCR but not in isolated colonies, *hlyA*- and *eaeA*-positive colonies were serotyped. Leftover raw venison was tested at MDA for STEC by PCR for *stx1*, *stx2*, and *uidA*; by immunomagnetic separation for STEC O103; and by O145 isolation (*7*). SAS software version 9.2 (SAS Institute, Cary, NC, USA) was used for analyses. $p \leq 0.05$ was considered significant.

Of 225 students from 5 class periods, 117 (52%) were interviewed. Twenty-nine case-patients (25%) were identified. Twenty additional students reported gastrointestinal symptoms that did not meet the case definition and were excluded from analysis. Twenty (69%) case-patients were male. Median incubation from the November 23 class date for 28 case-patients with illness onset after that class was 53.5 hours (range 22–121 hours) (Figure 1). All 29 case-patients reported diarrhea, 21 (72%) reported cramps, 5 (17%) vomiting, 5 (17%) bloody stools,

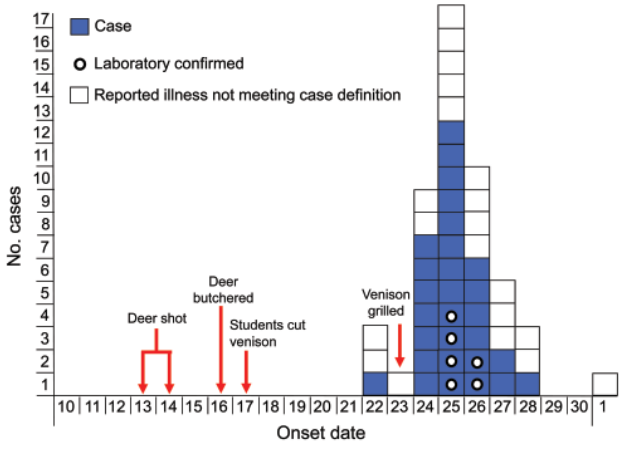

Figure 1. Non-O157 Shiga toxin–producing *Escherichia coli* infections associated with venison among students in a high school class, by illness onset date, November 2010, Minnesota, USA. The case-patient with illness onset on November 22 reported 1 instance of vomiting on that date, followed by a distinct onset of diarrhea on November 24, which suggests that the case-patient may have been co-infected with norovirus and non-O157 Shiga toxin–producing *E. coli*.

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DOI: http://dx.doi.org/10.3201/eid1802.110855

Table 1. Analysis of non-O157 pathogenic *Escherichia coli* from 6 high school students, Minnesota, USA, November 2010*

Case-patient no.	*stx1* sweep PCR	*stx1* isolated colony	*hlyA*	*eaeA*	*E. coli* O103:H2	*E. coli* O145:NM	Norovirus genogroup II
1	+	+	+	+	+	–	–
2	+	+	+	+	+	–	–
3	–	–	+	+	–	+	–
4†	+	–	+	+	–	+	+
5‡	+	–	+	–	–	–	–
6§	+	–	–	–	–	–	–

*+, positive; –, negative.
†100 colonies tested; none were *stx1* positive, and 5 were *hlyA* and *eaeA* positive.
‡122 colonies tested; none were *stx1* positive.
§Positive enzyme immunoassay results for Shiga toxin at clinical laboratory; *stx*1 positive by PCR of submitted broth. Submitted broth failed to grow *E. coli* colonies.

and 2 (7%) fever. Median duration of illness was 5 days (range 4–12 days). Two case-patients were hospitalized for 2 and 3 days, respectively. No case-patients showed development of hemolytic uremic syndrome and none died.

All 6 stool samples were negative for *stx2*, *E. coli* O157, *Salmonella*, *Shigella*, *Yersinia*, and *Campylobacter* spp. Five samples were positive for *stx1*, 5 for *hlyA*, and 4 for *eaeA* by sweep PCR (Table 1). Two of these samples did not yield additional findings. An *stx1*-positive *E. coli* O103:H2 was isolated from 2 samples (from the 2 hospitalized students). Both *E. coli* O103:H2 isolates were indistinguishable by pulsed-field gel electrophoresis (PFGE) (Figure 2). In another sample that was *stx1* positive by sweep PCR, *stx1* was not identified in isolated colonies, but serotyping of *hlyA*- and *eaeA*-positive colonies identified *E. coli* O145:NM (Table 1). A sixth sample was negative for *stx1* and *stx2* by sweep PCR but positive for *hlyA* and *eaeA*; serotyping of *hlyA*- and *eaeA*-positive colonies identified *E. coli* O145:NM (Table 1). Both *E. coli* O145:NM isolates were indistinguishable by PFGE (Figure 2). One of the samples that yielded *E. coli* O145:NM was also positive for norovirus genogroup II.

Six deer were shot and field dressed during November 12–14. A seventh deer was obtained after being hit by a vehicle. Students brought the deer to the school where they were stored in a shed packed in ice. On November 16, a butcher processed each deer by using tools that had never been used to butcher domestic ruminants. Tables, cutting boards, and tools were reportedly cleaned with a 10% bleach solution. Venison was wrapped in plastic, covered in ice, and stored overnight in the shed.

On November 17, students cut selected pieces of meat into cubes, which was wrapped in butcher paper and frozen. Remaining large cuts were returned to students who had provided deer. Students could have received venison from any of the 7 deer. On November 22, the venison was thawed and marinated in 5-gallon buckets. On November 23, several students used wooden bamboo skewers to assemble venison kabobs. The kabobs were grilled by several students on a gas grill for consumption during each class period. Students were instructed to wear gloves and wash their hands after handling raw venison on November 17, 22, and 23.

In the case–control study, consuming undercooked or pink venison was associated with illness (Table 2). Among students who handled raw venison or helped clean up on November 23, students who reported handwashing afterwards were less likely to become ill. Numerous students reported instances of potential cross-contamination or

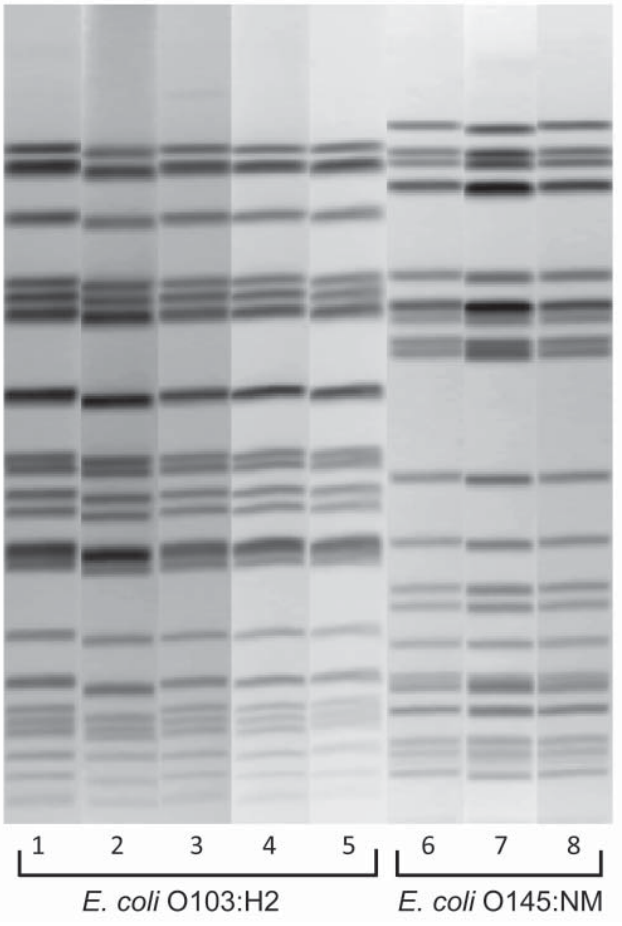

Figure 2. *Xba*I pulsed-field gel electrophoresis patterns of pathogenic *Escherichia coli* from humans and venison, Minnesota, USA, November 2010. Lanes 2, 4, 6, and 8, isolates from venison. Lanes 1, 3, 5, and 7, isolates from human case-patients.

Table 2. Risk factors for infection with non-O157 Shiga toxin–producing *Escherichia coli* for high school students, Minnesota, USA, November, 2010

Risk factor	No. positive patients/total (%)	No. positive patients/total (%)	Odds ratio (95% CI)
Consumption of undercooked venison	11/26 (42)	11/60 (18)	3.27 (1.18–9.03)
Handwashing*	5/9 (56)	15/16 (94)	0.08 (0.01–0.93)
Wearing gloves†	18/22 (81)	54/56 (96)	0.15 (0.03–0.94)

*Among 25 students who had contact with raw venison or helped clean up on November 23.
†Among 78 students who had contact with raw venison during the class on November 17.

other food handling errors, including using the same plate for raw and cooked venison, using the same tongs to handle raw and cooked venison, and not washing hands after bare-hand contact with raw venison.

Venison butchered at the school and collected from 2 households was positive for *E. coli* O103:H2, which was indistinguishable from the isolates from the 2 case-patients by PFGE. One sample of venison butchered at the school was positive for *E. coli* O145:NM and was indistinguishable from the isolates from the 2 case-patients by PFGE (Figure 2).

Conclusions

This outbreak of non-O157 STEC was associated with handling and consumption of venison from wild white-tailed deer in a high school class. Venison butchered at the school was positive for the outbreak PFGE subtype of STEC O103:H2 and non-Shiga toxin–producing (stx–) *E. coli* O145:NM.

The role of stx– *E. coli* O145:NM is unknown. Although *E. coli* O145:NM strains isolated from patients 3 and 4 and venison were stx–, other virulence factors, clinical illness, and an enterohemorrhagic *E. coli* serotype suggest a potentially pathogenic strain. Human infections with stx– *E. coli* serotypes may cause bloody diarrhea and hemolytic uremic syndrome (*8*). Further characterization of virulence determinants and phylogeny of these strains may shed light on their pathogenicity.

Multiple potential routes of transmission from venison to case-patients were identified, included consumption of venison and cross-contamination from raw to cooked venison. Handwashing after touching raw venison or contaminated surfaces was protective. Interviews with the butcher ruled out cross-contamination from domestic ruminants to venison during butchering. Therefore, we conclude that ≥1 deer were colonized with non-O157 STEC.

A study of white-tailed deer feces in Minnesota and Wisconsin found non-O157 STEC in 5% of samples (*9*). Studies have found non-O157 STEC contamination of deer meat ranging from 7.5% of roe deer meat in Germany to 22% of fallow deer meat in Belgium (*5*). Prevalence rates of *E. coli* O157 in deer have ranged from 0.25% to 2.4% (*10–12*). Previous outbreak investigations and case reports have linked *E. coli* O157 infections to deer (*13–15*). This outbreak indicates that white-tailed deer are a source of human non-O157 STEC infections. Venison should be handled and cooked with the same caution recommended for other meats.

Acknowledgments

We thank the Public Health Laboratory staff and Foodborne Disease Unit staff at the Minnesota Department of Health; and Laboratory Services Division staff and Dairy, Food, and Meat Inspection Division staff at the Minnesota Department of Agriculture for assistance during this study.

This study was supported in part through cooperative agreements with the Centers for Disease Control and Prevention Emerging Infections Program, Foodborne Diseases Active Surveillance Network (FoodNet) (U50/CCU511190-10) and the Centers for Disease Control and Prevention Epidemiology and Laboratory Capacity for Infectious Diseases Program (U50/CCU519683-04-4).

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High Seroprevalence of Enterovirus Infections in Apes and Old World Monkeys

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To estimate population exposure of apes and Old World monkeys in Africa to enteroviruses (EVs), we conducted a seroepidemiologic study of serotype-specific neutralizing antibodies against 3 EV types. Detection of species A, B, and D EVs infecting wild chimpanzees demonstrates their potential widespread circulation in primates.

Enteroviruses (EVs) form a diverse genus in the virus family *Picornaviridae*. EVs that infect humans are divided genetically into 4 species (EV A–D), and each contains numerous antigenically distinct serotypes (*1*). Although EVs were originally classified by serologic analysis and pathogenic properties in laboratory animals, sequences from the viral capsid region provide an alternative method for classification (*2*). More recently, classified variants have been assigned as chronologically numbered EV types (currently to EV-C116).

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DOI: http://dx.doi.org/10.3201/eid1802.111363

EVs also naturally infect other mammalian species, although most are in separate species from those that infect humans. However, EVs isolated from Old World monkeys (OWMs) (principally Asian macaques) are grouped into species A and B; a separate simian species (SEV-A); or are unassigned (EV-108, SV6, and EV-103) (*3*).

Although EV isolates from OWMs have been extensively characterized, little attention has been paid to EVs that circulate in apes. We recently detected EV-A76 (species A) and a new EV type in species B and D (EV-B110 and EV-D111) that infect a wild population of chimpanzees (*Pan troglodytes*) in Cameroon (*3*). Detection frequencies of 15% in fecal samples suggest that EV infections are relatively common in this species. We estimated population exposure of apes in Africa and OWM species to EVs.

The Study

To estimate population exposure of apes and OWM species in Africa to EVs, we conducted a seroepidemiologic study of serotype-specific neutralizing antibodies against 3 EV types. These seroprevalences were compared with seroprevalences in human populations in areas where primates also lived (Cameroon, Zimbabwe, and South Africa) and with those in control populations in Europe (United Kingdom and Finland). Ethical approval for the use of study samples was obtained from the University of Zimbabwe Institutional Review Board and the Medical Research Council of Zimbabwe; the Human Research Ethics Committee, South African National Blood Service; the ethics committees of the Cameroonian Ministry of Health; the Centre Hospitalier Universitaire de Sherbrooke, Canada; and Lothian Regional Ethics Committee, Edinburgh.

EV-D94 (E210), EV-A76 (KAZ00–14550) (*4*,*5*), and a clinical isolate of echovirus 11 from Edinburgh (E-11) were used for seroprevalence studies. Neutralization assays were performed in human rhabdomyosarcoma cells as described (*6*) with 1 minor change (inactivation at 56°C for 45 min). Serum specimens at 2 dilutions (1:16 and 1:64) were incubated with virus (one hundred 50% tissue culture infectious doses) in 96-well plates. Rhabdomyosarcoma cells were added to wells (≈2×10^5 cells/mL), and cultures were incubated at 37°C for ≤6 days. The highest dilution that completely inhibited viral replication was taken as the endpoint titer for the sample.

Plasma samples were collected from chimpanzees (*P. troglodytes*), gorillas (*Gorilla gorilla gorilla*), and several OWMs (Table 1). Sample shipments complied with the Convention on International Trade in Endangered Species of Wild Fauna and Flora. Samples were collected for veterinary welfare purposes from animals in 2 wildlife sanctuaries in Yaoundé and Limbe, Cameroon. Animals

Table 1. Seroprevalence of echovirus and enterovirus among apes and Old World monkeys, sub-Saharan Africa and Europe

Taxonomic name (common name)	No. tested	Date	No. (%) positive		
			Echovirus 11	Enterovirus A76	Enterovirus D94
Apes					
Pan troglodytes (chimpanzee)	40	2005–2009	23 (57.5)	16 (40.0)	5 (12.5)
Gorilla gorilla gorilla (gorilla)	9	2004–2008	8 (72.0)	1 (11.0)	0
Total	49	2004–2009	31 (63.0)	17 (35.0)	5 (10.0)
Old World Monkeys					
Genus *Cercopithecus*					
C. erythrotis (red-eared monkey)	3	2007–2008	0	0	0
C. preussi (Preussi's monkey)	3	2007–2009	0	1 (33.0)	0
C. pogonias (crowned monkey)	1	2009	0	0	0
C. mona (mona monkey)	7	2006–2009	4 (57.0)	1 (14.0)	0
C. nictitans (spot-nosed monkey)	3	2007–2009	0	0	0
Genus *Madrillus*					
M. sphinx (mandrill)	5	2006–2009	1 (20.0)	3 (60.0)	0
M. leucophaeus (drill)	16	2006–2009	3 (18.8)	5 (28.0)	1 (6.0)
Genus *Chlorocebus*					
C. tantalus (tantalus monkey)	1	2007	0	1 (100.0)	0
Genus *Erythrocebus*					
E. patas (patas monkey)	2	2008	0	2 (100.0)	0
Genus *Papio*					
P. anubis (olive baboon)	19	2006–2009	1 (5.3)	9 (47.0)	0
Total	60	2006–2009	9 (15.0)	22 (37.0)	1 (2.0)

were primarily wild born and brought to sanctuaries after confiscation by authorities or abandonment by owners. Human samples were obtained from 3 sub-Saharan African populations and control groups in the United Kingdom and Finland (Table 2). None had identifiable compounding risk factors that influenced their exposure to EVs. Plasma was separated from anticoagulated blood by centrifugation and stored at −70°C until testing.

The study was designed to determine the extent to which a human EV serotype (E-11) could spread into nonhuman populations, and conversely, the extent to which EV-A76 (previously recovered from chimpanzees) circulated in human populations in areas where chimpanzees also lived (Cameroon), elsewhere in Africa in regions without apes, and in nonprimate-exposed control populations in Europe. Species D viruses are frequently isolated from chimpanzees and gorillas (*3*), and we selected EV-D94, isolated from populations in central Africa, as a representative of this species.

Chimpanzees and gorillas showed evidence (Figure) of extensive previous exposure to E-11 (58% and 72%) and EV-A76 (40% and 11%). Lower levels of antibodies were detected against EV-D94 (13% and 0%). Conversely, OWMs showed higher seroreactivity with EV-A76 (37%) than with E-11 (15%) and EV-D94 (2%), which demonstrated wide circulation of this virus among OWM species. Seven samples from mona monkeys accounted for half of E-11–positive samples, and EV-A76 antibodies were widely distributed in baboons, mandrills, and other species.

Table 2. Human samples from sub-Saharan Africa and Europe tested for echovirus and enterovirus antibodies*

Country	Category	Mean age, y	Year
Cameroon	General population	>60	2007
Zimbabwe	General population	45	2007
South Africa	Blood donors	25	2009
Finland	Pregnant women	30	2002
UK	General population	55	2009

*Forty samples from each country were used.

Contrasting patterns of EV exposure to the 3 EV types were observed in humans (Figure). High seroprevalences of E-11 and EV-D94 were observed in all human populations (53%–95% and 63%–85%), whereas seroreactivity to EV-A76 was largely confined to Cameroon (55%) and Zimbabwe (35%), and uniformly <6% elsewhere.

Conclusions

Serologic testing of nonhuman primate samples provided unequivocal evidence of exposure to all 3 serotypes. Although primate sampling was restricted to animals held in sanctuaries under veterinary supervision and infections may have been acquired in captivity from human or dietary sources, epidemiologic observations support the hypothesis that EV infections may also be acquired in the wild. Many sampled animals were adults on entry to sanctuaries, while analogous to human infections, and EV exposure is frequent during infancy or childhood. Also, EV infections in apes are widespread in the wild, and active infections are found in more than one sixth of animals screened (*3*).

Another factor determining whether EVs can be indigenous to a species or originate from repeated external (cross-species) transmission is host population size. Perpetuation of nonpersistent viruses requires minimum population sizes large enough to sustain chains of transmission, which is dependent on duration of infectivity, seasonality of infections, duration of

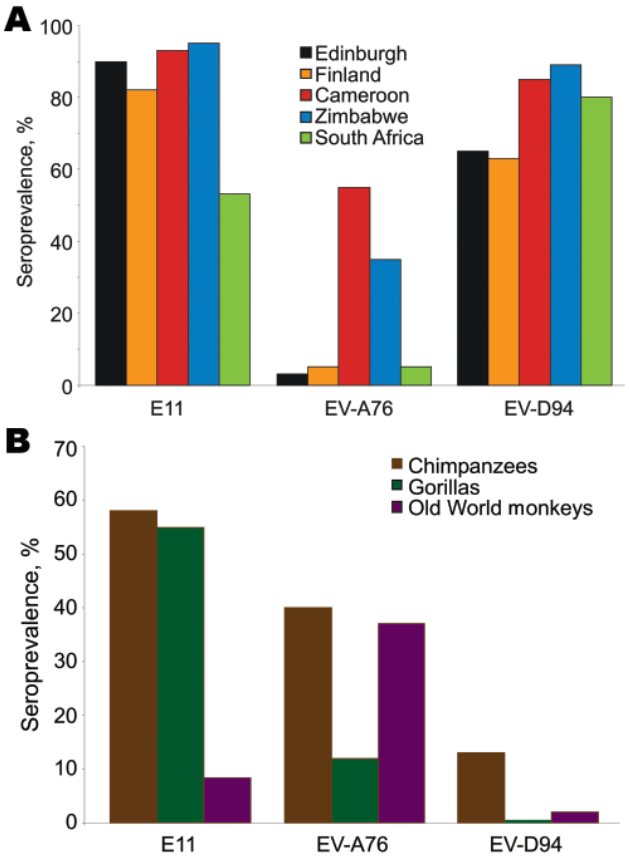

Figure. Seroprevalence of neutralizing antibody (titers ≥16) to echovirus 11 (E-11) and enteroviruses A76 (EV-A76) and D94 (EV-D94) in A) human populations and B) nonhuman primates.

immunity, generation time of the virus and host, rate of population turnover (*7*,*8*), and degree of fragmentation of populations. These parameters are difficult to estimate in nonhuman primates, although studies of isolated human communities show that population size needed for maintaining transmission of EVs may be large, e.g., poliovirus infections were not sustained among an Eskimo community of 450 persons (*9*). Therefore, chimpanzee and gorilla populations may be too small and fragmented to sustain EV infections. However, relatively long-term fecal excretion of EVs, the environmental stability of shed EVs, and contamination of nest sites may perpetuate infections within an established group.

In contrast to apes, the population size of several OWM species is large and with higher population connectivity, turnover, and supply of susceptible animals, may support indigenous circulation of EVs. Supporting this suggestion is genetic evidence that EVs isolated from OWMs in southern Asia and Africa group separately from most variants identified in humans elsewhere by phylogenetic analysis (*10*). EV variants in chimpanzees that matched OWM serotypes (e.g., EV-B110 most closely related to SA5, and EV-A76, EV-A89, and EV-A90 in the OWM species A group) (*3*; unpub. data) suggest that OWMs are a potential source of infection. Predation of the red colobus monkey by chimpanzees (*11*,*12*) may favor such cross-species transmissions, as documented in the genesis of simian immunodeficiency virus$_{CPZ}$ from OWM simian immunodeficiency viruses (*13*). Cross-species transmission from OWMs to apes is consistent with high seroprevalences of EV-A76 antibodies in apes, baboons, and other OWMs.

Overall, our serologic survey data and previous fecal sampling data (*3*) provide evidence for extensive circulation of EVs between primates and existence of human and OWM reservoirs of infection that may spill over into ape populations too small to maintain indigenous EV variants. Whether OWMs or apes represent a potential source of new EVs in humans (that may become pandemic in the absence of prior population exposure) is uncertain. However, the global outbreak of EV-D70 that originally centered on a cluster of human infections in central Africa (*14*,*15*) provides a potential example of this occurrence. Extensive past infection of a variety of EVs in apes and OWMs should lead to a reappraisal of the host range of what have been considered to be primarily human viruses and a potential source for the periodic emergence of new EV types into immunologically naive human populations.

Acknowledgments

We thank the Cameroon Ministry of Forestry and Fauna, Limbe Wildlife Centre, Ape Action Africa, and the US Embassy in Cameroon for support; the Ministry of Health and Child Welfare in Zimbabwe; the Provincial Medical Director of Mashonaland East; and the environmental health workers, residents, teachers, and school children in Mutoko and Rusike for their cooperation; and members of the National Institutes for Health Research (Zimbabwe) for technical support.

This study was supported by the Wellcome Trust, UK (grant no. WT082028MA), and the Thrasher Foundation. Global viral forecasting is supported by Google.org, the Skoll Foundation, the Henry M. Jackson Foundation for the Advancement of Military Medicine, the US Armed Forces Health Surveillance Center Division of Global Emerging Infections Surveillance Operations, and the US Agency for International Development Emerging Pandemics Threat Program (PREDICT Project) under Cooperative Agreement no. GHN-A-OO-09-00010-00. F.M. was supported by Medical Research Council UK (grant no G81/538). N.D.W. was supported by the National Institutes of Health Director's Pioneer Award (DP1-OD000370),

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Multiorgan Dysfunction Caused by Travel-associated African Trypanosomiasis

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We describe a case of multiorgan dysfunction secondary to *Trypanosoma brucei rhodesiense* infection acquired on safari in Zambia. This case was one of several recently reported to ProMED-mail in persons who had traveled to this region. Trypanosomiasis remains rare in travelers but should be considered in febrile patients who have returned from trypanosomiasis-endemic areas of Africa.

We describe a British safari tourist with multi-organ dysfunction and shock secondary to African trypanosomiasis. This case illustrates the complications associated with treatment of *Trypanosoma brucei rhodesiense* infection and highlights a recent increase in cases reported to ProMED (www.promedmail.org) of trypanosomiasis in travelers to Zambia.

The Case-Patient

A 49-year-old woman with a 5-day history of fever, malaise, headache, dizziness, abdominal discomfort, diarrhea, and vomiting sought treatment 1 day after returning to the United Kingdom from a 2-week safari in Zambia. During the safari, she spent 3 days in the South Luangwa National Park, 3 days in the Lower Zambezi National Park, and 6 days in Kafue National Park. Initial blood films examined at Furness General Hospital were negative for malaria parasites but positive for trypanosomes. Urgent transfer of the patient to the Tropical and Infectious Disease Unit in Liverpool, UK, was arranged.

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DOI: http://dx.doi.org/10.3201/eid1802.111479

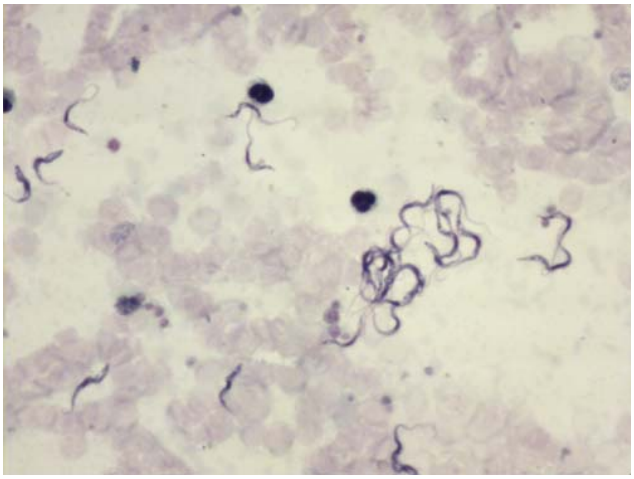

Figure. Thick film using Field's stain showing trypanosomes under ×400 magnification. Motile trypanosomes are shown in the online Video (wwwnc.cdc.gov/EID/article/18/2/11-1479-V1.htm).

Upon arrival, the patient was dehydrated and had jaundice and tachycardia, but she initially was normotensive. Examination revealed reduced breath sounds at the lung bases and a distended, nontender abdomen. There was a mild erythematous rash on the patient's abdomen, but no chancres. Results of a neurologic examination were unremarkable.

Repeat blood films confirmed numerous trypanosomes (Figure), which, given the patient's travel history, were considered likely to be *T. b. rhodesiense*. PCR results confirmed the trypanosomes positive for the *T. b. rhodesiense*–specific serum resistance–associated gene (*1*). Blood test results were as follows: urea 9.2 mmol/L, creatinine 146 μmol/L, leukocytes 3.7 × 10^9 cells/L, platelets 13 × 10^9/L, C-reactive protein 234 mg/L, alanine aminotransferase 179 U/L, bilirubin 38 μmol/L, and prothrombin time 13.5 s.

Despite fluid resuscitation, the patient became increasingly hypotensive over the next 12 hours, prompting transfer to the intensive care unit. A 100-mg test dose of suramin was well tolerated by the patient; however, the first full treatment dose was complicated by circulatory collapse and bronchoconstriction, which required administration of hydrocortisone and chlorphenamine and immediate discontinuation of the suramin infusion. Subsequent investigation showed that the suramin dose had been infused more rapidly than prescribed. Further doses administered as slow infusions were uncomplicated.

Hypotension persisted for 4 days but did not necessitate vasopressors. Results of a short synacthen test, electrocardiogram, and echocardiogram were normal.

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After the patient received 2 treatment doses of suramin and analysis of repeat blood films confirmed that parasitemia had cleared, a lumbar puncture was performed. The cerebrospinal fluid (CSF) had 4 leukocytes/μL and normal levels of protein and glucose, and no trypanosomes were detected after double centrifugation.

The patient received a full course of suramin for early-stage disease, (regimen in Table 1), which resulted in full recovery. As follow-up care, the patient will receive repeat lumbar punctures every 3 months for 2 years to exclude occult invasion of the central nervous system (CNS). Thus far, 3 repeat lumbar punctures have shown no evidence of CNS invasion.

African trypanosomiasis is caused by the protozoan parasite *T. brucei*, which is transmitted by tsetse flies. Two subspecies are pathogenic in humans: *T. b. gambiense* in central and western Africa, and *T. b. rhodesiense* in eastern and southern Africa.

Disease progresses in 2 stages. In the first stage, parasites spread in the blood to the lymph nodes, liver, spleen, heart, endocrine system, and eyes (*4*). Untreated, they invade the CNS, which leads to second-stage or meningoencephalitic disease with characteristic sleep disturbances. Progression to the second stage may take months in *T. b. gambiense* infection but only weeks in *T. b. rhodesiense* infection.

Although trypanosomiasis is uncommon in travelers, it should be considered in the differential diagnosis of patients with fever who have returned from trypanosomiasis-endemic areas of Africa (*5*). Recent reports suggest an increase in cases emerging from Zambia, particularly from the South Luangwa Valley (Table 2) (*6*). Whether these cases reflect an increased risk for infection in that region or increasing tourism in a trypanosomiasis-endemic area is unclear. Infection in travelers is usually characterized by an acute febrile illness, sometimes associated with a macular evanescent rash or chancre (*2*,*4*). Laboratory tests often indicate anemia, thrombocytopenia, leukopenia, impaired renal function, electrolyte disturbances, coagulation abnormalities, and elevation in hepatic transaminase and C-reactive protein levels (*2*,*7*).

Conditions that should be considered in patients with persistent hypotension are adrenal insufficiency and cardiac dysfunction. The prevalence of adrenal insufficiency was 27% in a study of Ugandan patients with trypanosomiasis (*8*). Myocarditis, pericarditis, and congestive cardiac failure have been described and should be excluded by electrocardiogram and echocardiography (*4*).

The treatment for first-stage *T. b. rhodesiense* infection is intravenous suramin, given as 5 injections of 20 mg/kg each over 3–4 weeks (*2*,*4*). Early hypersensitivity reactions to suramin (i.e., nausea, circulatory collapse, and urticaria) are described in 0.1%–0.3% of patients; thus, an initial test dose is advocated (*9*).

Second-stage *T. b. rhodesiense* infection is treated with melarsoprol, a highly toxic arsenical which causes a severe reactive encephalopathy in ≈10% of patients, half of whom die as a result (*10*). This toxicity among patients emphasizes the importance of accurate staging, which is determined by CSF examination. According to World Health Organization guidelines, the presence of >5 leukocytes/μL and/or the presence of trypanosomes in the CSF indicates second-stage disease (*11*). Lumbar puncture should be deferred until clearance of blood parasitemia has been confirmed.

In view of our patient's rapid onset of a high level of parasitemia, we investigated the possibility of a genetic susceptibility to trypanosomal infection. Human plasma contains a trypanosome lytic factor called apolipoprotein L-1 (APOL1) (*12*). This protein causes lysis of *T. brucei* subspecies other than *rhodesiense* and *gambiense,* both of which have acquired resistance to it (*13*). In 2006, Vanhollebeke et al. (*14*) described a patient infected with *T. evansi*, which is usually sensitive to APOL1. The patient's serum lacked APOL1 due to mutations in the *APOL1* gene, rendering him susceptible to a species regarded as nonpathogenic in humans. We sequenced the *APOL1* gene of our patient, but no substantial variations suggesting enhanced susceptibility were detected.

Conclusions

In summary, trypanosomiasis remains rare in travelers, but possible infection should be considered in patients with

Table 1. Treatment regimen for *Trypanosoma brucei rhodesiense* infection in adults*

Disease stage	Drug, route of administration	Regimen	Adverse effects
First	Suramin, intravenous	Test dose of 100 mg in 100 mL 0.9% saline over 30 min on day 0; and 5 doses of 20 mg/kg (maximum 1 g/dose) in 250 mL 0.9% saline over 3 h on days 1, 3, 7, 14, 21	Hypersensitivity reactions (early and late); nephrotoxicity, hepatotoxicity, hemolytic anemia, peripheral neuropathy, agranulocytosis, thrombocytopenia, and cutaneous reactions
Second	Melarsoprol, intravenous	2.0–3.6 mg/kg/d (maximum 180 mg/d) for 3 d; after 7 d, 3.6 mg/kg/d for 3 d; after 7 more d, 3.6 mg/kg/d for 3 d†	Encephalopathy, cutaneous reactions, peripheral neuropathy, cardiac arrhythmias, thrombophlebitis, fever, and gastric upset

*Source of drug regimen: Brun et al. (*2*) and Abramowicz (*3*).
†In frail patients, begin with 18 mg melarsoprol and progressively increase dose (*3*). Pretreatment with suramin for 2–4 d is recommended for debilitated patients.

Table 2. Reports to ProMED-mail of *Trypanosoma brucei rhodesiense* infections associated with travel to or bordering Zambia, 2010*

Month of report	ProMED-mail archive no.	Nationality of patient	Travel activity	Area visited
September	20100915.3338	Zambian	Visiting game ranch	South Luangwa Valley, Zambia
	20100915.3338	American	Hunting safari	South Luangwa Valley, Zambia
October†	20101022.3833	British	Camping safari	South Luangwa National Park, Lower Zambezi National Park, Kafue National Park, Zambia
	20101022.3833	British	Visiting national park	Mana Pools National Park, Zimbabwe (bordering Zambia)
November	20101111.4093	South African national of Scandinavian origin	Hiking	Luangwa River area, Zambia

*Reports in (*6*). Since the start of 2005, 11 other cases of *T. b. rhodesiense* infection in travelers have been reported in ProMED-mail; those cases were acquired in Uganda (1), Tanzania (3), and Malawi (7).
†Case presented in this report.

fever who have returned from trypanosomiasis-endemic areas of Africa. Early reporting of trypanosomiasis cases to ProMED-mail allows timely recognition of emerging safari destinations that present an increased risk for infection to travelers. In patients with *T. b. rhodesiense* infection, multiorgan dysfunction may develop in early-stage disease. Treatment of such cases should be managed with critical care support, and it should be remembered that rapid infusion of suramin may precipitate circulatory collapse.

Acknowledgments

We acknowledge Anthony Macheta from Furness General Hospital.

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Phylogeography of *Francisella tularensis* subsp. *holarctica*, Europe

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Francisella tularensis subsp. *holarctica* isolates from Austria, Germany, Hungary, Italy, and Romania were placed into an existing phylogeographic framework. Isolates from Italy were assigned to phylogenetic group B.FTNF002–00; the other isolates, to group B.13. Most *F. tularensis* subsp. *holarctica* isolates from Europe belong to these 2 geographically segregated groups.

Francisella tularensis is the etiologic agent of tularemia and a highly virulent category A biothreat agent (*1*,*2*). The most widely distributed subspecies is *F. tularensis* subsp. *holarctica*, which is found throughout much of the Northern Hemisphere and is the only subspecies found in Europe (*3*). Despite its wide geographic distribution, *F. tularensis* subsp. *holarctica* contains low genetic diversity, which indicates recent emergence (*4*). A recent global phylogeographic analysis (*5*), and several subsequent analyses (*6*–*9*), assigned most isolates from Europe to 2 phylogenetic groups: B.FTNF002–00 and B.13 (includes multiple subclades descended from branch B.13 [*5,6,8*]; branch and subclade nomenclature from [*5*] has been shortened by removing Br and extra 0s from individual branch and subclade names). These groups appear to be geographically segregated: only isolates from B.FTNF002–00 have been reported from the western European countries of Spain, France, and Switzerland, whereas B.13 is the only or dominant type reported from the Czech Republic, Finland, Georgia, Russia, Slovakia, and Ukraine (*5*–*9*). We provide additional information about the geographic distribution of these 2 groups using existing phylogenetic signatures (*5*,*8*) to place 45 isolates from Austria, Germany, Hungary, Italy, and Romania (online Appendix Table, wwwnc.cdc.gov/EID/article/18/2/11-

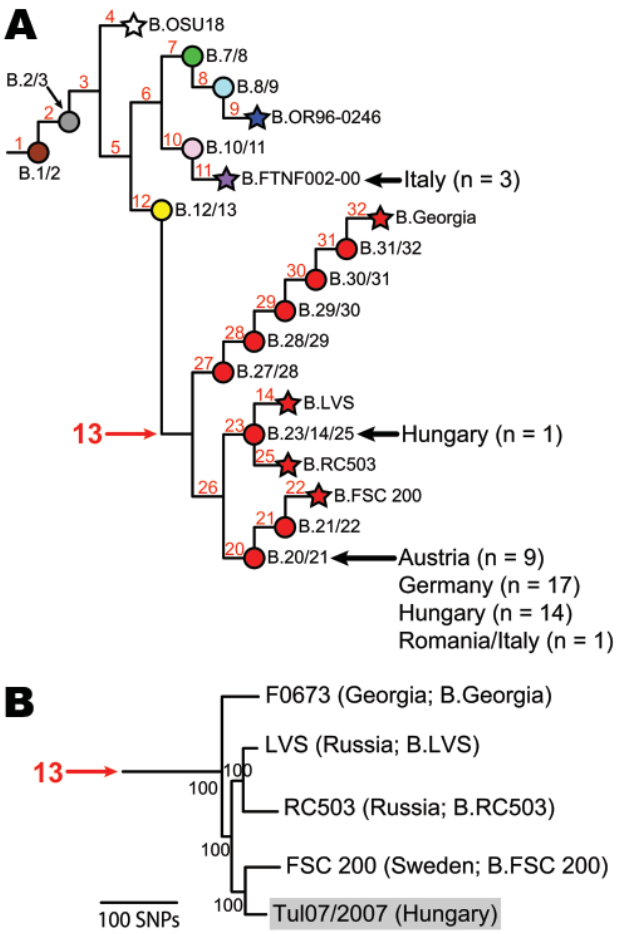

Figure 1. Existing phylogeny of *Francisella tularensis* subsp. *holarctica*. A) Single nucleotide polymorphism (SNP)–based phylogeny of *F. tularensis* subsp. *holarctica* derived from previous studies (*5,6,8*). Terminal subgroups representing sequenced strains are shown as stars, and intervening nodes representing collapsed branches are indicated by circles. Subclades within group B.13 are depicted in red. Isolates from Austria, Germany, Hungary, Italy, and Romania (n = 45) were assigned to existing subclades (black arrows) by using existing canonical SNP assays (*5,8*). B) Maximum parsimony phylogeny constructed by using SNPs discovered from 6 *F. tularensis* whole-genome sequences, including 5 strains from group B.13 and an outgroup strain, OSU18 (not shown). This phylogeny was rooted by using OSU18, and bootstrap values were based on 1,000 simulations by using a heuristic search. The newly sequenced Hungarian strain (Tul07/2007) is highlighted in gray.

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DOI: http://dx.doi.org/10.3201/eid1802.111305

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1305-TA1.htm) into the existing global phylogeographic framework.

The Study

All of the isolates were assigned to group B.FTNF002–00 or to group B.13. All 3 isolates from Italy were assigned to group B.FTNF002–00 (Figure 1, panel A). Although the sample size was small, these isolates were obtained in 3 different years (online Appendix Table), which suggests that this group is ecologically established in Italy. These results increase the known geographic distribution of this group, which appears to be the dominant clone in western Europe (Figure 2, panel A, purple shading). All 42 isolates from Austria, Germany, Hungary, and Romania were assigned to group B.13 (Figure 1, panel A), further demonstrating that B.13 is the most prevalent group of *F. tularensis* subsp. *holarctica* in central and eastern Europe (Figure 2, panel A, red shading). Within group B.13, one isolate from Hungary was assigned to subclade B.23/14/25 (Figure 1, panel A); isolates from Finland, Russia, and Sweden were previously assigned to this subclade (*6,8*) (Figure 2, panel B). However, the other 41 isolates were assigned to subclade B.20/21 (Figure 1, panel A).

We identified new genomic signatures to provide increased genetic resolution within subclade B.20/21. Next-generation sequencing technology (Illumina Inc., San Diego, CA, USA) was used to sequence the genome of an isolate from Hungary (Tul07/2007, GenBank accession no. SRX025133) assigned to subclade B.20/21. Putative single nucleotide polymorphisms (SNPs) were identified in the resulting sequence and the genomes of 4 other strains previously assigned to group B.13 (LVS, AM233362.1; FSC 200, AASP00000000; RC503, SRX000104; Georgia F0673, SRX025885) by using an existing bioinformatics pipeline (*5*). The more distantly related strain OSU18 (CP000437.1) genome was also included as an outgroup. A maximum-parsimony tree was constructed by using the resulting ≈700 putative SNPs and PAUP 4.0b10 software (Sinauer Associates, Inc., Sunderland, MA, USA) (Figure 1, panel B). Most of the putative SNPs separated OSU18 from the B.13 strains (data not shown), but the remaining putative SNPs provided resolution among the B.13 strains,

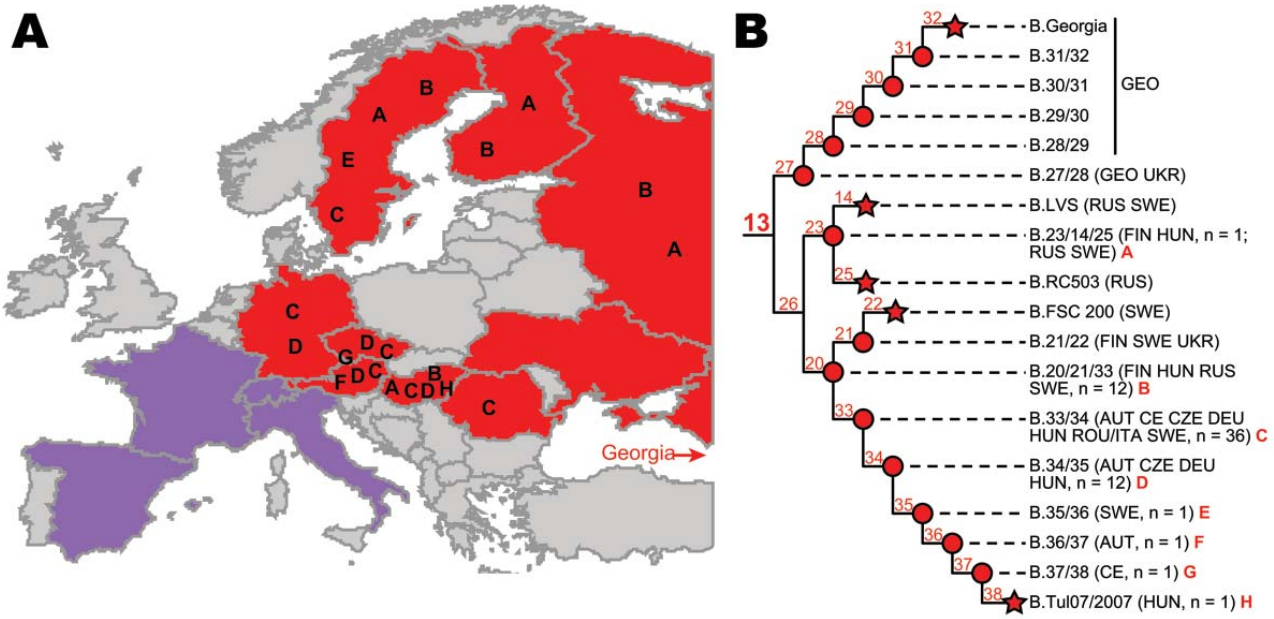

Figure 2. Detailed geographic distribution and phylogeny of *Francisella tularensis* subsp. *holartica* subclades within group B.13. A) Countries from which groups B.13 and B.FTNF002–00 have been reported. Countries of origin for isolates assigned to select subclades within group B.13 are indicated by the letters A–H. Red and purple shading indicates the known geographic distributions of groups B.13 and B.FTNF002–00, respectively, in this and previous studies (*5–9*). The country of Georgia, which also contains isolates from group B.13 but is not depicted in the map, is indicated by red text and a red arrow pointing toward its location. Isolates assigned to other phylogenetic groups within *F. tularensis* subsp. *holarctica* have been reported from some of these countries (*5,8*), but most isolates from these countries are from groups B.13 and B.FTNF002–00. B) Single nucleotide polymorphism–based phylogeny of previously (*5,6,8*) and newly identified subclades within the B.13 group of *F. tularensis* subsp. *holarctica*. Terminal subgroups representing sequenced strains are shown as stars, and intervening nodes representing collapsed branches are indicated by circles. The countries of origin for isolates assigned to each subclade are indicated: AUT, Austria; CE, central Europe, unknown country; CZE, Czech Republic; DEU, Germany; FIN, Finland; GEO, Georgia; HUN, Hungary; ITA, Italy; ROU, Romania; RUS, Russia; SWE, Sweden; UKR, Ukraine). For mapping purposes, letters are assigned to a previously identified subclade that contains a new isolate from Hungary now assigned to that subclade (A) and newly identified subclades (B–H). The number of isolates listed for each subclade refers only to isolates examined directly in this study (online Appendix Table, wwwnc.cdc.gov/EID/article/18/02/11-1305-TA1.htm).

including 20 putative SNPs specific to the branch leading to the strain from Hungary (Figure 1, panel B). Consistent with previous analyses (Figure 1, panel A), the strain from Hungary clustered as a sister taxon to strain FSC 200 (Figure 1, panel B).

To show additional phylogenetic structure within subclade B.20/21, we designed genotyping assays targeting the 20 putative SNPs along the branch leading to the strain from Hungary (Figure 1, panel B) and screened them across 64 isolates assigned to subclade B.20/21. This analysis included the 41 isolates from Austria, Germany, Hungary, and Romania, as well as 23 additional isolates from central Europe, the Czech Republic, Finland, Russia, and Sweden that were previously assigned to this subclade (*6,8*) (online Appendix Table). The assays were constructed and performed as described (*5*) by using an annealing temperature of 60°C. All 20 SNPs were laboratory confirmed, and 52 of the isolates were assigned to 6 new subclades (B.33/34, B.34/35, B.35/36, B.36/37, B.37/38, and B.Tul07/2007); the 12 other isolates remained in the basal subclade, now identified as B.20/21/33 (Figure 2, panel B; online Appendix Table). Information about assays targeting canonical SNPs for the branches leading to the 6 new subclades are presented in the Table.

Conclusions

Our results are consistent with complex dispersal patterns within the B.13 group of *F. tularensis* subsp. *holarctica*. Several of the B.13 subclades identified in this study are broadly distributed throughout central and eastern Europe (Figure 2, panel A), including subclades B.20/21/33, B.33/34, and B.34/35. All of the new subclades containing >1 isolate have representatives from multiple countries (Figure 2, panel B). Other previously identified B.13 subclades, including B.27/28, B.LVS, B.23/14/25, and B.21/22 are also broadly distributed (Figure 2, panel A).

This study and previous studies have increased understanding of *F. tularensis* subsp. *holarctica* in Europe by placing isolates from multiple countries into the existing global phylogeographic framework. As a result, the genetic background is becoming defined for each country (i.e., the specific subtypes reported from each country). This information can be useful for identifying intentional (e.g., bioterrorism) or unintentional movement of *F. tularensis* subsp. *holarctica* between countries. For example, the isolate from Romania examined in this study was actually isolated in Italy from an infected hare that was shipped from Romania for hunting. Genotyping results are consistent with a Romanian origin for this isolate because it was assigned to the B.13 group that is widespread in central and eastern Europe (Figure 2, panel A) and not to the B.FTNF002–00 group, to which the isolates from Italy were assigned (Figure 1, panel A).

Understanding global phylogeographic patterns is possible only if isolates from multiple geographic locations are placed within the same framework (i.e., examined with the same genomic signatures). Because *F. tularensis* is genetically monomorphic and highly clonal, SNPs are preferred signatures for determining phylogenetic structure within this species (*3*). Vogler et al. (*5*) conducted the first SNP-based global phylogeographic analysis of *F. tularensis*. Subsequent studies (*6*–*8*) have used the SNP

Table. Melt-MAMA primers targeting canonical SNPs for 6 new phylogenetic branches in a study of *Francisella tularensis* subsp. *holarctica*, Europe*

SNP	SCHU† S4 position	SNP state, D/A‡	Primers, 5′ → 3′§	Con, μM¶	T_m, °C
B.33	78,382	T/C	A: ATTGCTACTTCTATTTACGCCAACAG	0.20	74.3
			D: GGGGCGGGGCGGGGCATTGCTACTTCTATTTACGCCAAGAA	0.20	79.0
			C: TGTGAACAACCAAGTTGGCTT	0.20	
B.34	766,614	A/G	A: GTAGCGAGCATTATTTGCTGGTTC	0.40	69.2
			D: GGGGCGGGGCGGGGCTAGCGAGCATTATTTGCTGGGTT	0.20	78.6
			C: ATAAAACTATAAATTTACATAAAATGAAAACTTCTC	0.20	
B.35	239,479	A/C	A: GCCTTAATCTAGTATTTTCGCTTATCTCC	0.40	70.3
			D: GGGGCGGGGCGGGGCGCCTTAATCTAGTATTTTCGCTTATCACA	0.20	75.5
			C: CGGGCTCTAAAATAAGATTTAAGTTAGTAAGT	0.20	
B.36	1,599,292	A/C	A: TATTATAGTTTCTAAAAACAGTCTAATTAATTTTG	0.60	69.0
			D: GGGGCGGGGCGGGGCTATTATAGTTTCTAAAAACAGTCTAATTAATTGTT	0.20	73.9
			C: GTTCGACCATGACTACAGTGTTG	0.20	
B.37	318,602	T/C	A: AACATTTTAGGAACTCTACGATGATAAACTTAAC	0.20	69.7
			D: GGGGCGGGGCGGGGCCATTTTAGGAACTCTACGATGATAAACTTGAT	0.20	75.9
			C: GAAATATCTCAATGAAATCTAATTTAACTAAAATCAC	0.20	
B.38	166,885	C/T	A: ATGCCATCAGCCATTTACTACTCACA	0.20	73.7
			D: GGGGCGGGGCGGGGCCCATCAGCCATTTACTACTCCCG	0.20	80.1
			C: CTTCCCTGATTTTCTAAGTTCTGCTTG	0.20	

*Melt-MAMA, melt-mismatch amplification mutation assay; SNP, single nucleotide polymorphism; con, concentration; T_m, melting temperature for ancestral and derived Melt-MAMA amplification products.
†SCHU strain GenBank accession no. NC_006570.
‡SNP states are presented according to their orientation in the SCHU S4 reference genome (AJ749949.2): D, derived SNP state; A, ancestral SNP state.
§D, derived allele primer; A, ancestral allele primer; C, common primer; primer tails and antepenultimate mismatch bases are in lower case.
¶Final concentration of each primer in Melt-MAMA genotyping assays.

signatures described by Vogler et al. (*5*) and new SNPs discovered from new whole-genome sequences or multiple sequence typing data to further refine phylogeographic patterns within *F. tularensis*, particularly *F. tularensis* subsp. *holarctica*. These new signatures, when screened across diverse isolate collections, have identified new subclades within preexisting subclades. This pattern will continue as whole-genome sequencing becomes less expensive and more widely available. As a result, the nomenclature of phylogenetic groups within *F. tularensis* and the particular subclade to which a given isolate is assigned are constantly changing and will continue to change, which makes comparison of results and findings across different studies difficult. To address this problem, we have included all known *F. tularensis* subsp. *holarctica* SNP-based phylogenetic groups within our phylogenetic trees (Figure 1, panel A; Figure 2, panel B), including those discovered by other researchers. In addition, for the isolates analyzed in this study (online Appendix Table), where applicable, we have listed the phylogenetic groups to which they were assigned in previous studies.

Acknowledgments

We thank Talima Pearson for helpful discussions and Megan Shuey for technical assistance with whole-genome sequencing.

This work was supported in part by the US Department of Homeland Security Science and Technology Directorate through awards HSHQDC-10-C-00139 and 2010-ST-108-000015.

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Invasive Pneumococcal Pneumonia and Respiratory Virus Co-infections

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To confirm whether respiratory virus infections increase susceptibility to invasive pneumococcal pneumonia, we examined data from 11 influenza seasons (1994–2005) in the United States. Invasive pneumococcal pneumonia was significantly associated with influenza and respiratory syncytial virus activities in 5 seasons. Association strength was higher when strain H3N2 was the predominant influenza A virus strain.

Invasive pneumococcal pneumonia (IPP) diseases cause high rates of illness and death every year. Existing evidence supports the biological plausibility that preceding respiratory viral infections, particularly with influenza virus and respiratory syncytial virus (RSV), increase susceptibility to IPP diseases (*1*). Although it has been generally believed that the increases in IPP diseases in winter relate to increased activity of respiratory viruses, especially influenza virus and RSV (*2–6*), evidence of association of IPP diseases and respiratory virus infections is not conclusive. If such an association is likely, then public health authorities should emphasize that vaccination against influenza, as well as other interventions against influenza and RSV, can reduce incidence of IPP diseases. We believe that cold temperatures, lack of sunshine, and rainy and snowy weather are the main reasons that persons increase their indoor activities during the winter and that respiratory diseases are mainly transmitted by close person-to-person contact (*5,7,8*). Therefore, we conducted separate analyses of the association of IPP with seasonal influenza virus and RSV activities, adjusted by climate variables, for each of 11 influenza seasons from 1994–95 through 2004–05.

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DOI: http://dx.doi.org/10.3201/eid1802.102025

The Study

All data used in this study were recorded for each influenza season (surveillance weeks 40–20) from 1994–95 through 2004–05. Weekly IPP cases were obtained from the Active Bacterial Core Surveillance for Georgia Health District 3. Cases of IPP were defined by isolation of *Streptococcus pneumoniae* from normally sterile sites, e.g., cerebrospinal fluid or blood. Only IPP case-patients (defined as persons with *S. pneumoniae* isolated from pleural fluid or persons with a clinical diagnosis of pneumonia and *S. pneumoniae* isolated from blood or another sterile body site*)* were included in this study. Influenza virus surveillance data were obtained from the World Health Organization. Weekly data were collected from each state from October through May of each influenza season. The influenza virus–positive isolate percent was defined as the percentage of influenza virus–positive isolates out of all influenza specimens. Percentages of isolates positive for influenza A (H1N1), A (H3N2), and B viruses were also calculated.

Weekly RSV data were obtained from the National Respiratory and Enteric Virus Surveillance System (www.cdc.gov/surveillance/nrevss/). For each season, hospitals and laboratories reported to the Centers for Disease Control and Prevention the numbers of specimens tested for RSV by antigen detection each week. The RSV detection percent was defined as the percentage of RSV-positive isolates out of all RSV specimens. The influenza virus and RSV data used in this study were from the US Census South Atlantic Region (www.census.gov/geo/www/us_regdiv.pdf). Data on daily mean temperature (in °F), total sunshine (in hours), and total precipitation (in inches) were obtained from the National Weather Service Atlanta regional weather center at Hartsfield-Jackson International Airport (*9*). The weekly mean temperature, total sunshine, and total precipitation were calculated from daily data.

For each of the 11 influenza seasons, we applied negative binomial regression models with multiple predictors to relate the weekly IPP rates with the indicators of influenza and RSV activities while adjusting for the weekly mean temperature, total sunshine, and total precipitation. The full model used to explore the association of IPP with influenza and RSV was as follows:

$$\log(Y_t) = \alpha + \beta_0 X_t + \beta_1 X_{t-1} + \beta_2 X_{t-2} + \gamma_0 Z_t + \gamma_1 Z_{t-1} + \gamma_2 Z_{t-2} + \lambda W_t + \eta U_t + \theta V_t + \log(N_t)$$

where for week t, Y_t is the incidence of IPP, X_t is the influenza virus–positive isolate percent, Z_t is the RSV detections percent, W_t is the mean temperature, U_t is the total sunshine, V_t is the total precipitation, and N_t is the population size. The reduced model excluded the influenza and RSV terms. We

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Table 1. IPP incidence and associated factors, 11 seasons, Atlanta, Georgia, USA*

Season	No. IPP cases	No. IPP cases/10 million population	No. influenza isolates	No. influenza isolates/100 specimens	No. RSV detections	No. RSV detections/100 specimens	Temperature, °F†	Precipitation, inches‡	Sunshine, h‡
1994–95	633	26.75	1,203	12.67	2,116	23.41	57.44	0.81	79.46
1995–96	643	26.54	916	11.76	2,016	26.25	52.31	1.31	79.38
1996–97	653	26.33	1,382	15.87	1,635	18.64	55.24	0.89	79.30
1997–98	673	25.73	1,876	19.46	1,682	16.83	53.09	1.16	79.78
1998–99	599	23.04	2,073	21.01	1,672	15.61	56.42	0.59	79.60
1999–00	600	22.57	1,532	16.29	878	22.31	55.53	0.62	79.51
2000–01	516	18.97	1,409	15.31	3,458	21.45	52.97	0.85	79.43
2001–02	342	12.34	2,045	20.44	2,001	15.70	56.08	0.66	79.34
2002–03	336	11.90	1,287	14.81	1,713	14.23	53.85	1.18	79.27
2003–04	360	12.19	4122	28.69	4,227	18.50	55.07	0.63	79.75
2004–05	328	11.25	3813	23.34	3,415	14.34	54.94	1.08	79.56
Total	5683	NA	21,658	NA	24,813	NA	NA	9.78	874.37
Mean	517	19.78	1,969	NA	2,256	NA	54.81	0.89	79.49

*Virus data from US Census South Atlantic Region (www.census.gov/geo/www/us_regdiv.pdf). IPP, invasive pneumococcal pneumonia; RSV, respiratory syncytial virus; NA, not applicable.
†Seasonal mean calculated from weekly means.
‡Seasonal mean calculated from weekly totals.

calculated the difference in the log-likelihood ratio statistic between the full and reduced models and used it to test the hypothesis that IPP incidence is not associated with influenza and RSV activities when weekly temperature, sunshine, and precipitation were adjusted for.

For influenza seasons 1994–95 through 2004–05, the average annual incidence of IPP was 19.78 per 10 million persons (Table 1). Table 1 also shows the means of total number of IPP cases; influenza-positive isolates; RSV detections; and the average temperature, sunshine, and precipitation for each of these influenza seasons.

Table 2 shows the result of the negative binomial regression analysis for each influenza season. Significant associations between IPP and both influenza-positive isolate percent and RSV detection percent were found for 5 of the 11 seasons. Table 2 also shows the percentages of the influenza A (H1N1), A (H3N2), and B virus strains in each season. The predominant influenza virus strain (percentage ≥50%, [*10*]) varied across seasons. Influenza A (H3N2) virus was predominant in 8 seasons, and influenza B virus was predominant in 2 seasons. One season had no predominant influenza virus strain. Table 2 also shows a significant association between IPP incidence and influenza virus and RSV activities in 5 of the 8 seasons in which A (H3N2) was the predominant influenza virus strain; there was no significant association in any of the 3 seasons in which influenza A (H3N2) virus was not predominant ($p = 0.12$, 2-sided mid–p-value exact test).

To demonstrate the association of IPP incidence with influenza virus and RSV activities and the climate data, we displayed the weekly IPP, influenza virus, RSV, temperature, sunshine, and precipitation data for seasons 1998–99 and 2003/04 (Figure). Similar figures for the other 9 seasons can be found in the online Technical Appendix (wwwnc.cdc.gov/eid-static/spreadsheets/10-2025-Techapp.xls).

Conclusions

To explore the temporal variability in the association of IPP incidence with influenza virus and RSV activities over a long period, we conducted a season-by-season analysis over 11 influenza seasons. We adjusted the association of IPP with influenza virus and RSV by temperature, precipitation, and sunshine because these factors are

Table 2. Negative binomial regression analysis of the association of invasive pneumococcal pneumonia weekly incidence with influenza and respiratory syncytial virus activities *

Season	p value†	% Influenza A (H1N1)	% Influenza A (H3N2)	% Influenza B
1994–95	**0.029**	1.05	58.98‡	39.96
1995–96	0.2	36.58	30.39	33.03
1996–97	**0.002**	0.58	50.29‡	49.12
1997–98	0.09	0.19	98.66‡	1.15
1998–99	**0.015**	1.04	58.40‡	40.55
1999–00	0.424	3.8	95.75‡	0.45
2000–01	0.069	46.88	1.04	52.08‡
2001–02	0.641	12.09	74.53‡	13.38
2002–03	0.649	30.22	4.64	65.14‡
2003–04	**0.004**	0.03	98.75‡	1.22
2004–05	**<0.001**	0.19	69.97‡	29.85

*Analysis adjusted by temperature, sunshine, and precipitation. p values based on the likelihood ratio test for lack of association.
†**Boldface** indicates a statistically significant association.
‡Predominant (≥50%) influenza strain.

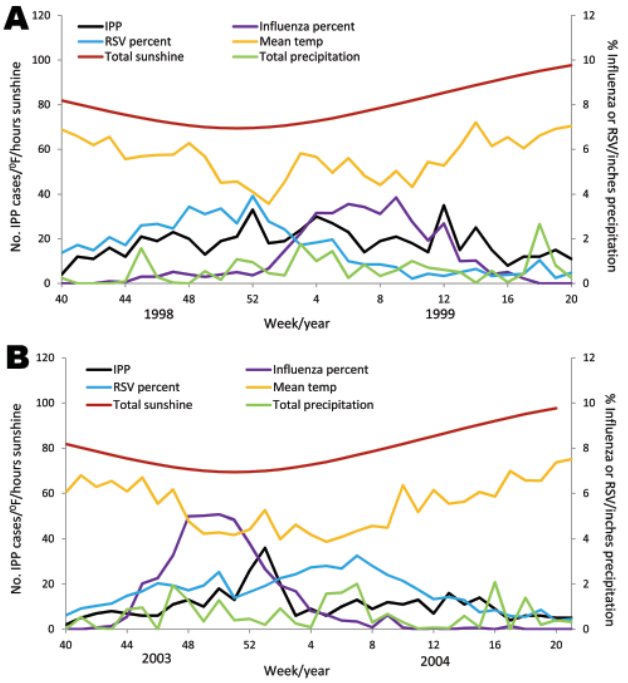

Figure. Trends for invasive pneumococcal pneumonia, virus, and climate data for 1998–99 (A) and 2003–04 (B), United States. IPP, invasive pneumococcal disease; influenza percent, percentage of influenza virus–positive isolates out of all influenza specimens; RSV, respiratory syncytial virus; RSV percent, percentage of RSV-positive isolates out of all RSV specimens.

believed to be associated with pneumococcal and viral respiratory infections (*5,7,8*).

The results indicated substantial variability across seasons in the strength of association of IPP incidence with influenza virus and RSV activities. This variability explains why findings from previous studies (*2–5,11*), which were based on data from a single season or on combined data from several seasons, are inconsistent. We found significant associations of IPP incidence with influenza virus and RSV activities for 5 of 11 influenza seasons from 1994–95 through 2004–05. Notably, in each of the 5 seasons for which we found a significant association, influenza A virus strain H3N2 was predominant (this strain predominated in 8 seasons). Alternatively, we did not find a significant association in any of the 3 seasons in which strain A (H3N2) was not the predominant influenza virus strain. Although this difference was not statistically significant, it suggests that the association between IPP and influenza virus and RSV activities might be stronger in seasons in which strain A (H3N2) is predominant. This finding is consistent with the idea that excess neuraminidase expression of strain H3N2 compared with strain H1N1 influenza viruses may lead to an excess number of pneumococcal superinfections (*12*).

In summary, in this season-by-season analysis, we found substantial variation in the strength of the association of IPP incidence with influenza virus and RSV activities across seasons. We also found that this association may be associated with the predominant influenza virus strain. More studies, using data from more seasons and from several geographic areas, are needed to better explain the variation in the association between invasive pneumococcal diseases and respiratory viral infections.

Acknowledgments

We thank David K. Shay, William W. Thompson, Lynnette Brammer, and 2 anonymous reviewers for their helpful comments.

Ms Zhou was an MPH student in biostatistics at Emory University at the time of this study. Her research interests focus on infectious diseases.

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Survey of Infections Transmissible Between Baboons and Humans, Cape Town, South Africa

Julian A. Drewe, M. Justin O'Riain, Esme Beamish, Hamish Currie, and Sven Parsons

Baboons on South Africa's Cape Peninsula come in frequent contact with humans. To determine potential health risks for both species, we screened 27 baboons from 5 troops for 10 infections. Most (56%) baboons had antibodies reactive or cross-reactive to human viruses. Spatial overlap between these species poses low but potential health risks.

The Cape Peninsula in South Africa is home to many species of wildlife, including ≈470 chacma baboons (*Papio ursinus*), which are a major tourist attraction and source of chronic conflict for local residents. Urban and agricultural land transformation has encroached markedly on the preferred natural habitat of baboons (*1*), and the 16 remaining troops on the Peninsula have been forced into marginal areas and are geographically isolated from all other baboon populations (Figure 1). The loss of preferred habitat, coupled with expanding numbers and a preference for high caloric food items, results in baboons entering residential areas daily to raid dustbins (garbage containers), enter homes, and attack humans in an effort to secure human-derived food (Figure 2).

The close contact between baboons and humans results in a high potential for the transmission of infectious diseases (*2*), from baboons to humans (zoonoses) and from humans to baboons (anthroponoses). Globally, disease transmission between humans and wildlife is occurring at an increasing rate, posing a substantial global threat to public health and biodiversity conservation (*3,4*). Although a study of baboon parasites in Kenya found none directly attributable to exposure to humans (*5*), the human parasite *Trichuris trichiura* has recently been identified in the Cape Peninsula baboon population; this finding represents the first evidence of likely anthroponotic infection of baboons (*6*). Diseases such as measles and tuberculosis are highly prevalent among the local human population (*7*) and have the potential to pass to baboons. The risks for infectious disease transmission between baboons and humans remain unclear. The aim of this study was to determine which diseases are currently present in the Cape Peninsula

Figure 1. Cape Peninsula in South Africa, showing position and name of the different regions that have baboon troops. Baboons were sampled from those regions denoted by an asterisk. Green denotes natural land, and gray shows the current extent of urban and agricultural land on the Peninsula.

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DOI: http://dx.doi.org/10.3201/eid1802.111309

Figure 2. Baboon raiding a dustbin in the residential suburbs of Cape Town, South Africa.

baboon population to inform decisions relating to baboon management, welfare and conservation, and the health risk to local humans and baboons. Ethical approval was gained from the Royal Veterinary College Ethics and Welfare Committee.

The Study

Twenty-seven baboons (15 male, 12 female) from 5 troops were screened for 10 zoonotic infections in April 2011. A nonstratified power analysis indicated that this sample would provide >95% confidence of detecting infections if they were present at a prevalence of ≥10%. Pathogens were chosen for screening according to a literature review of infections in primates of potentially serious anthroponotic or zoonotic risk. Older animals were preferentially sampled because these were thought most likely to have been exposed to diseases. Fourteen adult baboons (7 males >7 years of age, 7 females >5 years of age), 7 subadult baboons (2 males 5–7 years of age, 5 females 4–5 years of age), and 6 juvenile baboons (6 males <5 years of age) were sampled. Nonrandom sampling was done to increase the chances of detecting diseases, if present, and was considered appropriate because the aim of this study was to determine presence or absence of infection, not prevalence of infection.

Baboons were individually trapped in cages and anesthetized for blood sampling. Samples of feces from each baboon were collected from the cage floor. After reversal of anesthesia and a suitable recovery period, the baboons were released in sight of their troop.

Automated enzyme linked fluorescent assays (Vidas; bioMérieux, Marcy l'Etoile, France) were used to test for antibodies against measles, hepatitis A virus (HAV), cytomegalovirus (CMV), and Epstein-Barr virus (Table 1). The manufacturer's positive controls (human serum specimens containing IgG) were used. A serum neutralization test was used to screen samples for poliovirus antibodies. An interferon-gamma release assay for tuberculosis was conducted by using the QuantiFERON-TB Gold In-Tube test (Cellestis, Carnegie, Australia). This assay has been used previously for detection of *Mycobacterium tuberculosis* infection in chacma baboons (*8*). Test results were interpreted according to the manufacturer's criteria for human patients. Feces samples were stored at 5°C for up to 24 h before being cultured for *Salmonella* spp., *Shigella* spp., *Yersinia* spp., and *Campylobacter* spp. by using standard techniques (*9*).

Results are shown in Table 1. Fifteen (56%) baboons had antibodies reactive or cross-reactive to at least 1 human virus: CMV, HAV, and Epstein-Barr virus. Seven (26%) baboons had antibodies reactive or cross-reactive to 2 of these viruses. Baboons in every troop were positive for at least 1 viral infection, but considerable variation was found among troops (Table 2). One troop (Da Gama) showed a higher than average rate of exposure to HAV; 6 (75%) of 8 of the HAV antibody-positive baboons were in this 1 troop, despite this troop's representing just 7 (26%) of the 27 baboons in the sample. All 3 baboons sampled from another troop (Red Hill) had antibodies against CMV (Table 2). No pathogenic bacteria were found. Because intermittent shedding of fecal pathogens means that

Table 1. Results of diagnostic tests for exposure to 10 infectious diseases in 27 wild baboons, Cape Peninsula , South Africa, April 2011*

Infection	Diagnostic test	No. (%) baboons testing positive
CMV	Anti-CMV IgG ELFA	9 (33)
HAV	Anti-HAV total immunoglobulins ELFA	8 (30)
EBV	Anti-EBV early and nuclear antigens IgG ELFA	5 (19)
Measles virus	Anti-measles virus IgG ELFA	0
Polio virus	Serum neutralisation test	0
Tuberculosis	Whole blood gamma interferon test	0
Salmonella spp.	Fecal culture†	0
Shigella spp.	Fecal culture†	0
Yersinia spp.	Fecal culture†	0
Camplyobacter spp.	Fecal culture†	0

*CMV, cytomegalovirus; ELFA, enzyme-linked fluorescent assay; HAV, hepatitis A virus; EBV, Epstein-Barr virus.
†Single fecal cultures performed on samples from 21 baboons only.

Table 2. Distribution of antibody-positive baboons by troop, Cape Peninsula , South Africa, April 2011*

Baboon troop	Predominant human habitat type	No. baboons tested	No. (%) CMV positive	No. (%) HAV positive	No. (%) EBV positive
Red Hill	Urban residential	3	3 (100)	1 (33)	1 (33)
Da Gama	Urban residential	7	3 (43)	6 (86)	1 (14)
Smitswinkel Bay	Scenic tourist route	3	1 (33)	0	1 (33)
Tokai JT	Forest plantation	6	0	1 (17)	2 (33)
Tokai MT1	Forest plantation	8	2 (25)	0	0
Totals		27	9	8	5

*The locations of each baboon troop are indicated in Figure 1. CMV, cytomegalovirus; HAV, hepatitis A virus; EBV, Epstein-Barr virus.

sampling animals on a single occasion may miss cases of infection (*10*), negative fecal culture results should not be considered definitive.

Conclusions

This study provides evidence of the potential for cross-species trafficking of select pathogens. Widespread evidence of reactive or cross-reactive humoral immune responses to human pathogens was found in wild baboons. The detection of antibodies reactive or cross-reactive to HAV in 30% of baboons tested is a potential cause for concern. Because HAV is spread by the fecal–oral route, many opportunities might exist for direct and indirect transmission between baboons and humans; e.g., baboons frequent picnic sites and enter houses and cars in search of food. The frequency with which such contacts result in transmission of HAV should be investigated because of the potentially fatal consequences of human infection with HAV, particularly for immunocompromised persons such as those co-infected with HIV. Furthermore, as pathogens pass back and forth across species lines, the potential for changes in pathogenicity and host specificity exists, which can result in serious adverse effects on human and wildlife health.

The considerable variation in virus immunity among baboon troops (Table 2) warrants further study. The difference was particularly pronounced in the 2 most sampled troops, in which HAV antibody prevalence varied from 0% (0/8 baboons in the Tokai MT1 troop, in a forest) to 86% (6/7 baboons in the Da Gama troop, in an urban area). Future work should target these groups for more extensive sampling (ideally, all baboons should be sampled) to more accurately determine the prevalence of infection and investigate risk factors for virus exposure. A suitable hypothesis for testing would be that zoonotic infection prevalence in baboons is positively correlated with the proportion of urban land in their habitat.

The results of this study suggest that baboons on the Cape Peninsula pose a low but potential risk for transmitting zoonoses and that they might be at risk from anthroponoses. The findings should not be interpreted as definitively showing baboon exposure to human viruses because the serologic tests did not distinguish between human and baboon variants of the viruses and some cross-reactivity may have occurred. Virus isolation would be needed to determine the virus types. Nonetheless, there is ample evidence that disease of human origin can be devastating for primate populations (*11,12*). Further research is required on the Cape Peninsula to quantify the incidence of infections in baboons and humans, to examine the variation in levels of infection among baboon troops, and to measure the frequency of contact between species. Estimating the probability of cross-species disease transmission is challenging (*13*), but this information would be of tremendous use in informing baboon management plans with the aim of reducing the risks for infectious disease in humans and baboons.

Acknowledgments

We thank Bentley Kaplan and Matthew Lewis for their assistance with capturing baboons, Shahrina Chowdhury for help identifying baboons, the staff at Nature Conservation Corporation in Cape Town for the loan of traps and assistance in the field, and the Medical Research Council in Cape Town for the loan of recovery cages.

Permission to conduct this research was granted by the South African National Parks Cape Research Centre. This study was funded by the Royal Veterinary College and the University of Cape Town.

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Disseminated Infection Caused by Novel Species of *Microsporidium*, Thailand

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We describe a case of microsporidial myositis in a healthy man from Thailand. The small subunit rRNA sequence of this microsporidium is novel and has a close phylogenetic relationship with *Endoreticulatus*, a genus of lepidopteran microsporidia. Myositis could be caused by more genera of microsporidia than previously known.

Microsporidia are obligate intracellular eukaryotes of broad host range (*1*). At least 15 species have been implicated in human infections. Although few microsporidiosis cases have been reported, they have emerged as opportunistic infections in patients with HIV infection (*1*). In addition, microsporidiosis has increasingly been diagnosed in other immunocompromised patients, including those who have received an organ transplant, and in immunocompetent persons (*1*). Various clinical manifestations, ranging from localized (diarrhea, cholangitis, sinusitis, keratitis) to disseminated (myositis, osteomyelitis, encephalitis) infection have been observed, depending on the causative agent and host immune status (*1*). We report microsporidial myositis caused by a suspected new species of *Microsporidium* in an otherwise healthy man.

The Study

A 43-year-old man (a welder living in Lopburi Province, central Thailand) sought treatment at King Chulalongkorn Memorial Hospital, Bangkok, on November 30, 2010. He had experienced difficulty in swallowing for 2 weeks. Fifteen months before hospital admission, he experienced a low-grade fever and generalized muscle pain, especially in his lower back and both thighs. Despite several courses of medication, hospital, his condition had not improved.

Ten months before admission, weakness of lower and upper limbs developed in his proximal muscle, primarily on the left side. Two months before admission, his entire left leg was swollen, and he was confined to bed. During this illness, he had lost ≈29 pounds. He reported no travel abroad and had not taken any herbal medications. He did not smoke, drank alcohol occasionally, and did not use illicit drugs. He had no history of recurring infections during childhood and early adulthood.

Physical examination showed no keratoconjunctivitis, but indicated engorged jugular veins and right ventricular heave with loud pulmonic valve closure sound. Abdominal examination showed no hepatosplenomegaly. Non-pitting edema of the entire left leg was observed. Neurologic examination showed hyporeflexic proximal muscle weakness of grade III–IV/V in all limbs. Complete blood count showed the following: hematocrit 43.5%, leukocyte count 5,230/mm^3 (79% neutrophils, 14% lymphocytes, 4% eosinophils, and 3% monocytes), platelet count 209,000/mm^3. Liver function test showed the following results: total bilirubin 0.34 mg/dL, alkaline phosphatase 57 U/L (reference value 42 U/L–121 U/L), aspartate transaminase 268 U/L (4 U/L–36 U/L), alanine transaminase 101 U/L (4 U/L–36 U/L), albumin 3.1 g/dL, and creatine phosphokinase 4,308 U/L (60 U/L–400 U/L). Test results were negative for antibody against nuclear antigen and HIV (2 tests, 1 month apart). CD4 cell count was 368/μL (24%), and serum protein electrophoresis showed polyclonal gammopathy.

Chest radiograph showed pulmonary hypertension without significant pulmonary infiltration. Severe pulmonary hypertension without left ventricular dysfunction or left-to-right shunt was evident on electrocardiogram. Computed tomographic scan showed an enlarged pulmonary trunk (4 cm in diameter) and right and left main pulmonary arteries (thrombosis was not shown in the lumens). Electromyograph showed irritative myopathy without evidence of large-fiber sensory and motor polyneuropathy.

A biopsy of left deltoid and both vastus lateralis muscles showed necrotizing granulomatous inflammation without any organisms by Gram, acid-fast bacilli, Wright, and Gomori methenamine silver staining. Bone marrow biopsy showed normal cellularity with increased plasma cells and histiocytes and focal aggregation of microsporidial spores with characteristic belt-like stripe (Figure 1). A 24-hour urine sample (centrifuged) yielded characteristic microsporidial spores on modified trichrome stain, 1.0–1.5 μm × 1.2–2.2 μm, similar to those observed in the bone marrow sample. Because of an overgrowth of *Candida* yeast, we did not isolate the organism from the urine sample.

Analysis of the small subunit rRNA gene spanning 797 bp of DNA from microsporidial spores isolated from this patient showed a novel sequence (GenBank accession no. JN619406). Phylogenetic reconstruction using the maximum-likelihood method placed the

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DOI: http://dx.doi.org/10.3201/eid1802.111319

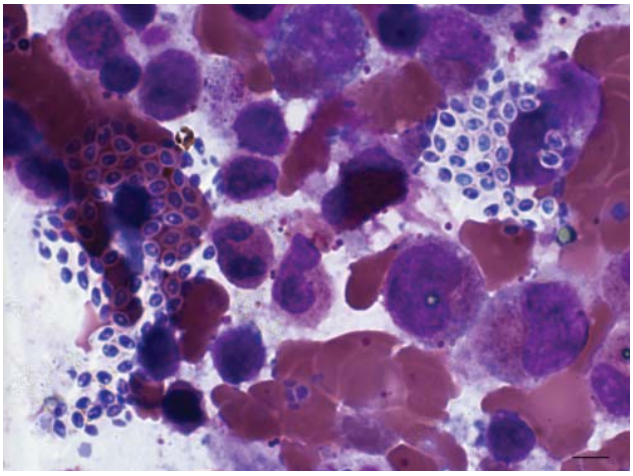

Figure 1. *Microsporidium* species in bone marrow aspiration specimen (Wright stain) from a 43-year-old man, Thailand. The image shows focal aggregations of microsporidia-like microorganisms with a belt-like stripe (1.0–1.5 μm × 1.2–2.2 μm). Original magnification ×100.

patient's *Microsporidium* sp. within the cluster of the genera *Endoreticulatus*, *Cystosporogene,* and *Vittaforma* (Figure 2) (*2*). This species has a close phylogenetic relationship with *Endoreticulatus* spp. Notably, the guanine-cytosine content of the SSU rRNA locus of microsporidia within the genus *Endoreticulatus* varied from 51.4% to 51.8%, with the base composition distance 0.001–0.029. However, the small subunit rRNA sequence of microsporidium from our patient (GenBank accession no. JN619406) contained 52.4% guanine-cytosine, and the base composition distance differed from *Endoreticulatus* spp. from 0.094–0.170, making it unlikely that the patient was infected with an organism from this genus.

The patient was treated with albendazole (800 mg/d continuously). Fever subsided after 1 week of treatment, and weakness gradually improved. He was discharged on a regimen of oral albendazole (800 mg/1×/d) but died unexpectedly from aspiration pneumonia 1 month later. An autopsy could not be performed.

Conclusions

Microsporidia can cause either localized or disseminated infection in humans. The true prevalence of microsporidiosis in humans may be underestimated because of asymptomatic infection in most healthy persons, clinician unawareness, and difficulties in diagnosis. Myositis caused by microsporidia is rare; only 12 patients (including our patient) have been reported in the literature (online Appendix Table, wwwnc.cdc.gov/EID/article/18/2/11-1319-TA1.htm; *3*–*12*). Among these 12 patients, 9 were male and 3 were female (age range 4 months to 67 years). Patients were from all continents except Europe: United States (7 patients), Australia (2), Haiti (1), the Gambia (1), and Thailand (1). The patient described here is the first report from Asia. *Trachipleistophora* sp. (6 patients) is the most common causative agent of myositis, followed by *Brachiola* sp. (3 patients), *Pleistophora* sp., *Tubulinosema acridophagus* sp., and *Microsporidium* spp. (1 patient each). However, the species of microsporidia originally described in 2 reports (*5,6*) was changed from *Pleistophora* sp. to *Trachipleistophora* sp. upon reexamination of the ultrastructures by electron microscopy.

Although ultrastructural study of the microsporidium from this patient has not been performed, the SSU rRNA sequence clearly supports the finding that it is a novel species, closely related to *Endoreticulatus* spp. (Figure 2). Most patients in previous reports had disseminated infection or isolated myositis. The patient described here had pulmonary hypertension, likely caused by microsporidia; several reports have shown that these organisms can cause pulmonary infection (*13*).

The routes of microsporidial infection are still uncertain, but the species that can infect humans have been identified in water sources and in farm, domestic, and wild animals. Furthermore, *Trachipleistophora* spp. have been found only in human hosts. However, on the

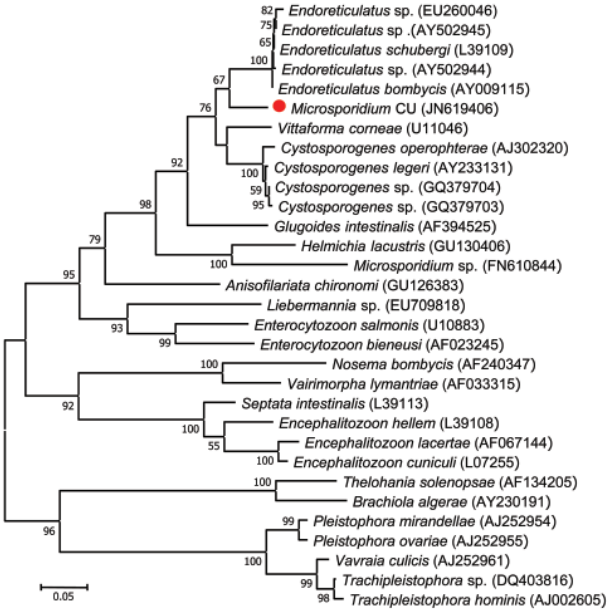

Figure 2. Phylogenetic tree inferred from the small subunit rRNA sequences of microsporidia in this study and those in the GenBank database by using the maximum-likelihood method as implemented in MEGA5.05 software (*2*). Red circle indicates a novel microsporidium identified in this study (Microsporidium CU) that caused myositis. GenBank accession numbers are listed in parentheses after each species. Bootstrap percentages >50% based on 1,000 replicates are shown on the branches. The tree is drawn to scale, with branch lengths measured in the number of nucleotide substitutions per site.

basis of the feature of bisporous sporophorous vesicles, *T. anthropophthera* was proposed to be related to *Telomyxa* spp., which are parasites of mayflies. *T. anthropophthera* was reported to be the causative agent of keratitis in an HIV-infected patient from Thailand (*14*). *Brachiola* spp. (formerly *Nosema* spp.) are known to be pathogens of several kinds of insects, including mosquitoes (*10*). *Pleistophora* spp. have been found in fish and reptiles, but *P. ronneafiei* has never been identified in any hosts other than humans. *Microsporidium* spp. that can infect humans are *M. ceylonensis* and *M. africanum*. Both species were reported from the patients with keratitis from Sri Lanka and Africa, respectively, although their natural hosts remain unknown.

To our knowledge, the microsporidium possessing a novel SSU rRNA sequence closely related to *Endoreticulatus* spp. has never been identified in patients with myositis. Notably, certain lepidopteran microsporidia can produce various responses in nontarget hosts, from refractory to severe infections. Although we have no evidence regarding the route or source of this patient's infection, the close phylogenetic relationship of this microsporidium and lepidopteran microsporidia suggests that these non–blood-sucking insects could be natural hosts. Water or foods contaminated with lepidopteran insects could not be excluded as the source of infection. Likewise, recent reports that *Trachipleistophora extenrec* can cause myositis in anteaters (*15*) and that *Tubulinosema* sp. can cause myositis in an immunosuppressed patient (*12*) further support the role of insect microsporidia as human pathogens.

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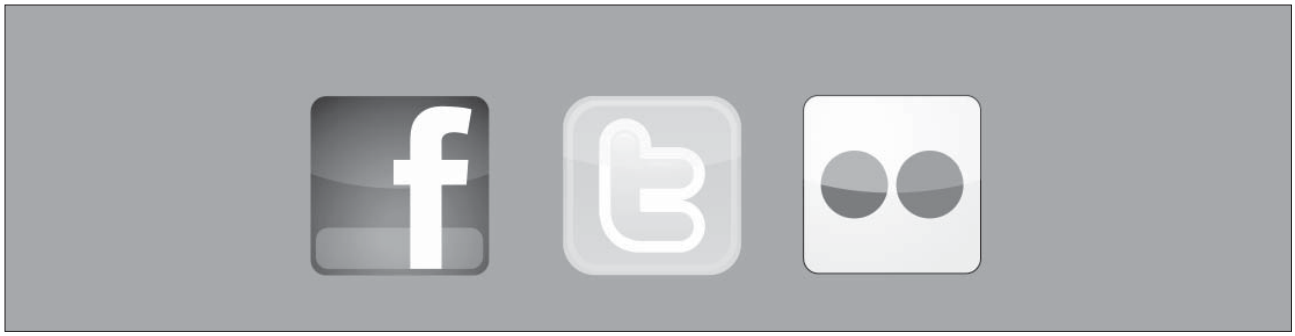

Determining Mortality Rates Attributable to *Clostridium difficile* Infection

Susy S. Hota, Camille Achonu, Natasha S. Crowcroft, Bart J. Harvey, Albert Lauwers, and Michael A. Gardam

To determine accuracy of measures of deaths attributable to *Clostridium difficile* infection, we compared 3 measures for 2007–2008 in Ontario, Canada: death certificate; death within 30 days of infection; and panel review. Data on death within 30 days were more feasible than panel review and more accurate than death certificate data.

Clostridium difficile infection (CDI) has emerged as a major health care–associated infection; incidence, hospitalizations, and mortality rates are increasing (*1*,*2*). Reported case-fatality rates are 6%–30% and seem to be rising (*3*,*4*). The reporting of CDI-associated deaths could be considered a quality indicator; however, the accuracy of death certificate data is questionable (*5*). We analyzed CDI deaths in 3 hospitals in Ontario, Canada, and compared 3 measures for attributing death to CDI: death certificate, death within 30 days of CDI, and a panel review process (considered the reference standard).

The Study

From April 2007 through February 2008, as independent quality initiatives, 3 hospitals in Ontario reviewed deaths among patients with CDI infection. Patients were identified by using existing surveillance data. To calculate the time from CDI diagnosis to death, we compared date of death with date of onset of CDI symptoms or date of the positive *C. difficile* test result if symptom onset was unclear. For recurrent CDI, date of recurrent symptoms nearest to date of death was considered. Patients with suggestive recurrent symptoms but no laboratory confirmation were included in a further analysis in this study.

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DOI: http://dx.doi.org/10.3201/eid1802.111611

For panel review, clinical data and cause of death indicated on death certificate were anonymously summarized for each patient. Each case summary was reviewed by 3 physicians with varying levels of expertise with CDI. Panel members were asked to independently categorize their interpretation as follows: a) death was directly attributable to CDI; b) CDI strongly contributed to death; c) CDI somewhat contributed to death; d) death was unrelated to CDI; or e) information was insufficient to determine the role of CDI in the death. Hospital A was the first facility to undertake a panel review and included only 4 categories (category b was excluded). Feedback from reviewers at hospital A led to development of category b.

After individually classifying each death, reviewers participated in a panel discussion to achieve consensus. For analysis, the 5 categories were subsequently collapsed into 3: CDI directly caused or strongly contributed to death; CDI somewhat contributed to death or was unrelated to death; and information was insufficient to determine the role of CDI in the death.

The κ statistic was used to determine the level of agreement on cause of death between death certificate and panel review. The 3 categories listed above were compared with the following death certificate categories: CDI, enterocolitis, or toxic megacolon as primary or contributory cause of death; death unrelated to CDI; and missing information.

The percentage agreement of death within 30 days of CDI and panel review consensus was calculated by using combined data from hospitals A and B. Hospital C was excluded from this analysis because only patients who died within 30 days of CDI diagnosis were included in that review. At hospital B, because data on individual physician assignment of categories were available, inter-rater reliability was analyzed by using the Fleiss κ statistic.

CDI was diagnosed for 501 patients, and 188 CDI patients died. Of these, 120 (64%) patients died within 30 days of CDI. The 30-day case-fatality ratios for hospitals A, B, and C were 20% (25/124), 35% (62/177), and 16% (33/200), respectively.

Panel reviews were conducted for all 31 in-hospital deaths in hospital A, 90 deaths in Hospital B, but only 30 of the deaths that occurred within 30 days of CDI diagnosis in hospital C. Among the 151 deaths included in the panel review process, CDI directly caused or strongly contributed to the death for 101 (67%) and somewhat contributed or was unrelated to death for 49 (32%). For 1 patient, information was insufficient for determining cause of death. Where data were available (hospital B), inter-rater reliability among panel members was satisfactory (κ = 0.71, 95% CI 0.59–0.83).

According to death certificate data, CDI was the primary cause of death for 7 (5%) patients and a contributory

Table 1. Proportion of deaths attributable to CDI, by panel review coding and death certificate classification, Ontario, Canada, April 2007–February 2008*

Reference category	Death certificate, no. (%)				Panel review, no.†
	CDI as primary cause of death	CDI as primary or secondary cause of death	CDI as unrelated cause of death	Missing information	
Directly/strongly attributable to CDI	6 (6)	37 (37)	61 (60)	3 (3)	101
Somewhat/unrelated to CDI	1 (2)	14 (29)	35 (71)	0	49
Insufficient information	0	0	1 (100)	0	1
Total	7 (5)	51 (34)	97 (64)	3 (2)	151

*CDI, *Clostridium difficile* infection.
†Categories determined by panel review. Because panel review was considered the reference standard, panel review percentages = 100%.

cause of death for 44 (29%). Of the 101 deaths classified by panel review as strongly attributable to CDI, 37 were classified by death certificate as having CDI as primary/secondary cause (Table 1). When panel review data were compared with death certificate data, κ was 0.07 (95% CI 0.05–0.20), indicative of poor agreement. The exclusion of hospital A, where deaths were originally classified into only 4 categories, did not alter the observed κ scores (κ = 0.04, 95% CI 0.09–0.17).

To compare the proportion of in-hospital deaths within 30 days with results of the panel review, we used only data from hospitals A and B because hospital C data only included patients who had died within 30 days. The panel concluded that CDI directly or strongly contributed to death within 30 days of onset for 80% (63/79) of patients (Table 2). If cases suggestive of recurrent CDI (not confirmed by testing) were included, this percentage rose to 86% (68/79). When panel review was used as the reference standard, the sensitivity of death within 30 days of CDI onset was 80%, specificity 41%, and positive predictive value 72%.

Conclusions

Agreement between causes of deaths categorized by review panel and causes listed on death certificates is poor. Ontario's vital statistics system currently codes only 1 cause of death, limiting the ability to identify deaths for which CDI might have been a contributing cause. These shortcomings suggest that death certificate data may be inaccurate for assigning CDI-attributable death.

Panel review of all deaths is an alternative approach that would enable clinical analysis of the circumstances surrounding the death. A panel review reduces individual reviewer bias; however, while arguably the most accurate method of determining cause of death other than autopsy, it is not feasible for wide-scale public reporting.

Our study supports the use of death within 30 days as a marker for CDI-attributable death because 80% of deaths identified by panel review as being directly or strongly attributable to CDI occurred within 30 days of diagnosis. This percentage increased to 86% if clinical recurrences were included. Compared with panel review, death within 30 days had reasonable sensitivity (80%) and positive predictive value (72%) but, as expected, was not specific (41%). Capturing data on death within 30 days would be more feasible than panel review and more accurate than death certificate data. However, data on death within 30 days could not be used to determine the contribution of CDI to any patient's death.

Our study has a few limitations. Because data were derived from 3 hospitals that undertook reviews for different purposes, the slight differences in inclusion criteria and death categorizations necessitated subanalyses for the death within 30 days comparison and the collapsing of the categorizations into 3 groups. Data quality also has inherent problems associated with retrospective chart audits because of limitations in the documentation of clinical events. Finally, we acknowledge that categorization of deaths by panel review is a subjective process based on interpretation of the clinical case summaries and expert opinion.

Table 2. Proportion of deaths within 30 days after CDI, by panel review coding, and hospital, Ontario, Canada, April 2007–February 2008*

Reference category†	No. (%) deaths for hospital A within 30 d after		Panel review, no.	No. (%) deaths for hospital B within 30 d after		Panel review, no.	No. (%) deaths for hospitals A and B within 30 d after		Panel review, no.
	Toxin-positive result	Clinical onset‡		Toxin-positive result	Clinical onset‡		Toxin-positive result	Clinical onset‡	
Directly/strongly attributable to CDI	17 (94)	17 (94)	18	46 (75)	51 (84)	61	63 (80)	68 (86)	79
Somewhat/unrelated to CDI	8 (62)	8 (62)	13	16 (57)	18 (64)	28	24 (59)	26 (63)	41
Insufficient information	–	–	–	0	0	1	0	0	1
Total	25 (81)	25 (81)	31	62 (69)	69 (77)	90	87 (72)	94 (78)	121

*CDI, *Clostridium difficile* infection.
†Categories determined by panel review. Because panel review was considered the reference standard, panel review percentages = 100%.
‡Because recurrent CDI is not always supported by laboratory or pathology confirmation, those patients with recurrent symptoms suggestive of CDI but no toxin confirmation were included by using the date of clinical onset of symptoms nearest to the date of death.

Our findings suggest that cause of death on death certificate is an inaccurate measure of death attributable to CDI. However, death from CDI within 30 days should be considered a feasible measure for the purposes of aggregate public reporting.

This study was supported by internal funds at the Ontario Agency for Health Protection and Promotion and the University Health Network.

Dr Hota is an infectious diseases and infection prevention and control medical specialist at the University Health Network. Her research interest is CDI.

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Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus Infections, United States, 2010–11

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During October 2010–July 2011, 1.0% of pandemic (H1N1) 2009 viruses in the United States were oseltamivir resistant, compared with 0.5% during the 2009–10 influenza season. Of resistant viruses from 2010–11 and 2009–10, 26% and 89%, respectively, were from persons exposed to oseltamivir before specimen collection. Findings suggest limited community transmission of oseltamivir-resistant virus.

During the 2009–2010 influenza pandemic, when pandemic (H1N1) 2009 virus was the predominant circulating influenza virus (*1*), the prevalence of oseltamivir-resistant pandemic (H1N1) 2009 viruses in the United States was 0.5%. Of the patients with oseltamivir-resistant virus infection, 89% had been exposed to oseltamivir before specimen collection (*2*). We describe patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infections during the 2010–11 influenza season.

The Study

During October 1, 2010–July 31, 2011, the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia, USA) asked all state public health laboratories to submit pandemic (H1N1) 2009 virus–positive respiratory specimens and virus isolates for antiviral susceptibility testing. Laboratories were asked to provide the first 5 specimens of any type/subtype collected during each 2-week period for virus isolation. Comprehensive antiviral testing, including neuraminidase inhibition (NI) assay, was performed on all isolates, and sequencing was performed on all isolates with elevated 50% inhibitory concentration values. CDC also requested that laboratories provide 5 additional specimens every 2 weeks for pyrosequencing to identify the H275Y substitution in the neuraminidase, a change associated with oseltamivir resistance (*3*). In addition to (or instead of) participating in the national surveillance, state laboratories in California, Maine, Maryland, Minnesota, New York, Texas, and Washington performed pyrosequencing on state surveillance specimens to detect the H275Y substitution. We included those state-specific data in the national surveillance data for this report. State health departments used a standard case form to collect demographic and clinical information for patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infection and their ill close contacts.

Oseltamivir resistance was determined by use of an NI assay or pyrosequencing for the H275Y substitution. For NI testing on isolates, we used the NA-Fluor kit (Applied Biosystems, Foster City, CA, USA) as described (*4*). We performed pyrosequencing, as described (*5*), on all oseltamivir-resistant pandemic (H1N1) 2009 isolates identified by NI assay to confirm the presence of the H275Y substitution. We performed pyrosequencing for H275Y, without the NI assay, to screen pandemic (H1N1) 2009 clinical specimens (*5*). For the national surveillance, NI testing was performed at CDC and pyrosequencing was performed at CDC and at state laboratories in Wisconsin, New York, and California. State laboratories followed pyrosequencing protocols provided by CDC; when possible, CDC confirmed results for resistant viruses by use of pyrosequencing and NI assay. Most oseltamivir-resistant viruses in this report were included in the weekly FluView report prepared by CDC (*6*).

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DOI: http://dx.doi.org/10.3201/eid1802.111466

[1]Additional members of the US Antiviral Resistance Surveillance Working Group who contributed data are listed at the end of this article.

We tested a total of 3,652 pandemic (H1N1) 2009 virus isolates and specimens from every state and the District of Columbia; 35 (1.0%) isolates/specimens from a total of 18 states were oseltamivir-resistant (Figure). Overall, 8 (1.3%) of 609 isolates tested by NI assay and 27 (1.0%) of 3,043 specimens tested by pyrosequencing were resistant to oseltamivir. The state-specific prevalence of oseltamivir-resistant pandemic (H1N1) 2009 viruses varied; however, the number of viruses and specimens tested also varied markedly between states, and several states submitted only a few specimens. Forty-four states submitted >20 specimens for antiviral resistance surveillance. The prevalence of oseltamivir resistance among these specimens ranged from 0% to 5.6%. None of the 609 pandemic (H1N1) 2009 isolates tested by NI assay were resistant to zanamivir. The ranges of 50% inhibitory concentration values for oseltamivir-resistant and -susceptible isolates were 166.17–230.37 nmol/L and 0.10–0.80 nmol/L, respectively.

The median age of the 35 patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infections was 33 years. Of 34 patients with available information, 26% reported receiving oseltamivir before providing a specimen for antiviral susceptibility testing (Table 1). Among 33 patients with a completed case form, 67% had at least 1 preexisting chronic medical condition, 24% had an immunocompromising medical condition, 42% required hospitalization, and 9% died. Most patients with oseltamivir-resistant virus infection for whom housing information was available lived in a single-family household. Two siblings from 1 household had oseltamivir-resistant virus infection; neither child had received oseltamivir.

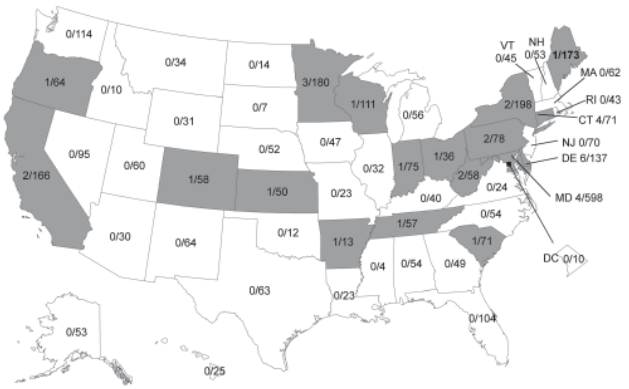

Figure. Geographic distribution of oseltamivir-resistant pandemic (H1N1) 2009 viruses in the United States, October 1, 2010–July 31, 2011. Numerators are number of oseltamivir-resistant viruses identified by state public health laboratories; denominators are number of pandemic (H1N1) 2009–positive specimens submitted by each state for susceptibility testing. Gray shading indicates states that had ≥1 infection with oseltamivir-resistant virus.

All oseltamivir-resistant pandemic (H1N1) 2009 viruses were identified from specimens collected during December 2010–April 2011; the prevalence of resistance did not change significantly over time (test for trend, p = 0.18) (Table 2). In addition, the proportion of patients with oseltamivir-resistant virus infections who did not have exposure to oseltamivir before specimen collection did not change significantly over time (test for trend, p = 0.48); however, the number of specimens tested each month was small.

The number of oseltamivir-resistant pandemic (H1N1) 2009 virus–infected patients was small during the 2009–10

Table 1. Characteristics of patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infection, United States, October 1, 2010–July 31, 2011*

Characteristic	No. with characteristic/total no. (%), n = 35
Male sex	19/35 (54)
Exposed to oseltamivir before specimen collection†	9/34 (26)
Exposed to another person using oseltamivir	0/15 (0)
Received 2010–11 influenza vaccine	6/22 (27)
Any chronic medical conditions	22/33 (67)
Chronic pulmonary disease, including asthma	10/33 (30)
Chronic cardiac disease	6/33 (18)
Diabetes mellitus	8/33(24)
Immunocompromising condition‡	8/33(24)
Pregnancy	1/33 (3)
Other§	10/33 (30)
Lived in a single-family household	14/22 (64)
Lived in a residential facility	1/22 (4)
Others in the household/residence were ill before patient's illness	2/15 (13)
Traveled within 7 d before illness	2/18 (11)
Hospitalized during influenza illness	14/33 (42)
ICU admission	8/14 (57)
Died	3/34 (9)

*The median age of patients was 33 y (range 1 mo–78 y). ICU, intensive care unit.
†Nine patients began oseltamivir treatment prior to specimen collection; none received oseltamivir chemoprophylaxis.
‡Includes HIV/AIDS, malignancy, autoimmune disorder, solid organ transplant, stem cell transplant, and history of taking immunosuppressive therapy in the past year.
§Includes morbid obesity, obstructive sleep apnea, chronic liver disease, and neurologic or developmental disorders.

(April 2009–September 2010) and 2010–11 influenza seasons. However, the prevalence of oseltamivir-resistant virus–infected patients was slightly higher during 2010–11 compared with 2009–10 (35/3,652 [1.0%] vs. 37/6,740 [0.5%], respectively, p = 0.02 by χ^2 test) (*2*). Also, during 2010–11, compared with 2009–10, more patients with oseltamivir-resistant virus infection had no history of oseltamivir exposure before specimen collection (25/34 [73.5%] vs. 4/35 [11.4%], respectively; p<0.0001 by χ^2 test).

Conclusions

During the 2010–11 US influenza season, the prevalence of oseltamivir-resistant pandemic (H1N1) 2009 viruses remained low, and most persons with oseltamivir-resistant virus infection had no prior oseltamivir exposure. This is a notable difference from surveillance findings both globally and in the United States during the 2009–10 season, when most patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infection had a history of oseltamivir exposure, and many were severely immunocompromised, a condition that may increase the risk for resistance developing during oseltamivir therapy (*2,7*). These data suggest a low level of community transmission of oseltamivir-resistant pandemic (H1N1) 2009 virus in the United States during the 2010–11 influenza season. The United Kingdom also reported that the proportion of oseltamivir-resistant pandemic (H1N1) 2009 virus–infected patients without prior oseltamivir exposure was higher during 2010–11 than 2009–10 (*8*).

The increase during the 2010-11 influenza season in the proportion of patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infections without prior oseltamivir exposure causes concern in light of the recent history of oseltamivir resistance among seasonal influenza A (H1N1) viruses that circulated before pandemic (H1N1) 2009 virus emerged. In the United States before the 2007–08 influenza season, the prevalence of oseltamivir resistance among seasonal influenza A (H1N1) viruses was <1% (*9,10*). However, during the 2007–08 season, the prevalence of oseltamivir resistance among seasonal influenza A (H1N1) viruses increased to 12%, and by the 2008–09 season, resistance dramatically increased to >99% (*9,11,12*). No association was found between this increase in oseltamivir resistance and prior oseltamivir use (*11,13*). Oseltamivir resistance in pandemic (H1N1) 2009 and seasonal influenza A (H1N1) viruses was conferred by the H275Y substitution in the neuraminidase. However, unlike seasonal influenza A (H1N1) viruses, which retained susceptibility to the M2-blocking adamantanes (amantadine and rimantadine), >99% of circulating pandemic (H1N1) 2009 viruses are inherently resistant to adamantanes (*14*). Thus, inhaled zanamivir or investigational drugs, such as intravenous zanamivir, are the only treatment options for patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infection.

Our conclusions are limited by the small number of patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infection. Variability in state surveillance and the number of specimens tested from each state may also have limited the representativeness of our data. Despite these shortcomings, our findings emphasize the importance of ongoing surveillance for oseltamivir-resistant pandemic (H1N1) 2009 viruses in the United States and globally and of close monitoring for changes in the epidemiology of oseltamivir resistance among pandemic (H1N1) 2009 viruses. Updated information about antiviral resistance in influenza viruses in the United States is available at www.cdc.gov/flu/professionals/.

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Table 2. Temporal trends of oseltamivir-resistant pandemic (H1N1) 2009 viruses and history of patient exposure to oseltamivir, by month, United States, October 1, 2010–July 31, 2011

Year and month of specimen collection	No. specimens tested	No. (%) oseltamivir-resistant specimens	No. (%) patients with oseltamivir-resistant virus but no oseltamivir exposure
2010 Oct	9	0	0
2010 Nov	17	0	0
2010 Dec	172	1 (0.6)	0
2011 Jan	1,074	6 (0.6)	4 (66.7)
2011 Feb	1,475	18 (1.2)	15 (83.3)
2011 Mar	801	9 (1.1)	6 (66.7)
2011 Apr	95	1 (1.0)	1 (100.0)
2011 May	8	0	0
2011 Jun	0	0	0
2011 Jul	1	0	0
Total	3,652	35 (1.0)	25 (73.5)*

*Oseltamivir exposure known for 34 of 35 patients.

Webber (Maine Department of Health and Human Service); René Najera (Maryland Department of Health and Mental Hygiene); Karen Martin (Minnesota Department of Health); Jennifer Lapplante (New York State Department of Health); Matthew Laidler (Oregon Health Authority); Owen Simwale and Kumar Nalluswami (Pennsylvania Department of Health); Brittanni Wright (South Carolina Department of Health and Environmental Control); Robb L. Garman (Tennessee Department of Health); Crystal Van Cleave (Texas Department of State Health Services); Julie Tseng-Crank (Washington State Department of Health); Julie Freshwater (West Virginia Bureau of Public Health); Mary Wedig and Thomas Haupt (Wisconsin Department of Health Services); Shikha Garg, Marnie Levine, Katrina Sleeman, Kristina Ballenger, Angie Trujillo, and Ha Nguyen (Centers for Disease Control and Prevention).

Acknowledgments

We thank all patients and health care providers who participated in these surveillance activities, as well as state and local public health officials and laboratory staff who assisted in compiling data for this report.

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Extended Outbreak of Cryptosporidiosis in a Pediatric Hospital, China

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Four *Cryptosporidium* spp. and 6 *C. hominis* subtypes were isolated from 102 of 6,284 patients in 3 pediatric hospitals in People's Republic of China. A cryptosporidiosis outbreak was identified retrospectively. The outbreak lasted >1 year and affected 51.4% of patients in 1 hospital ward, where 2 *C. hominis* subtypes with different virulence were found

Since the 1980s, ≈20 outbreaks of cryptosporidiosis have been reported in health care facilities (*1*–*9*). Thus far, to our knowledge, genotyping and subtyping tools have not been used in the investigation of this type of outbreak (*10*). We used subtyping in a molecular epidemiologic study of endemic cryptosporidiosis to retrospectively identify an extended outbreak among children in a hospital ward.

The Study

During September 2007–October 2009, fecal specimens were collected from children in hospitals I (3,245 patients), II (489), and III (2,550), in Shanghai, People's Republic of China. The children (1 month–19 years old, median 36 months) were hospitalized primarily for nongastrointestinal illnesses. For each patient, information was collected on age; sex; occurrence of diarrhea; and, later in the study, ward assignment in hospital I. The study was approved by the ethics committee of East China University of Science and Technology, Shanghai.

Cryptosporidium spp. were detected in the specimens and differentiated by PCR and restriction fragment length polymorphism analysis of the small subunit rRNA gene (*11*). *C. hominis* was subtyped by sequence analysis of the 60-kDa glycoprotein gene (*12*). Each specimen was analyzed at least 2× by PCR, with positive and negative controls in each run. Prevalence rates and 95% CIs were computed; the χ^2 test was used to test differences. Odds ratios (ORs) and 95% CIs were calculated.

Among the 6,284 patients, 102 were positive for *Cryptosporidium* spp.: 90 from hospital I (2.8%, 95% CI 2.2–3.3), 3 from hospital II (0.6%, 95% CI 0–1.3), and 9 from hospital III (0.4%, 95% CI 0.1–0.6) ($p<0.01$). Ward assignment was available for 1,592 of 3,245 patients in hospital I. In most of the 12 wards, the infection rate was 0%–2.3%; in ward A, it was 51.4% ($p<0.01$) (Table).

In hospital I, children <6 months old had a significantly higher positive rate (8.4%, 95% CI 5.6–11.2) than older children (1.9%, 95% CI 1.4–2.4) ($p<0.01$; data not shown). This was mainly because of a high infection rate among the age group in ward A (61.5%, 95% CI 36.9–86.2) versus those in other wards (40.0%, 95% CI 19.0–61.0). No age-associated difference in infection rates was found in other wards ($p = 0.80$; data not shown).

Cryptosporidiosis was more prevalent during February–July 2008 ($p<0.01$). Prevalence rates remained at ≈6% in the monthly distribution of the 2 main *C. hominis* subtypes in hospital I; however, when adequate numbers of patients were sampled, rates of *Cryptosporidium* infection in ward A remained >28% in most study months.

C. hominis was identified in 90.2% (92/102) of *Cryptosporidium*-positive patients in the 3 hospitals, of whom 86 were patients in hospital I. In hospital I, *C. hominis* was detected only in ward A; *C. meleagridis* was isolated from 4 patients in other wards (Table). In contrast, *C. canis* (1 case) and *C. hominis* (2 cases) were identified in hospital II patients, and *C. canis* (1 case), *C. hominis* (4 cases), *C. felis* (2 cases), and *C. meleagridis* (2 cases) were identified in hospital III patients (Table).

Six *C. hominis* subtypes were found at the 3 hospitals; 4 were in 73 specimens from hospital I (Table). Of those 73 specimens, 71 (97.3%) were subtype IaA14R4 or IdA19, and they were mostly found in ward A and unknown wards (Table). Other subtypes (IbA19G2 and IdA14) were not found in ward A (Table). With 1 exception, subtypes in hospital I were not found in other hospitals; subtype IaA14R4 was found in 2 patients in hospital III. Likewise, subtypes IaA18R4 (in 1 patient in hospital II) and IgA14 (in 1 patient in hospital III) were not found in hospital I.

In hospital I, 44 of 1,084 patients with diarrhea (4.1%, 95% CI 2.9–5.3) and 46 of 2,161 without diarrhea (2.1%, 95% CI 1.5–2.7) were positive for *Cryptosporidium* spp. ($p = 0.002$, OR 1.95, 95% CI 1.28–2.96). *C. hominis* subtype IaA14R4 (21 diarrheic and 13 nondiarrheic cases) was significantly associated with diarrhea ($p = 0.0004$, OR 3.29, 95% CI 1.64–6.59), but subtype IdA19 (11 diarrheic and 26

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DOI: http://dx.doi.org/10.3201/eid1802.110666

nondiarrheic cases) was not (OR 0.86, 95% CI 0.42–1.75, p = 0.68).

Conclusions

Our data indicate that a cryptosporidiosis outbreak occurred among children in ward A of hospital I. This conclusion was supported by the following findings: the rate of *Cryptosporidium*-positive cases in ward A (51.4%) was significantly higher than the overall rates in hospitals I (2.8%), II (0.6%), and III (0.4%); less *Cryptosporidium* diversity was found in ward A (only *C. hominis*) than in other wards/hospitals (4 *Cryptosporidium* spp.); only *C. hominis* subtypes IaA14R4 and IdA19 were present among 38 ward A patients (vs. 6 subtypes in 12 patients in other wards/hospitals); and a high rate (61.5%) of *Cryptosporidium*-positive cases occurred in ward A among children <6 months old, an age that usually has a low prevalence of cryptosporidiosis (*13*).

The source of the cryptosporidiosis outbreak is unknown. Most of the 12 wards in hospital I were located in the main building; ward A, the smallest ward, was in an adjacent building and was for children from a welfare institute. Hired caregivers cared for children in ward A; family members were the primary caregivers for patients in other wards. Thus, poor diaper-changing and hand-washing practices by caregivers could be responsible for the persistence of *C. hominis* infections in ward A. However, the facts that most of the patients were examined for *Cryptosporidium* infection only once and that many of the specimens were not submitted immediately after patients were hospitalized prevented us from concluding with certainty whether the infections were acquired in the hospital or in the welfare institute. The likelihood for widespread foodborne and waterborne transmission of cryptosporidiosis in hospital I was small because children in ward A and other wards shared the same source for food and drinking water. The likelihood of direct transmission of cryptosporidiosis among ward A patients was also small because 80% of patients were <1 year old and mostly stayed in cribs and beds.

This cryptosporidiosis outbreak has several key features. First, it was lengthy, lasting ≥14 months (November 2007–December 2008); only limited sampling was done before November 2007; and *Cryptosporidium* spp. were still present in December 2008. The longest previous outbreak was 4 months (*14*). Second, the number (≥38) of involved patients was high. Judged by the low occurrence of the 2 subtypes in other wards, most of the 32 IaA14R4- and IdA19-positive patients with missing ward information were probably also from ward A. Thus, >60 children might have been part of the outbreak. Third, this outbreak was caused concurrently by 2 *C. hominis* subtypes, of which IaA14R4, but not IdA19, was significantly associated with diarrhea. The observed difference in virulence is consistent with data from a community study in Peru (*15*), in which subtype family Ia, of which IaA14R4 is a member, was more virulent than Id, of which IdA19 is a member.

We retrospectively identified the outbreak by subtyping; the delay in detection prevented us from doing a thorough investigation, and continued sampling in the hospital and welfare institute and detailed epidemiologic and environmental investigation became impossible after we reported the outbreak to hospital I. Despite not knowing the source of infections, hospital I took measures to reduce hospital-acquired infections, including better training of caregivers and moving ward A to a new location. Thus, study data highlight the power of molecular epidemiologic tools in the surveillance and control of cryptosporidiosis and the need for prompt identification and investigation of outbreaks in health care facilities.

Acknowledgments

We thank the hospital personnel for assistance in specimen collection.

This work was supported in part by the National Natural Science Foundation of China (31110103901, 30928019 and 81041078); Fundamental Research Funds for the Central Universities, China (WB0914044); open funding projects of the State Key Laboratory of Bioreactor Engineering and the State Key Laboratory of Veterinary Etiological Biology.

Table. Distribution of *Cryptosporidium* spp. and subtypes among wards in a hospital in Shanghai, People's Republic of China, September 2007–October 2009*

Ward	No. patient samples	% Positive (95% CI)	No. patients positive					
			C. meleagridis	*C. hominis*	IaA14R4	IdA19	IbA19G2	IdA14
A	74	51.4 (35.0–67.7)	0	38	19	14	0	0
B	348	0.6 (0–1.4)	1	1	0	1	0	0
C	283	1.8 (0.2–3.3)	2	3	0	1	0	1
D	216	2.3 (0.3–4.3)	1	4	2	1	1	0
E	266	1.1 (0–2.4)	0	3	0	1	0	0
F	56	1.8 (0–5.3)	0	1	0	0	0	0
Others	349	0	0	0	0	0	0	0
Unknown	1653	2.2 (1.5–2.9)	0	36	13	19	0	0
Total	3,245	28 (2.2–3.3)	4	86	34	37	1	1

*Among 86 specimens positive for *C. hominis*, 78 were gp60 positive and 73 were subtyped. In 2 other hospitals, II and III, among 6 *C. hominis*–specimens, 1 was IaA18R4 (hospital II), 1 was IgA14 (hospital III), 2 were IaA14R4 (hospital III), and 2 were gp60 negative (hospitals II and III); 2 patients each were positive for *C. meleagridis*, *C. canis*, and *C. felis*.

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Pandemic (H1N1) 2009 in Captive Cheetah

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We describe virus isolation, full genome sequence analysis, and clinical pathology in ferrets experimentally inoculated with pandemic (H1N1) 2009 virus recovered from a clinically ill captive cheetah that had minimal human contact. Evidence of reverse zoonotic transmission by fomites underscores the substantial animal and human health implications of this virus.

Case Report

In November 2009, during the surge of pandemic (H1N1) 2009 cases among humans and ≈7 months after A/California/04/2009(H1N1) was first reported in a child in southern California (*1*), nasal swab specimens from a cheetah (*Acinonyx jubatus*) were submitted to the California Animal Health and Food Safety Laboratory. The 8-year-old cheetah, referred to as animal D, belonged to a privately operated wild animal park in northern California. The animal park veterinarian collected the specimens from animal D for diagnostic testing after severe respiratory symptoms, ptyalism, anorexia, and lethargy were observed in the animal and 3 other cheetahs housed in the same area. The 4 animals had been showing clinical signs of an influenza-like illness the previous week and were being treated with a combination of amoxicillin, enrofloxacin, famotidine, and omeprazole.

At the recommendation of a volunteer worker who was professionally affiliated with the California Public Health Department, a nasal swab sample was obtained from animal D 2–3 days following the onset of clinical signs. The California Public Health Department supplied a sampling kit consisting of cotton-tipped swabs, viral transport media, and a shipping container. A swab sample also was obtained from animal C, another 8-year-old cheetah, ≈4–6 days after it showed clinical signs. At the time of sampling, the remaining 2 cheetahs (animals A and B), which were housed in a separate but conjoining area, had clinically recovered from their respiratory illness. To avoid the additional handling and sedation required for sample collection from the nondomesticated animals, park personnel decided not to collect samples from the recovered cats.

The nasal swab samples were processed according to a standardized procedure distributed by the United States Department of Agriculture National Veterinary Services Laboratory through the National Animal Health Laboratory Network. The protocol used was an approved deviation of the standard operating procedure used for testing swine in the United States. In brief, RNA was recovered by using the MagMAX Viral RNA Isolation Kit (Applied Biosystems, Austin, TX, USA) following the manufacturer's recommendations. Real-time reverse transcription PCR (qRT-PCR) individually targeting the influenza A matrix (M) gene and the neuraminidase (N1) gene were performed as described (*2*). Positive qRT-PCR test results, with cycle thresholds of 26.83 and 30.23 for the M and N1 genes, respectively, were obtained for animal D. PCR results were confirmed as pandemic (H1N1) 2009 virus by sequence analysis of the PCR amplicon. Animal C had negative test results for M and N1 genes.

A second aliquot of the nasal sample from animal D was inoculated onto trypsin-treated MDCK cells (*3*), and the propagated cytolytic virus was forwarded to St. Jude Children's Research Hospital for complete genome sequence analysis. The influenza virus isolate was inoculated into ferrets for further characterization of the virus. The United States Department of Agriculture National Veterinary Services Laboratory (Ames, Iowa, USA) was additionally provided an aliquot of the nasal swab specimen. Sequence analyses of the M, N1, and hemagglutinin genes were performed (GenBank accession nos. HMO12479, HMO 12480, and HMO12481) and confirmed the initial diagnostic detection of pandemic (H1N1) 2009 virus.

Whole genome sequencing was performed by using methods recommended by the World Health Organization (www.who.int/csr/resources/publications/swineflu/sequencing_primers/en/). Sequence analysis confirmed the existence of the pandemic (H1N1) 2009 virus (A/Cheetah/California/D0912239/2009, GenBank accession nos. CY092750–CY092757). No unique molecular signatures were detected in the cheetah virus as compared with other pandemic subtype H1N1 viruses isolated from humans. Except for 1 synonymous base-pair mismatch at position 1440 in the hemagglutinin gene, the sequence data matched in the 3 genes sequenced in 2 locations.

To characterize and further assess the pathogenicity profile of the virus, we inoculated 3 groups of 5 ferrets each

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DOI: http://dx.doi.org/10.3201/eid1802.111245

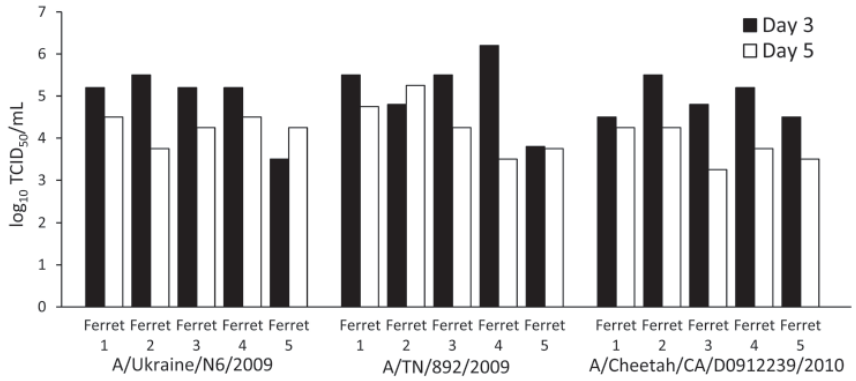

Figure. Virus concentration (50% tissue culture infectious dose) in nasal secretions of 3 groups of ferrets (5 animals/group) experimentally infected with different strains of pandemic (H1N1) 2009. In all 3 groups, viral shedding was detected on days 3 and 5, with the virus being cleared by day 7. NW, nasal wash.

intranasally with a $10^{5.5}$ 50% tissue culture infectious dose of A/Cheetah/California/D0912239/2009 or one of the representative strains from humans, A/Tennessee/1–560/09 and A/Ukraine/N6/2009. Animal studies were approved by the St. Jude Animal Care and Use Committee (protocol 428) and were conducted according to applicable laws and guidelines. Ferrets were observed daily, and temperature and weight measurements and nasal washes were obtained for analysis on day 0 and on postexposure days 3, 5, and 7. Weight measurements were performed until day 14.

In all 3 groups, viral shedding was detected on days 3 and 5, with the virus being cleared by day 7. The concentration of shed virus ranged from a $10^{3.5}$ to a $10^{6.3}$ tissue culture infectious doses for all 3 tested viruses; differences in the measured virus concentration were not statistically significant (Figure). Slight elevations in body temperature were detected in each group. The bodyweight per day for each ferret was analyzed as the percentage of the original weight; with all 3 viruses, the highest weight loss occurred within the first 7 days after inoculation. Overall, the clinical and virologic course of infection did not differ substantially between infecting viruses (Table).

Conclusions

The pandemic (H1N1) 2009 virus was recovered from a captive cheetah showing clinical signs compatible with influenza-like illness. Approximately 7 days before onset of clinical signs in 2 of the 4 affected cheetahs (animals C and D, the animals affected last), it was reported that an animal caregiver, who was not in direct contact with the cheetahs but who had contact with their food and environment, had influenza-like symptoms for 2 days before taking sick leave from work. Attempts to retrospectively confirm the presence of the novel subtype H1N1 virus in this particular worker were not successful, as no specimens were obtained from the worker's primary care physician at the time or immediately following clinical illness. The 4 cheetahs recovered completely under veterinary care, and a convalescent-phase sample, which tested negative by PCR and virus isolation, was available for only 1 of the 2 earliest affected cheetahs (animal B).

Reverse zoonotic transmission by fomites from contact with an ill animal caregiver is the highly likely scenario for transmission within the cheetah's restricted environment. Whole-genome sequence analysis showed a single-pair mismatch and 100% amino acid identity between the virus isolated from the cheetah and the pandemic (H1N1) 2009 virus isolated from humans. In addition, the similar data generated from experimentally inoculated ferrets suggest direct transmission of the virus rather than an evolutionary event necessary for species adaptation. The pandemic influenza A (H1N1) virus has been shown to have a high replication rate and expanded tissue tropism pattern that differ from those for seasonal influenza viruses (*4*), which may help explain the observed interspecies transmission of the virus to the cheetah in the reported absence of direct human-animal contact. In the subsequent 2 years, naturally acquired disease has been reported in 10 domestic and wildlife animal species (*5*), including the cheetah reported here.

This case demonstrates the need for a close collaboration between public health and veterinary health agencies in monitoring and understanding the transmission potential of zoonotic infectious agents, including pandemic

Table. Clinical and virologic course of infection in 3 groups of ferrets experimentally infected with different strains of pandemic (H1N1) 2009 virus, by strain*

Virus	Average maximum % weight loss (range)	Average maximum increase in temperature, °C	Observed clinical signs
A/Cheetah/CA/D0912239/2010	6.2 (2.3–10.3)	0.9 (0.9–2.7)	Sneezing (2 animals), nasal discharge (1 animal)
A/Ukraine/N6/2009	4.6 (2.8–7.1)	1.2 (0.9, 1.5)	None
A/TN/892/2009	7.0 (3.5–11.0)	1.4 (1.2–1.8)	None

*Five ferrets in each group were intranasally inoculated with a $10^{5.5}$ 50% tissue culture infectious dose of virus.

(H1N1) 2009 virus, that can be transmitted from animals to humans and from humans to animals.

Acknowledgments

We acknowledge Ben Sun and colleagues at the Sonoma County and California Departments of Public Health for their support in identifying the case and for obtaining the diagnostic specimens. We also acknowledge the timely laboratory support provided by the Laboratory Diagnostic Virology Section, United States Department of Agriculture National Veterinary Services Laboratory.

Work conducted at St. Jude Children's Research Hospital was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (contract no. HHSN266200700005C); and the American Lebanese Syrian Associated Charities.

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Shuni Virus as Cause of Neurologic Disease in Horses

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To determine which agents cause neurologic disease in horses, we conducted reverse transcription PCR on isolates from of a horse with encephalitis and 111 other horses with acute disease. Shuni virus was found in 7 horses, 5 of which had neurologic signs. Testing for lesser known viruses should be considered for horses with unexplained illness.

Several mosquito-borne alphaviruses, flaviviruses, and orthobunyaviruses, including West Nile, Rift Valley fever, and chikungunya viruses, with zoonotic potential have emerged from Africa to cause major outbreaks in previously unaffected areas. (*1*). Horses are highly sensitive to some of these viruses and have been used as sentinels for the identification of arboviruses associated with neurologic disease in South Africa (*2*). During the seasonal occurrence of common vector-borne diseases such as African horse sickness and equine encephalosis, many horses have febrile, neurologic, and fatal infections for which the etiology remains undetermined.

We report a case in which a virus isolated in cell culture from the brain of a euthanized horse that had severe encephalitis was identified as Shuni virus (SHUV), a member of the family Bunyaviridae, genus Orthobunyavirus, serogroup Simbu. SHUV-specific primers were designed and used to perform reverse transcription PCRs (RT-PCRs) on specimens from an additional 111 horses with fever and nervous disease that had been screened for the more common pathogens over 18 months. The study was conducted in accordance with the recommendations of the Faculty of Health Sciences Ethics Committee of the University of Pretoria under protocols 129/2006 and H016-09.

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DOI: http://dx.doi.org/10.3201/eid1802.111403

The Study

In January 2009, a crossbreed yearling horse (case SAE 18/09) was found wandering aimlessly in its paddock in the Vaalwater District of Limpopo Province, South Africa. The horse became progressively ataxic and, when recumbent, was referred to the hospital at the Faculty of Veterinary Science, University of Pretoria. When examined, the horse was unaware of its surroundings and paddled constantly (front legs swinging inward in their trajectory). Sedation, including the use of ketamine as a last resort, failed to calm the animal. The yearling experienced several episodes of muscle spasm interspersed with tremors and was euthanized when its condition was deemed terminal. Cytologic examination of a cerebrospinal fluid sample taken at euthanasia found pleocytosis with 98% lymphocytes, suggestive of viral infection. An autopsy was performed, and various specimens were submitted for histopathologic and virologic examination. A cytopathic agent isolated from the brain of the horse could not be identified as one of the common horse pathogens, but electron microscopic examination of negative-stained preparations of culture fluid and resin sections of infected cells showed 80-nm to100-nm particles resembling bunyaviruses (Figure 1).

RNA was extracted from infected cultures by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). RT-PCRs were performed by using the Titan One Tube RT-PCR Kit (Roche, Mannheim, Germany) with published primers Bunya 1 and 2, which amplify a 550-bp fragment of the N gene of the S RNA segment of orthobunyaviruses (*3*). Blast search analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the fragment showed that the amplicon was related to members of the Simbu serogroup of the genus Orthobunyavirus, and neighbor-joining phylogenetic analysis indicated high bootstrap support (>91%) for placement of SAE 18/09 within the Shuni, Aino, and Kaikular branches of the serogroup. The isolate shared 95.9% identity with SHUV and 91.1% identity with Aino virus (results not shown).

SHUV-specific amplification was then performed by using SHUV-specific primers designed for the present study, i.e., SHUVS111+ (5′-CGA TAC CGT TAG AGT CTT CTT CC-3′) and SHUVS688- (5′-CGA ATT GGG CAA GGA AAG T-3′). Nested PCRs were performed by using primers SHUVS178+ (5′-CCG AGT GTT GAT CTT ACA TTT GGT-3′) and SHUVS611- (5′-GCT GCA CGG ACA GCA TCT A-3′) and the Expand High FidelityPLUS PCR System (Roche, Mannheim, Germany) to produce 430-bp amplicons. Sequences were edited by using Sequencher version 4.6 (Gene Codes Corp., Ann Arbor, MI, USA) and aligned by using the ClustalW subroutine (www.ebi.ac.uk/Tools/msa/clustalw2/), which forms part of the Bioedit program (www.mbio.ncsu.edu/BioEdit/BioEdit.html). Phylogenetic trees were generated by using maximum-

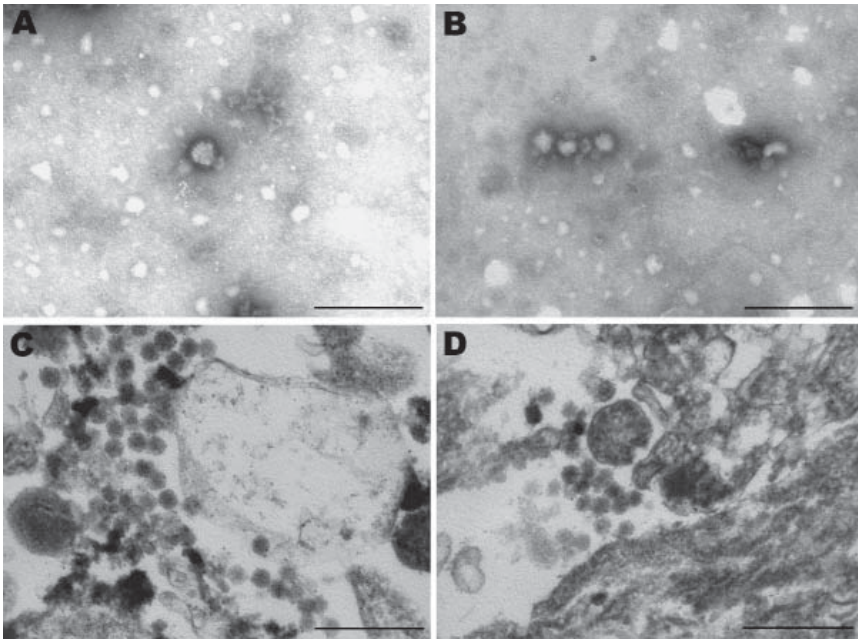

Figure 1. Electron micrographs of Vero cells infected with virus from horse SAE 18/09. A, B) Negative stain showing fringed particles (bunyavirus size) with bleb formation. C, D) Resin section showing spherical and pleomorphic bunyavirus particles in the range of 80–100 nm. Scale bars = 250 nm.

likelihood estimation in PhyML (http://code.google.com/p/phyml/) with 100 bootstrap replicates. P-distance analyses were carried out for nucleotide and amino acid sequences by using MEGA4 (www.megasoftware.net).

Specimens from an additional 111 horses were submitted by veterinarians throughout South Africa, the Onderstepoort Veterinary Institute, and the Faculty of Veterinary Science, University of Pretoria, to the Department of Medical Virology, University of Pretoria, for investigation of febrile or nervous disease. These specimens were included in the SHUV study. Specimens had been screened as appropriate for poisons and specific pathogens, including rabies, equine herpes virus, African horse sickness virus, and equine encephalosis virus (*4,5*). Specimens found to be negative were subjected to RT-PCRs with alphavirus and flavivirus generic primers and West Nile virus–specific and SHUV-specific primers (*6,7*).

SHUV infection was identified in 7 horses, 2 (8%) of 26 with unexplained fever and 5 (6%) of 86 with nervous disease (Table). In 1 of the SHUV-infected horses with febrile illness, horse SAE 38/10 (Table), co-infection with an alphavirus was found, and the virus identified as Middelburg virus. Three of the 5 horses that showed signs of nervous disease had to be euthanized when they were near death. The 2 horses with febrile disease and 2 with mild nervous disease recovered fully.

Maximum-likelihood analysis of 330-nt fragments of the amplicons with corresponding sequences of representatives of the Simbu serogroup of orthobunyaviruses confirmed that all strains clustered with SHUV virus (Figure 2). The nucleotide sequences differed from the prototype SHUV isolate by 3.6%–4.8% (average 4.2%) and from each other by 0%–1.8% (average 0.7%). The full N gene was determined for the

Table. Clinical signs in horses with Shuni virus infection, South Africa, 2009–2010

Case no.	Age, y	District, province	Specimen	Date received	Clinical signs	Outcome
SAE 18/09	1	Vaalwater, Limpopo	Brain, cell culture	2009 Jan 16	Ataxia, tremors, convulsions, recumbent with paddling of legs	Euthanized
SAE 72/09	5	Bapsfontein, Gauteng	Brain	2009 Jul 2	Depression, anorexia, ataxia, recumbent with paddling of legs	Euthanized
SAE 27/10	18	Unrecorded, Gauteng	Blood	2010 Apr 13	Tremors, petechiae, quadriplegia	Euthanized
SAE 38/10*	4	Kimberly, Northern Cape	Blood	2010 Apr 14	Fever, anorexia, leukopenia	Survived
SAE 39/10	4	Kimberly, Northern Cape	Blood	2010 Apr 14	Fever, anorexia, leukopenia	Survived
SAE 48/10	4	Norvalspont, Northern Cape	Blood	2010 Apr 23	Depression, anemia, icterus, hepatitis, anorexia, ataxia, partial paresis	Survived
SAE 109/10	13	Bronkhorstspruit, Gauteng	Blood	2010 Jul 27	Depression, anorexia, ataxia, tremors, hyperaesthesia	Survived

*Co-infected with Middelburg virus.

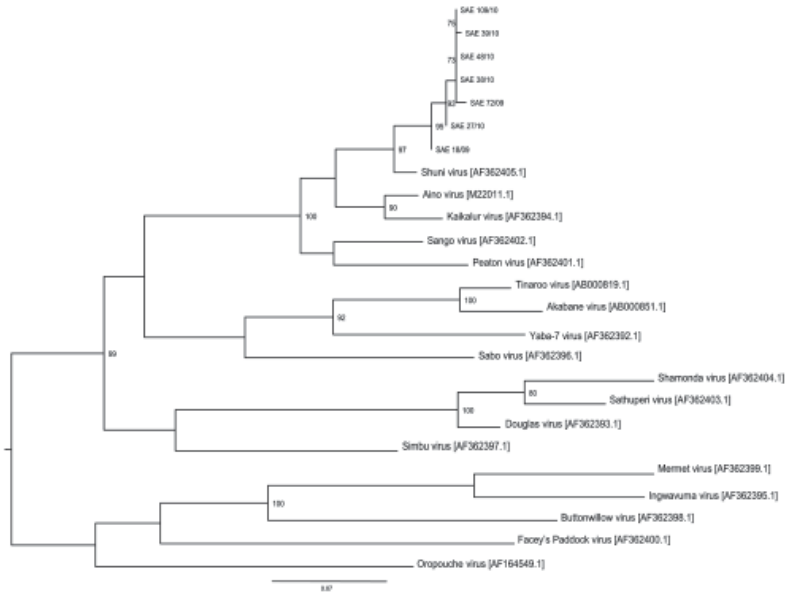

Figure 2. Maximum-likelihood tree constructed under the HKY codon position substitution model using PhyML (http://code.google.com/p/phyml/) of a 330-bp fragment of small segment RNA of Shuni virus (SHUV) identified in horses in South Africa, with representative sequences of selected other orthobunyaviruses. Scale bar = 0.07 nt substitutions. Estimates were based on bootstrap resampling conducted with 100 replicates. Only values >70 are shown. All SHUV amplicons were sequenced, and the data were deposited in GenBank, accession nos. SAE 18/09–HQ610137, SAE 72/09–HQ610138, SAE 27/10–HQ 610139, SAE 38/10–HQ 610140, SAE 39/10–HQ 610141, SAE 48/10–HQ 610142, and SAE 109/10–HQ 610143. Reference strains and GenBank accession numbers are indicated.

isolate from horse SAE 18/09, and analysis showed it to differ from the original Shuni isolate by 2.9% and to Aino virus by 6.2% at the nucleotide level.

Conclusions

SHUV was first isolated in the 1960s from cattle and sheep in abattoirs (*Culicoides* spp. midges tested as part of arbovirus surveys and in 1 instance from a febrile child in Nigeria) (*8–10*). Subsequently, the virus was isolated from pools of *Culex theileri* mosquitoes caught near Johannesburg and from cattle and a goat in KwaZulu-Natal Province, South Africa (*11,12*). In 1977, the virus was isolated from the brains of 2 horses that died of nervous disease, 1 in South Africa and 1 in Zimbabwe (*13,14*). Despite these data, no further investigations were undertaken to determine the role of the virus as a cause of neurologic disease in humans or animals. Identification of SHUV from a horse with severe neurologic signs prompted us to design specific Shuni virus primers and screen further cases of acute disease.

Over 18 months we identified 7 cases of SHUV infection, 5 of which were associated with neurologic signs. Our findings suggest that the role of SHUV as a pathogen may be underestimated and that it should be investigated routinely as a possible cause of unexplained nervous disease in humans and other animals in Africa. Most cases were identified in the autumn and winter months, which overlap with African horse sickeness, equine encephalosis, and West Nile virus outbreaks in South Africa (*5,15*), which have similar clinical signs. Such overlaps may contribute to the underrecognition of lesser known viruses, such as SHUV, because routine diagnostic investigation is limited to the more common viruses.

The discovery of a co-infection with Middelburg virus in 1 of the horses implies that broad screening for arbovirus infections in unexplained illnesses is warranted, and consideration should be given to inclusion of generic RT-PCRs for alphaviruses, flaviviruses, othobunyaviruses, and vesiculoviruses, in addition to African horse sickness and equine encephalosis viruses in future studies. Moreover, the inclusion of tests for immune response would improve the success rate for establishing diagnoses because viremia is fleeting in most arbovirus infections.

Acknowledgments

We thank the veterinarians for contributing specimens and members of the zoonosis group, Department of Medical Virology, University of Pretoria, for their involvement in the differential diagnostic screening.

The National Health Laboratory Service funded this study.

Ms van Eeden is a PhD student investigating zoonotic vector-borne viruses associated with neurologic disease in South Africa.

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Plesiomonas shigelloides Infection, Ecuador, 2004–2008

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Diarrheal risk associated with *Plesiomonas shigelloides* infection was assessed in rural communities in northwestern Ecuador during 2004–2008. We found little evidence that single infection with *P. shigelloides* is associated with diarrhea but stronger evidence that co-infection with rotavirus causes diarrhea.

Plesiomonas shigelloides (family Enterobacteriaceae) has been implicated in gastroenteritis outbreaks in travelers to tropical regions and in persons who have ingested contaminated food or water (*1*–*3*). For persons native to tropical regions, however, case–control studies have found little or no association between *P. shigelloides* infection and diarrhea (*4*–*6*). Although these studies have been conducted in areas where mixed infections are generally common, to our knowledge, none examined co-infections. We assessed the pathogenicity of *P. shigelloides* in the context of co-infections and across all age groups in a province in northwestern Ecuador.

The Study

During 2004–2008, serial case–control studies were conducted in 22 remote communities in Esmeraldas Province, Ecuador. Complete study design and laboratory procedures for pathogen detection have been described (*7*). Briefly, each community was visited 4–6 times on a rotating basis; each visit lasted for 15 days, during which all cases of diarrhea were identified by a visit to each household every morning. Household residents with cases had ≥3 loose stools in a 24-hour period, and controls had no symptoms of diarrhea during the past 6 days. Fecal samples were collected from 3 healthy controls per person with diarrhea. These samples were plated on selective agar media, and 5 lactose-fermenting colonies were screened by PCR for enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *E. coli*, and enteroinvasive *E. coli* (EIEC). Lactose-negative isolates that were identified as either *Shigella* spp. or *E. coli* were also screened by PCR for the same molecular marker used for EIEC. All non–lactose-fermenting pathogens, including *P. shigelloides*, were biochemically identified by API 20E system (bioMèrieux, Marey l'Etoile, France). Because shigellae are phylogenetically similar to *E. coli* pathotypes, we combined data from persons infected with *E. coli* and those infected with shigellae in our analysis. We tested for *Giardia lamblia* by using an ELISA kit (RIDASCREEN *Giardia*; R-Biopharm, Darmstadt, Germany), and rotavirus was detected with an enzyme immunoassay kit (RIDA Quick Rotavirus; R-Biopharm). We chose a molecular method for detecting *E. coli* pathotypes because they cannot be differentiated solely on the basis of biochemical tests; the metabolic homogeneity of *P. shigelloides*, however, makes this organism easily and clearly identifiable by biochemical test. Similarly, immunologic methods used for *Giardia* sp. and rotavirus detection are specific and sensitive enough to accurately detect these pathogens, and use of molecular methods would be justified only for deeper analysis. Institutional review board committees at the University of California, Berkeley; University of Michigan; Trinity College; and Universidad San Francisco de Quito approved all protocols.

During March 2004–March 2008, a total of 2,936 fecal samples were collected from persons of all ages (168 [6%] were <1 year of age, 597 [20%] were 1–4 years, 753 [26%] were 5–12 years, 1,362 [46%] were ≥13 years, and 56 [2%] were missing a birth date), corresponding to 775 cases and 2,161 controls. *P. shigelloides* was isolated in 253 (8.6%) samples. This number exceeded isolation rates for all of the pathogens analyzed except *G. lamblia*, which was present in 701 (23.9%) samples. Rotavirus was detected in 225 (7.7%) samples and EIEC/shigellae in 188 (6.4%) samples. *P. shigelloides* was detected in 11.4% of case-patients with diarrhea (case prevalence), which is more than the 7.2% estimated in the community (weighted control prevalence; Figure). However, once we stratified by persons infected only with *P. shigelloides* and those infected with *P. shigelloides* plus ≥1 of the other marker pathogens for which we tested, single infections with *P. shigelloides* were almost equally prevalent in the case-patients and in the community; in contrast, co-infections with *P. shigelloides* and other pathogens were more frequent in persons with diarrhea (Figure).

To determine whether *P. shigelloides* infection was associated with diarrhea, we estimated risk ratios (RRs) and bootstrapped 95% CIs for single and co-infection exposures (Table). A single infection with *P. shigelloides* was not associated with diarrhea (RR 1.5, 95% CI 0.9–2.2). Persons co-infected with *P. shigelloides* and another pathogen, however, had almost 6× the risk for diarrhea

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DOI: http://dx.doi.org/10.3201/eid1802.110562

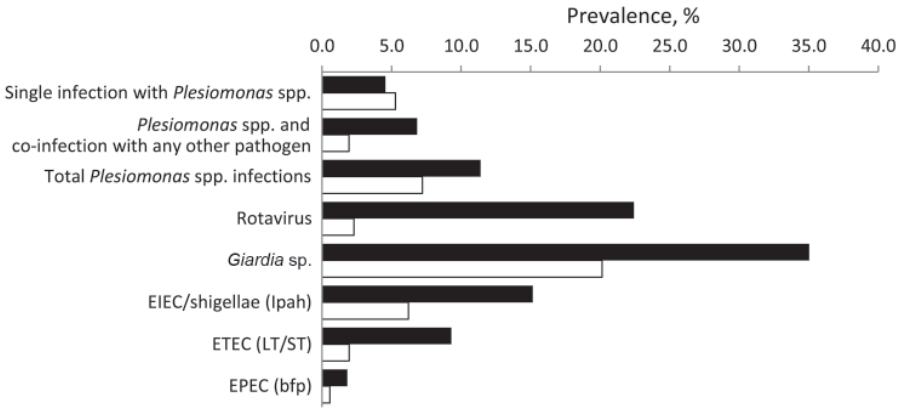

Figure. Case prevalence (black) and weighted community prevalence (white) of enteric pathogens, Ecuador, 2004–2008. Identification of pathogenic *Escherichia coli* was based on the genes given in parentheses. EIEC, enteroinvasive *E. coli*; Ipah, invasion plasmid antigen gene; ETEC, enterotoxigenic *E. coli*; LT, heat-labile toxin; ST, heat-stable toxin; EPEC, enteropathogenic *E. coli*; bfp, bundle-forming pili.

than those with no infection (RR 5.6, 95% CI 3.5–9.3) and simultaneous occurrence of *P. shigelloides* and rotavirus increased the risk for diarrhea to 16.2 (Table). We found no evidence for confounding of the association between *P. shigelloides* and diarrhea by co-infecting pathogens ($RR_{crude} = RR_{MH\text{-}pooled}$; where RR_{crude} is the unadjusted RR and $RR_{MH\text{-}pooled}$ is the pooled Mantel-Haenszel RR estimate). However, we found some evidence for confounding by age of *P. shigelloides* co-infection ($RR_{MH\text{-}pooled}$ 4.2 [95% CI 2.1–8.1], compared with the crude estimate of 5.6) but no evidence for confounding by age for single infection with *P. shigelloides*.

Conclusions

A single infection with *P. shigelloides* resulted in a moderately increased risk for diarrheal disease, which suggests that this microorganism plays a minor role as a pathogen. This result agrees with findings of previous studies (*4*,*8*,*9*). Analysis of the co-infections, however, suggests that *P. shigelloides* may be pathogenic in the presence of another pathogen. Specifically, co-infections of *P. shigelloides* with either rotavirus or pathogenic *E. coli* (including shigellae) were 16.2× (95% CI 5.5–62.3) and 13.8× (95% CI 3.3–69.3) more likely to result in diarrhea, respectively. We cannot, therefore, rule out the pathogenic capacity of *P. shigelloides* even though single infection may not be sufficient to cause disease.

This co-infection analysis might be limited by the number of pathogens considered (*Giardia* sp., rotavirus, pathogenic *E. coli*, and shigellae). However, the high isolation rates suggest we are detecting the major pathogens in the region. Other pathogens that may be useful to consider, given their attention in the literature, include *Entaemobae histolytica* and *Cryptosporidium* spp.

Although we found nothing in the literature that addresses the role of co-infection in the pathogenicity of *P. shigelloides*, co-infection with enteric pathogens is a well-known phenomenon, especially in tropical regions (*6*). Co-infection with ETEC and enteropathogenic *E. coli* increases virulence (*10*). Other studies have shown that the severity of disease is increased when rotavirus infections occur alongside another infection with another enteric pathogen (*11*).

P. shigelloides may take advantage of the disruption of the normal gut microbiota and gut physiology because of the concurrent presence of other pathogens, establishing a pathology in the human gut. For example, diarrhea caused by enterotoxins produced by pathogens, such as ETEC, and *Vibrio cholerae* (*12*), may remove normal gut microbiota, enabling *P. shigelloides* to establish an infection. The disruption of gut microbiota that facilitates gut colonization has been demonstrated in murine models infected with *Citrobacter rodentium* and *Salmonella enterica* serovar Typhimurium (*13*).

Table. RRs and bootstrapped 95% CIs for single infections and co-infections with *Plesiomonas shigelloides*, Ecuador, 2004–2008*

Co-infection	$RR_{Single\ P.shig}$ (95% CI)	$RR_{Co\text{-}Infection}$ (95% CI)	RR_{Crude} (95% CI)	$RR_{MH\text{-}Pooled}$ (95% CI)	Wald test for heterogeneity	p value
Any pathogen	1.5 (0.9–2.2)	5.6 (3.5–9.3)	2.6 (1.9–3.5)	2.7 (1.9–3.6)	32.1	<0.001
Rotavirus	1.5 (0.9–2.2)	16.2 (5.5–62.3)	1.7 (1.1–2.5)	1.9 (1.2–2.9)	61.8	<0.001
Giardia sp.	1.5 (0.9–2.2)	2.1 (1.0–3.9)	1.5 (1.0–2.2)	1.6 (1.1–2.3)	1.3	0.2
Escherichia coli/ shigellae	1.5 (0.9–2.2)	13.8 (3.3–69.3)	1.6 (1.1–2.4)	1.7 (1.1–2.6)	32.8	<0.001

*RR, risk ratio. RR_{crude} = the unadjusted RR and $RR_{MH\text{-}pooled}$ is the pooled Mantel-Haenszel RR ratio estimate. The Wald test assesses whether the strata $RR_{Single\ P.shig}$ and $RR_{co\text{-}infection}$ differ. Because of the clustered study design and the unequal sampling probabilities of controls, we chose not to use logistic regression models. Instead, we applied a nonparametric approach by using sampling weights to estimate RRs, as one would for a cohort study. We bootstrapped 1,000 samples from the original dataset, and with each new sample, we estimated the RR associated with single infection and co-infection. The lower 0.025 and upper 0.975 percentiles of the bootstrap distribution are reported as 95% CIs. Statistical analyses were conducted by using R version 2.11.1 (www.r-project.org).

Most medical literature considers infectious diarrhea as a monopathogenic phenomenon (*12*,*14*). In the data presented here, the crude risk ratio suggests that *P. shigelloides* is pathogenic. When looking at single infections, we found no evidence that *P. shigelloides* is pathogenic. When looking at co-infection data, however, we found associations between infection and diarrhea. Our findings suggest that multipathogenic infections may play a role in the pathogenesis of infectious diarrhea.

This study was supported by the US National Institute of Allergy and Infectious Disease, grant no. RO1-AI050038.

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Melioidosis in Animals, Thailand, 2006–2010

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We retrospectively estimated the incidence of culture-proven melioidosis in animals in Thailand during 2006–2010. The highest incidence was in goats (1.63/100,000/year), followed by incidence in pigs and cattle. The estimated incidence of melioidosis in humans in a given region paralleled that of melioidosis in goats.

Melioidosis is a serious infection caused by the gram-negative bacillus and biothreat organism, *Burkholderia pseudomallei*. It is the third most frequent cause of death from infectious diseases in northeastern Thailand (after HIV/AIDS and tuberculosis) (*1*) and the most common cause of community-acquired bacteremic pneumonia in northern Australia (*2*). Melioidosis also occurs in a wide range of animal species; most cases reported in the literature are in livestock in northern Australia (*3*–*8*). In Thailand, serologic studies that use the indirect hemagglutination test (IHA) have indicated that pigs, sheep, goats, and cattle are exposed to *B. pseudomallei* (*9*,*10*), but to our knowledge, culture-confirmed melioidosis in animals has not been reported in the literature (*11*). We describe the findings of a study to estimate incidence of melioidosis in animals in Thailand and compare the geographic distribution of melioidosis in animals with that in humans.

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DOI: http://dx.doi.org/10.3201/eid1802.111347

The Study

A retrospective study was performed to collect data on all animals recorded to have died of melioidosis and the total number of livestock in Thailand during January 1, 2006–December 31, 2010. This information was obtained from the Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand. Information about animal melioidosis was derived from necropsies on animals that died of unknown causes which are taken to the National Institute of Animal Health in central Thailand or to 1 of 8 veterinary research and development centers throughout Thailand. Necropsy is performed principally to monitor for infectious diseases that may be associated with outbreaks in farm animals. During the study period, IHA, blood culture, and pus culture (if available) for *B. pseudomallei* were performed if melioidosis was suspected as the cause of death.

Melioidosis was diagnosed as the cause of death in 61 animals. For 49 (80%) of these animals, diagnosis was based on a culture positive for *B. pseudomallei* from ≥1 clinical specimens; for 12 (20%) animals, cultures were negative but samples were IHA positive for melioidosis (based on a cutoff value of ≥320). Cases diagnosed by IHA, but culture negative, were excluded from further analysis because apparently healthy animals in melioidosis-endemic areas can have high bacterial titers (*9*,*10*). The animal species affected were goats (31), pigs (8), cattle (4), deer (1), horse (1), and wild animals in captivity (camel, crocodile, monkey, and zebra [1 each]). Thirty-one (61%) of the 49 cases were identified during the rainy season (June–November). The estimated incidence rate of melioidosis was highest in goats (1.63/100,000/year), followed by incidence in pigs and cattle (0.02 and 0.01/100,000/year, respectively) (Table 1).

We mapped the geographic distribution of melioidosis in goats by province and compared the meloidosis distribution with the total number of goats in the country during the same period (Table 2; Figure). The average number of total goats per year for 2006–2010 was 381,405. Most (41%) were in provinces in the south, with the remainder in western (19%), central (16%), northern (16%), northeastern (5%), and eastern (3%) Thailand. Although goats were not numerous in northeastern Thailand, provinces with the first

Table 1. Estimated incidence rates of culture-proven animal melioidosis, Thailand, 2006–2010*

Animals	Total population†	No. melioidosis cases over 5 y	Incidence rate
Goats	381,405	31	1.63
Pigs	8,187,332	8	0.02
Cattle	8,944,662	4	0.01
Others‡	NA	6	NA

*Cases/100,000 animals/year. NA, not available.
†Average number of animals per year during 2006–2010.
‡One each of the following animals: camel, crocodile, deer, horse, monkey, and zebra.

Table 2. Estimated incidence rates* of melioidosis in goats and humans, Thailand, 2006–2010

		Goat			Human		
Province†	Region	Total population‡	No. cases over 5 y	Incidence rate	Total population§	No. cases over 5 y	Incidence rate
Sakon Nakhon	Northeast	397	2	100.76	1,115,900	1,082	19.39
Ratchaburi	West	9,027	16	35.45	834,107	50	1.20
Khon Kaen	Northeast	1,372	1	29.15	1,757,772	2,122	24.14
Chachoengsao	East	1,433	2	27.91	664,184	151	4.55
Chonburi	East	2,106	1	9.50	1,262,661	76	1.20
Songkhla	South	18,355	4	4.36	1,335,832	Unknown	Unknown
Trang	South	7,058	1	2.83	614,797	Unknown	Unknown
Phetchaburi	West	7,823	1	2.56	459,398	8	0.35
Nakhon Si Thammarat	South	10,009	1	2.00	1,513,936	Unknown	Unknown
Patthani	South	19,768	1	1.01	643,718	Unknown	Unknown
Yala	South	33,325	1	0.60	476,437	Unknown	Unknown

*Per 100,000/year.
†Provinces are ordered by incidence rate of goat melioidosis.
‡Average number of goats per year during 2006–2010.
§Average human population per year during 2006–2010.

and third highest incidence of goat melioidosis (Sakon Nakohn and Khon Kaen) were situated in the northeast, a region with the highest reported incidence of human melioidosis (*1*). The incidence rate of goat melioidosis was low in the south, where the incidence of human infection has not been defined but appears to be low according to cases reported in the literature (*12–14*). The relative incidence of goat melioidosis was also high in western and eastern Thailand, regions where human melioidosis is not considered endemic. No reports of human melioidosis in the west have been published, and the 1 report from the east described 78 blood culture–positive cases during 3 years in Sa Keao Province, from which an annual incidence rate was calculated of 4.9 per 100,000 persons (*15*). To further evaluate this finding, we contacted 4 provincial hospitals in eastern and western Thailand, where cases in animals were observed, to request information about the number of culture-confirmed melioidosis cases in humans. Culture-confirmed human melioidosis cases were observed each year in all 4 hospitals (Table 2). Our findings indicate that that the geographic area of Thailand affected by melioidosis is much greater than appreciated previously.

Conclusions

The cases of animal melioidosis in Thailand reported here probably underestimate actual cases because necropsies are performed on a small minority of animals that die of natural causes. Goats are a major domestic animal in Thailand, particularly in western and southern Thailand. We demonstrated that the estimated incidence of melioidosis in goats relative to other animals is high and might represent a substantial cause of economic loss for goat farmers. The high susceptibility of goats to melioidosis relative to that of pigs and cattle is consistent with a previous report from Australia (*5*). Mapping of goat melioidosis demonstrated concentrations of cases in specific provinces and large areas of the country with no apparent disease. This finding might represent an artifact, based on the availability of information from specific veterinary centers, and we propose that animal melioidosis might be underestimated and widely distributed across the country. We predicted that the incidence of melioidosis in goats would parallel that in humans because both would be exposed to similar levels of environmental *B. pseudomallei*. This finding proved to be the case in northeastern Thailand, where the incidence of human melioidosis is high and where a small population of goats also had a high relative incidence. Such was not the case in the west, however, where the relative incidence of goat melioidosis was high, but human cases have not been reported in the literature. Possible explanations include a more active approach to determine causes of death in goats or failure to diagnose or report human disease in the west and, less plausibly, the presence of an animal-adapted *B. pseudomallei* strain that fails to infect humans. Contact with hospitals in western and eastern Thailand indicated that melioidosis was more common than the literature suggests. Further studies to determine an accurate incidence of human and animal melioidosis throughout Thailand are required. The ability to culture and detect *B. pseudomallei* in clinical microbiology laboratories countrywide also needs to be improved (*1*,*15*).

Our findings support the recommendation for pasteurization of goat milk before consumption in Thailand, which is necessary to prevent human brucellosis and also will prevent ingestion of live *B. pseudomallei* in milk. Our study was not designed to detect evidence for animal-to-human transmission in potentially at-risk populations, such as herdsmen, veterinarians, or abattoir workers. Such detection is a future objective in Thailand because presumptive zoonotic infection has been reported in Australia (*5*). Melioidosis is not currently part of the animal disease control program in Thailand, but its inclusion may now warrant review.

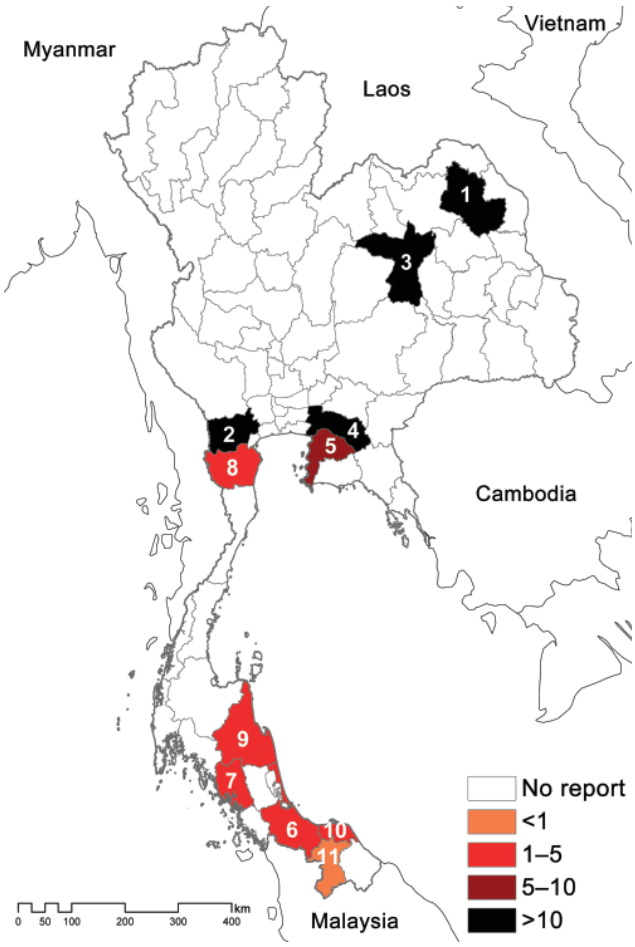

Figure. Map of estimated incidence rates for goat melioidosis, Thailand, 2006–2010. Provincial codes: 1, Sakon Nakhon; 2, Ratchaburi; 3, Khon Kaen; 4, Chachoengsao; 5, Chonburi; 6, Songkhla; 7, Trang; 8, Phetchaburi; 9, Nakhon Si Thammarat; 10, Patthani; 11, Yala. Provinces are ordered by estimated incidence of goat melioidosis.

Acknowledgments

We are grateful to the staff of National Institute of Animal Health, Department of Livestock Development, and the Wellcome Trust–Oxford University–Mahidol University Tropical Medicine Research Program. We thank Patiwat Sa-angchai and the Tropmed GIS Unit, Department of Tropical Hygiene, Mahidol University for technical support.

This study is funded by the Wellcome Trust (090219/Z/09/Z). S.J.P. is supported by the National Institute for Health Research, Cambridge Biomedical Research Centre.

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Rickettsia felis Infection in Febrile Patients, Western Kenya, 2007–2010

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To determine previous exposure and incidence of rickettsial infections in western Kenya during 2007–2010, we conducted hospital-based surveillance. Antibodies against rickettsiae were detected in 57.4% of previously collected serum samples. In a 2008–2010 prospective study, *Rickettsia felis* DNA was 2.2× more likely to be detected in febrile than in afebrile persons.

Rickettsioses are a major human health problem in many parts of the world, including sub-Saharan Africa (*1*,*2*). Awareness of rickettsiae as causes of public health problems has been increasing; several novel or emerging diseases caused by these pathogens have been recognized. In Kenya, recent reports have documented human infections with *Rickettsia conorii* (*3*,*4*) and *R. felis* (*5*) and tick infection with *R. africae* (*6*). Our objectives were to assess previous human exposure to rickettsiae and to determine the incidence of rickettsial infections among febrile and afebrile persons in western Kenya.

The Study

The study was conducted among patients visiting the Lwak Mission Hospital, a rural health care facility in western Kenya in the Asembo area, Rarieda District, in western Kenya (Figure 1). Lwak Mission Hospital serves as the field clinic for population-based infectious disease surveillance conducted by the Kenya Medical Research Institute and the US Centers for Disease Control and Prevention as described (*7*). The study was conducted with ethical approval from these institutions (protocol nos. 932 and 4566, respectively). To assess previous exposure to rickettsiae, we examined a randomly selected subset of 357 serum specimens collected January 2007 through October 2008 from patients participating in population-based infectious disease surveillance. Samples were screened at a dilution of 1:128 for IgG against spotted fever group (SFG) and typhus group (TG) rickettsiae by using an indirect fluorescence antibody assay (Fuller Laboratories, Fullerton, CA, USA).

Blood specimens were collected from the first 2 outpatients ≈5 years of age and the first 2 outpatients <5 years of age seen each day for acute febrile illness (recorded axillary temperature ≥38.0°C without an obvious cause, defined as cough, difficulty breathing, chest pain, signs of meningitis, or bloody diarrhea) from November 2008 through February 2010. A positive malaria smear was not an exclusion criterion. During the same period, blood specimens were also collected from controls: a group of outpatients who did not have febrile, respiratory, or diarrheal illness during the preceding 2 weeks and asymptomatic persons who accompanied patients to the clinic.

To detect rickettsial DNA, we performed 3 quantitative PCRs: a genus-specific assay selective for a 74-bp segment of the citrate synthase (*gltA*) gene, a group-specific assay that detects a 128-bp segment of the outer membrane protein (*ompB*) gene for tick-borne rickettsiae, and a species-specific assay that detects a 129-bp segment of the *ompB*

Figure 1. Locations of villages (brown shading in inset map) in Asembo area of western Kenya where the study was conducted, January 2007 through October 2008. Used with permission of Kenya Medical Research Institute/Centers for Disease Control and Prevention Research and Public Health Collaboration.

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DOI: http://dx.doi.org/10.3201/eid1802.111372

gene for *R. felis* (*5*,*8*). To identify which *Rickettsia* sp. was present in the positive specimens, we PCR amplified and sequenced segments of 4 rickettsial genes—*17-kDa*, *ompB*, and 2 *R. felis* plasmid genes (*pRF* and *pRFδ*)—by using primers and procedures as described (*5*).

Overall, 205 (57.4%) of 357 specimens had antibodies against rickettsiae. Of 357 serum specimens tested, 200 (56.0%, 95% exact binomial CI 50.7%–61.2%) had detectable IgG against SFG antigen preparation. Antibodies against TG antigen preparation were detected in 52 (14.5%, 95% CI 11.0%–18.6%) of 357 specimens tested; 47 (90.4%) of these specimens that reacted to TG antigens were also positive for SFG antigens, and 5 (1.4%) of the 357 specimens were positive for TG antigens alone. Presence of antibodies against SFG or TG antigens was not associated with patient sex (p>0.05). In addition, patient age was not significantly associated with TG seropositivity (χ^2 for linear trend 3.41, df 1, p = 0.065). However, an incremental linear association was demonstrated between age and IgG seropositivity to SFG (χ^2 for linear trend 45.46, df 1, p<0.001) (Figure 2).

A total of 699 febrile patients who sought care at Lwak Mission Hospital from November 2008 through February 2010 and 236 afebrile persons enrolled during this same period were tested for rickettsiae (Table 1). Overall, 50 (7.2%, 95% CI 5.4%–9.3%) of the febrile patients and 8 (3.4%, 95% CI 1.5%–6.6%) of the afebrile persons had positive rickettsiae results according to the genus-specific *gltA* assay. Univariate logistic regression indicated that febrile patients were more likely than afebrile persons to have positive PCR results (odds ratio 2.20, 95% CI 1.03–4.70, p = 0.04). According to the *ompB* assay, all specimens tested were negative for tick-borne rickettsiae. BLAST searches (www.ncbi.nlm.nih.gov/blast/Blast.cgi) for homologous sequences determined that the segments amplified from the 3 genes had 100% nt homology with *R. felis* URRXWCal2 (Table 2).

In addition to fever, the most common clinical manifestations among patients with positive PCR results for ricketttsiae were headaches (100%), chills (93.8%), muscle aches (68.8%), and joint pains (68.8%). Rash was reported for 4.4% of rickettsiae-positive patients. Among febrile patients, no statistically significant associations were found between specific signs or symptoms and positive PCR results for rickettsiae (p>0.05). Samples from all febrile patients were Giemsa stained and examined; malaria parasites were detected in 79.2% and 73.4% of samples from patients who had PCR-positive and PCR-negative results for rickettsiae, respectively.

Conclusions

The 2007–2008 serosurvey found prevalence of IgG against rickettsiae to be high. Other countries in Africa have

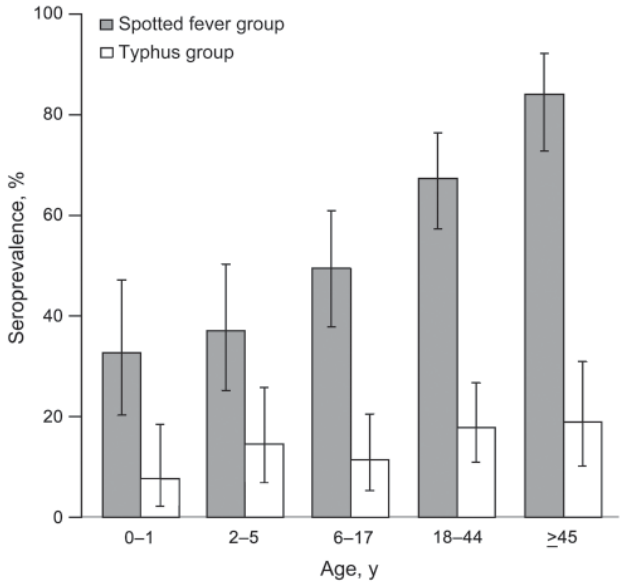

Figure 2. Age-stratified seroprevalence of IgG to rickettsiae among patients participating in population-based infectious disease surveillance, January 2007 through October 2008. Vertical lines indicate 95% binomial CIs.

reported similar (28%–58%) seroprevalence (*9*,*10*). The finding that prevalence of IgG to SFG rickettsiae increased with age can, in part, be explained by cumulative exposure to the pathogen and lifelong persistence of IgG. The high number of patients seropositive for SFG and TG rickettsiae may be attributed to cross-reactivity between SFG and TG rickettsial antigens (*11*), although results from studies in animal models show that cross-reactivity between SFG and TG rickettsiae is not consistent (*12*). Znazen et al. (*13*) speculate that antibodies against *R. felis* may be the major cause of cross-reactions to TG-rickettsiae–specific and SFG-rickettsiae–specific antigens.

The identification of *R. felis* DNA sequences in febrile patients confirms the previous finding of this pathogen among febrile patients in Kenya (*5*). Our findings are also similar to those from northern Tanzania, where acute

Table 1. Demographic characteristics of participants in hospital-based survey, western Kenya, 2008–2010

Characteristic	No. (%) febrile, n = 699	No. (%) afebrile, n = 236	p value*
Age group, y			<0.001
0–1	61 (8.7)	15 (7.0)	
2–5	345 (49.4)	30 (14.1)	
6–17	214 (30.6)	63 (29.6)	
18–44	61 (8.7)	72 (33.8)	
≥45	18 (2.6)	33 (15.5)	
Missing	0	23	
Sex			<0.001
M	352 (50.6)	73 (30.4)	
F	344 (49.4)	163 (69.1)	
Missing	3	0	

*By χ^2 test.

Table 2. Genetic sequence analysis results of rickettsial DNA amplified from *17-kDa*, *ompB*, and *pRF* genes from 21 human specimens, Lwak Mission Hospital, Western Kenya, 2008–2010*

DNA no.	*17-kDa* gene sequence	*ompB* gene sequence†	*pRF* gene sequence	*pRFδ* gene sequence
1	+	+	–	–
2	+	+	+	–
3	+	+	–	–
4	+	+	–	–
5	+	+	–	–
6	+	+	–	–
7	+	+	–	–
8	+	+	–	–
9	+	–	–	–
10	+	+	+	–
11	+	–	–	–
12	+	–	–	–
13	+	–	–	–
14	+	+	–	–
15	+	+	+	–
16	+	+	–	–
17	+	–	–	–
18	+	+	+	–
19	+	+	+	–
20	+	–	+	–
21	+	+	+	–

*omp, outer membrane protein; pRf, *R. felis* plasmid; +, positive; –, negative.
†*Rickettsia* DNA from fleas, dogs, and cats had 93%–99% nt sequence homology with *R. felis.*

rickettsiosis was serologically confirmed for 8% of febrile hospital inpatients (*14*), and from rural Senegal, where 6% of febrile patients without malaria had positive *R. felis* test results (*15*). Our findings suggest that rickettsial infections should be considered in the differential diagnosis of febrile cases in western Kenya and that diagnostic capacity should be established. Clinicians in malaria-endemic areas should consider rickettsial co-infections in diagnostic protocols and treatment of patients with malaria and other febrile illnesses.

Dr Maina is a researcher at the Kenya Medical Research Institute and a PhD student at Jomo Kenyatta University of Agriculture and Technology, Kenya. Her research interests are the epidemiology and diagnosis of emerging zoonotic diseases in multihost systems to identify reservoir hosts.

Acknowledgments

We thank Immaculate Amadi, Kabura Wamburu, and Sylvia Omulo for their assistance and all the reviewers for their helpful comments.

This research was supported by the US State Department Biosecurity Engagement Program, Wellcome Trust UK (grant no. 081828/B/06/Z), the US Centers for Disease Control and Prevention, and the US Department of Defense Global Emerging Infections Surveillance and Response System Program.

Ms Maina was a researcher at the Kenya Medical Research Institute and a PhD student at Jomo Kenyatta University of Agriculture and Technology, Kenya, at the time of this study. Her research interest is in epidemiology and diagnosis of emerging zoonotic diseases in multihost systems to identify reservoir hosts.

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1918 Influenza, a Puzzle with Missing Pieces

David M. Morens and Jeffery K. Taubenberger

Shanks and Brundage offer thought-provoking hypotheses about influenza pathogenesis during the catastrophic 1918–1919 pandemic (*1*). Although we neither agree nor disagree with their views, its central hypothesis of T-cell–mediated immunopathogenesis begs examination of past events in light of modern immunologic and virologic understanding. We also emphasize that effects of the pandemic virus should not be measured only by illness and death in 1918–1919, but should also take into account disease caused by its descendent seasonal and pandemic influenza viruses up to the present (*2*). Thus, for human influenza history to be better understood, it must be continually reevaluated.

Specifically, Shanks and Brundage hypothesize that high mortality rates in 1918 resulted from immunopathogenic effects of cell-mediated immune responses elicited by previously circulating influenza viruses. They also suggest that clues to immunopathogenic mechanisms are found in the unique, well-documented, W-shaped age-specific mortality curve of the 1918 pandemic (*3*) (Figure) in which the typical (U-shaped) curve of pandemic influenza, featuring mortality rate peaks in young and old persons, was augmented by an unprecedented third mortality rate peak in persons 20–40 years of age.

A complicating fact about 1918–1919 mortality patterns and pathogenesis hypotheses is that for ≈98% of infected persons, influenza was clinically unremarkable in its traditional signs and symptoms (fever, cough, myalgia) and severity (*4*). Clinical and epidemiologic differences were confined to 2 aspects: higher frequency of its long-appreciated post-illness complication—bacterial pneumonia (*5*)—and an unusual peak in fatal or nonfatal pneumonia cases in persons 20–40 years of age. In 1918, a higher percentage of persons of all ages, and especially those 20–40 years old, experienced influenza that led to cases of secondary bacterial pneumonia, which were caused by highly prevalent pneumopathogenic bacteria (especially pneumococci, streptococci, and staphylococci). These bacteria had been continuously causing primary pneumonia and pneumonia after influenza and other respiratory illnesses, and had long been exacting a substantial death toll.

These 1918 postinfluenza cases of pneumonia produced case-fatality rates similar to those of noninfluenza pneumonia caused by the same organisms. Moreover, antibacterial vaccines administered in 1918–1919 seem to have prevented postinfluenza deaths (*6*). Influenza mortality rates in 1918–1919 were most strongly associated with increased case incidence of, not increased severity of, common complicating bacterial pneumonia, and this finding was seen especially in persons 20–40 years of age. The epidemiology of 1918 influenza mortality is predominantly, almost entirely, the epidemiology of a single postonset complication: secondary bacterial pneumonia. Therefore, pathogenesis theories of severe or fatal 1918 influenza must account for why the 1918 virus predisposed more persons to secondary bacterial pneumonia, and also look beyond the virus to address bacterial cofactors. The hypotheses of Shanks and Brundage should be considered with these observations in mind.

An interesting aspect of the epidemiology of fatal 1918 influenza is demonstrated by epidemics in US military training camps, in which increased mortality rates were strongly associated with carriage epidemics of pneumopathogenic bacteria (*5*). An eerily analogous phenomenon had happened a year earlier (winter of 1917–1918) in deadly epidemics of measles/postmeasles bacterial pneumonia (*5*). Therefore, bacterial carrier status at the time of influenza virus introduction should be considered in interpreting mortality rate differences in soldiers and examined with respect to epidemiologic variables that could affect carriage (e.g., length of service, rural or urban differences, and health care worker status). Such simple exposure variables might explain at least some of the mortality rate differences pointed out by Shanks and Brundage.

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DOI: http://dx.doi.org/10.3201/eid1802.111409

With regard to possible immunoprotection afforded by earlier circulating influenza viruses, in our view, the picture is not fully interpretable. Epidemiologic information about the 1889 global pandemic suggests that the unidentified causative virus was novel in persons born after ≈1830 (*4*), if not before 1830. However, what the 1889 virus was, how long it may have circulated after 1889, in what form it may have drifted, and what level of population immunity in what age groups may have resulted are all speculative. Making various assumptions about post-1889 viral circulation patterns in an attempt to find epidemiologic evidence of protective or amplifying effects on incidence or mortality rates of 1918 influenza has not, to our knowledge, shown anything suggestive, let alone definitive.

Given that no age group in 1918 seems to have been protected by influenza exposures in 1889, some 1918 data are consistent with partial protection in persons >60 years of age (i.e., alive during and after the influenza pandemics of the 1830s and 1840s), even though the viruses involved in these pandemics had no discernible effect on 1889 influenza incidence (*4*). To further complicate the picture, major antigenic changes in the 1889 pandemic virus around 1900 have been postulated on the basis of epidemiologic/serologic evidence, and data from the 1957 (H2N2) and 1968 (H3N2) pandemics are each consistent with partial protection in persons alive during 1889–1918. Taken together, this information produces more questions than it answers, which suggests that only further virologic or serologic evidence based on examination of specimens from an earlier era can clarify the situation.

A related issue addressed by Shanks and Brundage concerns interpreting data on protection during the fatal October–November 1918 fall wave by influenza viruses circulating earlier in 1918 (we avoid the term spring wave on the grounds described below). In the 9 months before the 1918 fall wave, from which influenza (H1N1) viruses have been sequenced, 2 seemingly different types of influenza phenomena were observed. The first phenomenon was in January–May 1918 when scattered, explosive local outbreaks and epidemics of influenza-like illness occurred in various locations in Europe, and episodic outbreaks occurred in several other countries, which in virtually all cases showed lower than expected mortality rates for influenza. (Shanks and Brundage classify this spring activity, along with summer activity, as a spring wave.) If this wave was influenza, it was not a wave as the term had been used since 1889 to indicate global pandemic mortality.

The second phenomenon was a wave of moderate mortality rates that occurred not in the spring of 1918, but in the summer (July–August), mostly in a few countries in northern Europe. This summer wave seems consistent with a first major occurrence of the 1918 virus (H1N1), which may have found a tenuous foothold in the normally unfavorable summer months, predominantly in northern climes where temperature and humidity might be less restrictive of virus circulation. If this wave was the 1918 pandemic virus, simple arithmetic dictates that to have reached moderate explosiveness by July it must have been circulating for at least many weeks beforehand (*7*). Prepandemic circulation of virus (H1N1) in early 1918 could have caused at least some circumscribed outbreaks that elicited protection.

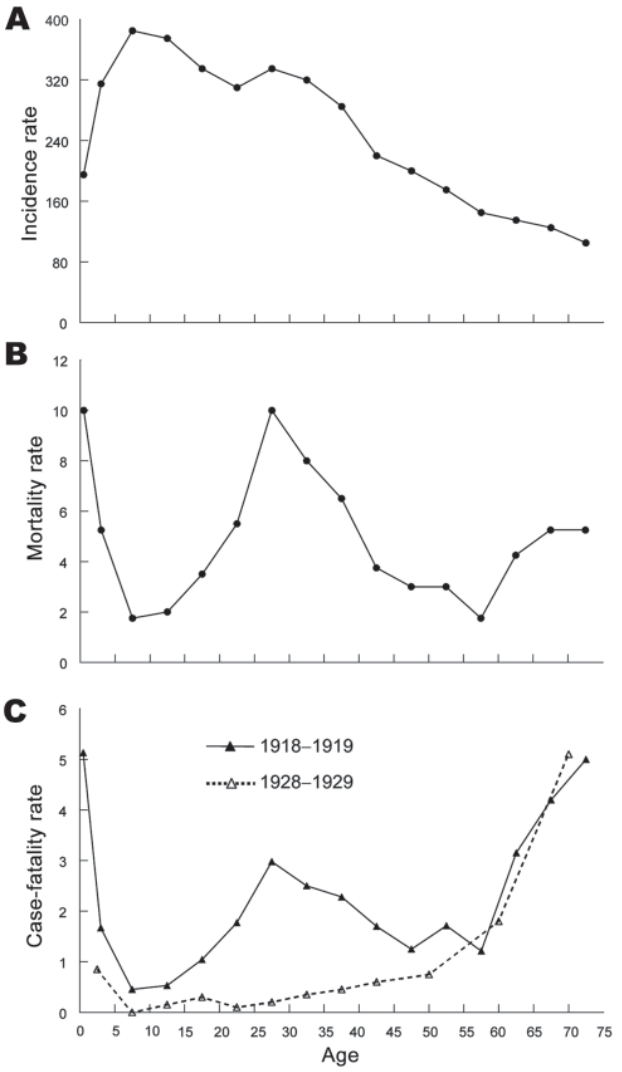

Figure. Combined influenza plus pneumonia (P&I) age-specific incidence, mortality, and case-fatality rates, per 1,000 persons/age group, US Public Health Service house-to-house surveys, 8 states, 1918, and US Public Health Service surveys during 1928–1929. A) P&I incidence for 1918; B) mortality rate for 1918 (ill and well persons combined); C) P&I case-fatality rates for 1918 (solid line) compared with a more typical curve of age-specific influenza case-fatality rates (dotted line) from 1928–1929. Reprinted from (*3*).

However, if all winter–spring prepandemic 1918 activity had been caused by the pandemic virus, we are left with the conundrum of why it did not become pandemic then, when environmental circumstances were seemingly more favorable, and when it was being locally transmitted within the war zone in Europe at more explosive levels than the fall wave pandemic virus would later be. We must also explain the frustratingly contradictory protection data from spring or summer influenza-like illness during the fall occurrence of influenza.

Astute observers of the time considered the 1918 protection data uninterpretable (*8*). Because influenza viruses of different subtypes are now understood to protect against each other for prolonged periods (e.g., H1N1 against H2N2 and H2N2 against H3N2), interpreting 1918 protection data has become even more problematic. One or more viruses unrelated to the fall wave virus (H1N1) (e.g., an 1889 viral descendant) may have caused at least some of the observed protection and nonprotection phenomena in 1918. Less plausibly, the pandemic virus could have lost transmissibility while gaining pathogenicity after early 1918. However, in the absence of virologic evidence, the identity of early 1918 viruses that may have caused or failed to cause protection remains speculative.

Finally, despite whatever degree of immunopathogenesis or immunoprotection may have occurred in 1918, we see no particular reason to focus hypotheses on T-cell immunity over immunity conferred by antibody to viral antigens. The extremely high 1918 influenza infant mortality rate cannot easily be linked to cell-mediated immunity because infant T cells would presumably have never been exposed to influenza viruses. It is also noteworthy that mortality rates across the entire 1918 age spectrum were higher than in any other year between 1889 and the present time. In looking at the W-shaped mortality curve, we believe that the findings are striking for persons ≈5–14 years of age, the age range of persons with the lowest mortality rates in virtually all influenza pandemics and epidemics studied to date. In 1918, this age group appears to have had an ≈4-fold higher mortality rate than in 1889, conceivably indicating inherent viral virulence or, more correctly, viral–bacterial copathogenicity because most of the relatively few deaths in this age group seem also attributable to secondary bacterial pneumonia.

Although it is intriguing to speculate about the role of severe and fatal primary viral pneumonia, we are unaware of data suggesting that primary viral or viral immunopathogenic mechanisms accounted for high mortality rates in any 1918 age group; results of reported experimental animal studies are of uncertain relevance for humans. Almost all of the tens of thousands of autopsies reported in 1918 indicated classic bacterial pneumonia as the most prominent feature, which was different in frequency, but not in kind, from the familiar cases of pneumonia seen year in and year out, before and after 1918 (*5*,*7*). The data appear most consistent with some unidentified property of the 1918 virus (e.g., respiratory cell cytopathicity) that potentiated pneumonia with common bacteria carried in the upper respiratory tract (*5*). The cause of the middle peak of the W-shaped mortality curve remains a fascinating mystery that so far seems inexplicable by any hypothesis.

In summary, Shanks and Brundage have addressed 3 major mysteries of the 1918 influenza pandemic: high mortality rates/unexplained pathogenesis, unexplained age-specific mortality rate patterns, and evidence for wave-to-wave protection, with a unifying hypothesis. In our view, they justifiably point out that highly inconsistent wave-to-wave protection data from different 1918 observers represent essential clues to what happened 94 years ago. However, these clues have not yet led to satisfactory answers. They also draw attention to the W-shaped age-specific mortality curve, still unexplained we would argue, and hypothesize that it, as well as disease pathogenesis and protection, results from cell-mediated immune responses. Although we are not fully persuaded by all aspects of this hypothesis, it does suggest avenues for experimental and perhaps serologic and immunologic research. It should also stimulate us to rethink old mysteries in light of modern and evolving understanding of influenza. Questions about 1918 persist, and critical pieces of the puzzle, in our view, are still missing.

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Rapid Diagnosis of Pandemic (H1N1) 2009 in Cuba

To the Editor: During 2005–2008, the Cuban National Influenza Center (NIC) at the Pedro Kourí Institute in Havana, Cuba, implemented a protocol for influenza surveillance proposed by the Pan American Health Organization and the US Centers for Disease Control and Prevention (*1*). One of the most essential features of this protocol was strengthening laboratory capacity for surveillance of seasonal influenza and timely detection of a new influenza virus with pandemic potential.

On April 26, 2009, in response to an outbreak of pandemic (H1N1) 2009 in Mexico, NIC proposed an algorithm with 2 phases for processing specimens. The purpose of the algorithm was to identify the novel virus, effectively monitor its circulation in Cuba, and make these data available to the national health authorities and the World Health Organization (WHO) (*2*).

The first phase of the algorithm was used during April–September 2009. Following the recommendations of WHO (*3*), this phase included fluorescent antigen tests, nucleic acid extraction by using QIAamp Viral RNA and QIAamp Viral DNA Kits (QIAGEN, Hilden, Germany), and 3 reverse transcription PCR (RT-PCR) assays for typing and subtyping of influenza A viruses. RT-PCRs were used for differential diagnoses, which included 15 other respiratory viruses (*4*–*7*).

At the same time, on the basis of pandemic (H1N1) 2009 virus sequences published on the Global Initiative on Sharing Avian Influenza Data website (http://platform.gisaid.org/dante-cms/struktur.jdante?aid=1131), we designed a primer set specific for the hemagglutinin gene and we developed an in-house, conventional RT-PCR was designed to enable virus-specific identification. Confirmation was performed by subsequent sequencing of hemagglutinion, nucleoprotein, or neuraminidase genes by using the BigDye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter Inc., Krefeld, Germany).

Several sequences obtained were submitted to GenBank under accession nos. HM159409–159418 and HM176606–HM17639) (*8*,*9*). BLAST (www.ncbi.nlm.nih.gov/BLAST/) search analysis on sequences obtained from the first cases identified indicated the highest alignment score (≥98% identity) with pandemic (H1N1) 2009 virus strains.

The second phase of the algorithm was used during September 2009–August 2010. This phase included automated nucleic acid extraction (QIAcube; QIAGEN) and real-time RT-PCR kits (Pan American Health Organization, Washington, DC, USA, and Centers for Disease Control and Prevention, Atlanta, GA, USA) for detection and characterization of pandemic (H1N1) 2009 virus (*10*).

During January 3–July 31, 2009, a total of 2,156 specimens were submitted to the NIC for influenza surveillance. During January 2009–August 10, 2010, a total of 14,692 clinical samples were processed. In the next 6 months, the number of specimens submitted to NIC doubled. More specimens (7,978) were submitted during January 1–August 10, 2010, than during all of 2009.

Most (5,601) clinical specimens processed were from patients with influenza-like illness. The highest percentage (45.9%) of influenza-positive samples was detected in specimens from these patients, followed by specimens from patients during outbreaks (18.0%).

Pandemic (H1N1) 2009 virus infection peaked during epidemiologic weeks 39–41, 2009 (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/2/11-0547-FA1.htm) and co-circulated with influenza A virus (H3N2). This period coincided with the start of school. A total of 285 and 211 specimens were positive for pandemic (H1N1) 2009 virus and influenza A virus (H3N2), respectively. During April 2010, a second peak of pandemic (H1N1) 2009 was detected, and 303 cases were confirmed during epidemiologic weeks 13–16 (online Appendix Figure).

By the time WHO declared the end of the pandemic on August 10, 2010, public health authorities recognized 1,805 cases of pandemic (H1N1) 2009 in Cuba. On the basis of laboratory results, during April 2009–2010, two peaks of pandemic (H1N1) 2009 were observed in Cuba.

During August 2010, Cuba experienced active transmission of seasonal influenza A (H3N2) virus, which displaced pandemic (H1N1) 2009 virus as the predominant virus. Similarly, seasonal influenza virus (H1N1) was displaced by pandemic (H1N1) 2009 virus. The last case of seasonal influenza (H1N1) detected in Cuba was in June 2009.

In this context, we believe that implementing national diagnostic algorithm enabled timely identification of the novel virus and effective monitoring of its circulation, even before international diagnostic protocols and reagents were available in Cuba. This study shows the need for nucleic acid amplification tools in laboratory diagnosis and surveillance of influenza viruses. As we prepare for future influenza pandemics, new and appropriate diagnostic methods and periodic assessment of influenza surveillance methods are needed as new information becomes available.

Acknowledgments

We thank the physicians and laboratory personnel for providing samples and clinical data; the technicians and researchers of the Virology Department and Microbiology Division of the Pedro Kourí Institute, Havana, Cuba, for

collaborating in the diagnosis of pandemic influenza; Thais dos Santos and Mauricio Cerpa for revising the manuscript; and the US Centers for Disease Control and Prevention, the Chinese Centers for Disease Control (Beijing, China), and the Carlos III Institute (Madrid, Spain) for providing test kits and positive controls.

This study was supported in part by the Cuban National Program of Surveillance and Control for Acute Respiratory Infection of the Ministry of Health and the Pan American Health Organization.

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DOI: http://dx.doi.org/10.3201/eid1802.110547

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Hand, Foot, and Mouth Disease Caused by Coxsackievirus A6, Japan, 2011

To the Editor: Coxsackievirus A6 (CVA6) belongs to human enterovirus species A of the genus *Enterovirus*. According to a Japanese Infectious Agents Surveillance Report, this virus is one of the major causes of herpangina, an acute febrile disease characterized by vesicles, ulcers, and redness around the uvula, which occurs mainly in young children and infants. (*1*).

In June 2011, a sudden increase in cases of hand, foot, and mouth disease (HFMD) at pediatric sentinel sites (≈3,000 pediatric hospitals and clinics) was reported to the National Epidemiologic Surveillance of Infectious Diseases System in Japan. Compared with past numbers of cases over 30 years of surveillance, the number of cases of HFMD per sentinel site peaked in week 28 (July) of 2011 (10.97 cases per sentinel), particularly in western Japan (*2*). According to the Infectious Agents Surveillance Report (as of September, 18, 2011), CVA6 was detected in 709 HFMD cases and 156 herpangina cases throughout Japan (*1*).

Clinical samples (throat swab specimens and feces) obtained from sentinel sites in Shimane, Hyogo, Hiroshima, and Shizuoka, Japan, were screened for enteroviruses by using an enterovirus-specific reverse transcription PCR and sequence analysis of the partial viral protein (VP)4/VP2 or VP1 region (*3*). Among 93 clinical samples from 108 HFMD case-patients, we identified 74 case-patients as CVA6 positive by sequence analysis.

On the basis of sequence analysis of the entire VP1 region (GenBank accession nos. AB649286–AB649291), the consensus sequence

had 82.3%–82.5% nt identity (94.8%–95.4% aa identity) with the prototype CVA6 Gdula strain (GenBank accession no. AY421764). CVA6 was not isolated from clinical samples in a cell culture system. Therefore, most CVA6 strains were identified by molecular detection directly from clinical samples and sequence analysis. Some CVA6 strains were grown and isolated in suckling mice; these strains were antigenically identified as CVA6 by a neutralization test with specific antiserum against CVA6 (*4*).

In Japan, HFMD and herpangina are classified as category V infectious diseases. On the basis of clinical diagnosis, suspected infections were reported by pediatric sentinel sites on a weekly basis to the Infectious Disease Surveillance Center of the National Institute of Infectious Diseases (Tokyo, Japan). Typical clinical signs and symptoms of HFMD cases caused by CVA6 were fever, mild vesicles in oral mucosa, and skin blisters on hands, arms, feet, legs, buttocks, and nail matrixes (Figure). Some patients with HFMD had onychomadesis (periodic shedding of the nails) 1–2 months after onset of HFMD. Most cases of HFMD were self-limited. However, additional follow-up may be necessary for patients with onychomadesis who are treated at dermatology clinics.

As in other countries in the Asia–Pacific region, major causes of HFMD in Japan were CVA16 and enterovirus 71. In 2010, enterovirus 71 was identified as a major cause of HFMD (*1*). In contrast, CVA6 was consistently associated with herpangina, as were CVA2, CVA4, CVA5, and CVA10, but CVA6 was occasionally detected in HFMD case-patients. CVA6 was the major cause of herpangina in 2007, but an increase in the detection rate of CVA6 in HFMD case-patients was reported in Japan in 2009 (*1*).

HFMD outbreaks caused by CVA6 were reported in Singapore, Finland, and Taiwan in 2007–2009 (*5*–*8*). Recent HFMD outbreaks in Finland and Spain were associated with cases of onychomadesis 1–2 months after onset of HFMD (*6*,*8*,*9*). In Japan, cases of onychomadesis after onset of HFMD were reported in 2009 (*10*). Therefore, changes in clinical outcomes of CVA6-associated diseases should be investigated.

Although most HFMD cases caused by CVA6 in Japan were mild, CVA6 was also detected in other clinical samples, including cerebrospinal fluid from a patient with acute encephalitis in Hiroshima, which reaffirmed possible additional clinical manifestations during an HFMD outbreak caused by CVA6. Careful surveillance of disease and infectious agent activities are crucial in monitoring CVA6-associated HFMD, onychomadesis, and neurologic diseases. Nucleotide identity between CVA6 strains in Finland (2008) (*7*) and Japan (2011) was ≈95% in the partial VP1 region. More detailed genetic, phenotypic, and epidemiologic analyses of CVA6 are needed to determine the role of CVA6 in HFMD outbreaks with or without onychomadesis.

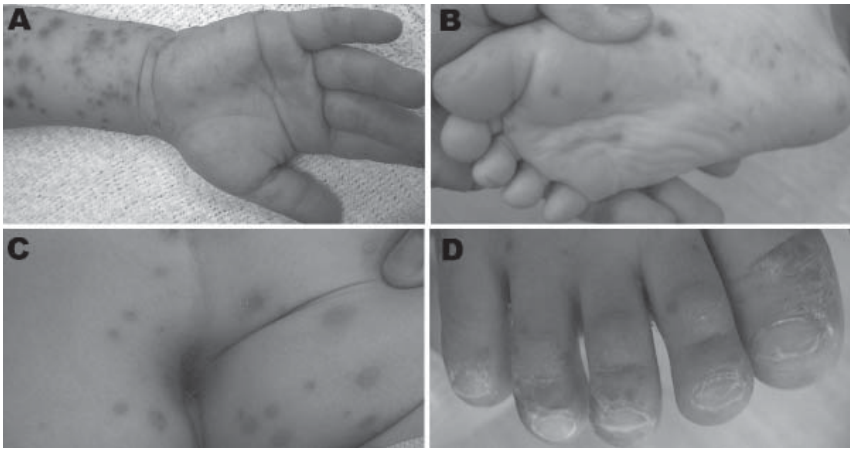

Figure. Typical clinical manifestations of hand, foot, and mouth disease associated with coxsackievirus CVA6 in Shizuoka, Japan, June–July, 2011. A) Hand and arm of a 2.5-year-old boy; B) foot and C) buttocks of a 6-year-old boy; D) nail matrix of a 20-month-old boy. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/2/11-1147-F1.htm).

Acknowledgments

We thank the staff of prefectural and municipal public health institutes in Japan for virus detection, identification and molecular analysis, and Grant Hansman for critical review of the manuscript.

This study was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and for research on emerging and re-emerging infectious diseases from the Ministry of Health, Labour and Welfare, Japan.

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DOI: http://dx.doi.org/10.3201/eid1802.111147

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Human and Porcine Hepatitis E Viruses, Southeastern Bolivia

To the Editor: Hepatitis E virus (HEV) genotypes 3 and 4 are considered to be primarily zoonotic (*1*). However, recent data indicate that both genotypes can be transmitted among humans through other routes (*2*,*3*). Observations of genetic distinctiveness between swine and human HEV strains circulating within the same region argue against exclusivity of zoonotic transmission (*4*). A recent report presented a remarkable example of such distinction between genotype 3 isolates in rural communities in southeastern Bolivia (*5*).

We examined HEV sequences obtained in that study to show the independent genetic origin of swine and human variants. Findings suggest disjunction between human and swine HEV strains in this epidemiologic setting, despite the potential for extensive cross-species exposure.

Using reference sequences from Lu et al. (*6*), we conducted subtype analysis of HEV open reading frame 2 sequences at nucleotide positions 826–1173 (GenBank accession no. AF060668) from isolates from 2 rural communities in southeastern Bolivia (*5*). Analysis showed that swine sequences belonged to subtype 3i and that the human sequences belonged to 3e.

We collected all available GenBank genotype 3 sequences covering this genomic region for which the dates of collection were documented. Sequences were used to estimate the time from the most recent common ancestor (tMRCA) by using BEAST version 1.6.1 (*7*). Estimated tMRCA for GenBank sequences was longer than for sequences from Bolivia alone (Table) or for all genotype 3 sequences together (Table).

To reduce the effect of close relatedness among human or swine HEV sequences from Bolivia on the tMRCA estimate, we used only 1 representative sequence per species from each community in the final analysis. This analysis identified an estimated tMRCA similar to that seen for GenBank sequences alone (Table, model F vs. model D). This estimate indicates that human and swine HEV isolates from southeastern Bolivia last shared a common ancestor ≈275 years ago (Table, model F). Thus, swine HEV strains from both rural communities belonged to subtype 3i, and the human HEV strains identified from the community of Bartolo, Bolivia, belonged to subtype 3e and shared an ancestor with swine strains almost 3 centuries ago.

This finding is surprising because the community of Bartolo has several potential risk factors for zoonotic transmission of HEV. There are ≈200 humans and ≈70 swine in Bartolo (*8*). Residents are mainly native Quechua and Guarani with some of mixed Spanish ancestry who subsist at a low socioeconomic level. Their main livelihood activities are agriculture and breeding of animals. Free-range pig farms are family owned. Because of its impoverished state, the community has no running water, and few houses have toilets. No facilities are suitable for safely slaughtering

Table. Model estimates of time to most common recent ancestor for HEV ORF2 nucleotide sequences, southeastern Bolivia*

Model	Start position	Stop position	Bolivia sequence	GenBank sequence	Mean tMRCA, y	95% HPD, y		Mean ± SD rate of substitutions/site/y
						Lower	Upper	
A	826	1173	X	NU	55.31	16.26	108.05	$3.27 \times 10^{-3} \pm 9.44 \times 10^{-6}$
B	826	1173	NU	3i and 3e	148.63	3.01	291.46	$1.88 \times 10^{-2} \pm 6.41 \times 10^{-4}$
C	826	1173	X	X	144.45	38.66	298.20	$3.43 \times 10^{-3} \pm 4.74 \times 10^{-5}$
D	826	1173	NU	X	328.05	45.59	681.90	$2.12 \times 10^{-3} \pm 7.33 \times 10^{-5}$
E	1	1980	NU	X	296.06	163.40	467.97	$9.27 \times 10^{-4} \pm 1.25 \times 10^{-5}$
F	826	1173	3 seqs	X	275.45	40.06	635.32	$2.40 \times 10^{-3} \pm 5.06 \times 10^{-5}$

*HEV, hepatitis E virus; ORF, open reading frame; tMRCA, time to the most recent common ancestor; HPD, highest posterior density boundary; X, sequences was used; NU, not used; 3i and 3e, subtype 3i and 3e sequences were used; 3 seqs, sequences (CV2, CB9, and HB2BA053) from Bolivia were used.

animals (*5*,*9*). These conditions appear to create a setting in which zoonotic transmission of HEV should be common, and infection should be caused by a strain shared between swine and humans. However, the data suggest host-specific infection with distinct HEV subtypes.

Although specimens were collected from 172 humans (≈86%) and 67 swine (≈96%) in Bartolo (*8*), zoonotically transmitted isolates may have been missed because of the sample-pooling technique used (*5*). Nevertheless, detection of distinct HEV strains in human and swine populations indicates possible nonzoonotic, human-to-human transmission in this community. Detection of antibodies against HEV among 7% of residents and HEV genomes in persons without serologic markers of HEV infection indicate a higher HEV prevalence in Bartolo (*5*). Subclinical infection detected by PCR among Bartolo residents (*5*), rapid decrease of HEV antibody, and uncertain sensitivity of commercial serologic assays (*10*) suggest that the reported extent of HEV infection is most likely an underestimate.

High prevalence may generate conditions in this community that effectively prevent cross-species transmission because of frequent exposure to HEV early in life when contacts between humans and animals are limited, thus promoting host-specific transmission. This supposition is supported by the higher seropositivity seen among children 1–5 years of age and adults 41–50 years of age in Bartolo (*5*). Implications of these observations for understanding HEV evolution and epidemiology of HEV infections warrant further research on genetic heterogeneity of HEV strains in this region and other epidemiologic settings.

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DOI: http://dx.doi.org/10.3201/eid1802.111453

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Baylisascaris procyonis Infection in Elderly Person, British Columbia, Canada

To the Editor: *Baylisascaris procyonis* is an ascarid roundworm that commonly parasitizes the intestine of North American raccoons (*1*,*2*). As intermediate hosts, humans may develop visceral, ocular, and neural larva migrans (NLM) (*1*,*3*). Despite the high potential for exposure, only 20 cases of human cerebral *B. procyonis* infection have been reported, most causing devastating neurologic disease in young children (*1*,*4*–*6*). Here we expand the currently recognized spectrum of human disease by describing an unusual case of pathologically proven cerebral *B. procyonis* infection, which caused no apparent symptoms, in an elderly patient from British Columbia, Canada, with Alzheimer dementia.

The estimated prevalence of *B. procyonis* infection in North American raccoon populations is 68%–82% in the western United States (*2*) and 61% in southwestern British Columbia, Canada (*7*). Infected animals shed millions of *B. procyonis* eggs each day; the eggs embryonate in soil and are highly resilient in harsh environmental conditions (*1*,*2*). Raccoons tend to defecate in communal locations that are often in areas of human activity (*8*). Accidental or incidental human contact with contaminated soil may result in fecal-oral transmission. Following ingestion by an intermediate host, the infective larvae hatch in the small intestine and then penetrate the intestinal wall, migrate through the liver and lungs, and undergo extensive somatic dissemination (*1*–*3*). Migration causes mechanical tissue damage and provokes an inflammatory response. *B. procyonis* larvae grow in size but do not undergo further maturation in humans. The severity of disease is hypothesized to be related to the number of eggs ingested, the path of larval migration, and the extent of the host inflammatory response (*1*–*4*).

Despite the apparent potential for human disease, to our knowledge, only 20 cases of *B. procyonis* NLM have been reported (*1*,*4*–*6*). Most cases occurred in children <2 years of age or in older children and young adults with developmental delay. The only previously reported case of *B. procyonis* NLM in an adult was in a developmentally disabled 21-year-old adult known to exhibit geophagia and pica (*4*). Hemorrhagic necrotizing eosinophilic meningoencephalitis associated with large numbers of intact larvae has been described in patients with fatal cases (*1*,*4*), and all but 1 patient who survived (*9*) were left with severe neurologic deficits (*1*,*4*–*6*).

We report the case of a 73-year-old female nursing home resident with a 10-year history of moderately severe Alzheimer-type dementia. She was well-educated, had no other medical problems, and had previously resided with her husband in a rural part of British Columbia. Apart from mild confusion and poor memory, she was in good health and able to ambulate and communicate. There had been no recent change in her medical condition, and she voiced no concerns about medical problems before dying suddenly of cardiopulmonary arrest.

Autopsy findings showed a large pulmonary embolus as the cause of death. Mild, diffuse cerebral atrophy was the only gross brain abnormality (brain weight 1,210 g). Examination by microscopy was restricted to the brain and demonstrated Alzheimer-type pathology that was sufficiently severe to account for the patient's dementia. In addition, sections of deep white matter from the left frontal lobe showed a small number of lesions, each consisting of a single larval nematode surrounded by mild chronic reactive changes and inflammation (macrophages, lymphocytes, plasma cells, and rare eosinophils) (Figure, panel A). Inflammation and reactive changes were restricted to the tissue immediately surrounding the larvae.

The larvae measured 65 μm in maximum transverse diameter and had a 1-μm thick striated cuticle that formed sharply pointed paired single lateral alae (Figure, panel B). The centrally located intestine measured 25 μm in diameter, was laterally compressed, and was lined by columnar cells with microvilli and abundant cytoplasmic granules. The intestine was flanked by smaller paired triangular excretory columns. There were 4–6 muscle cells per quadrant. These morphologic features

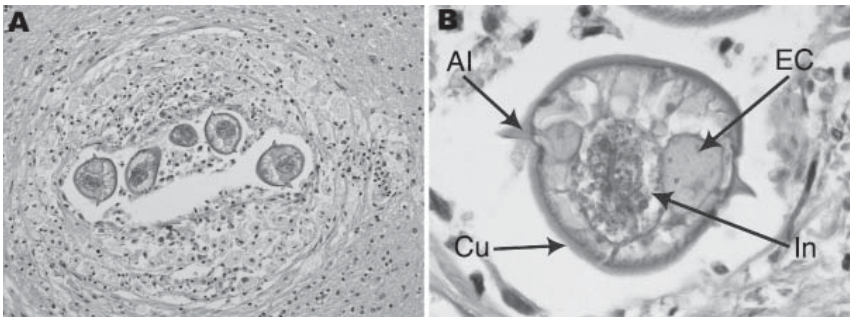

Figure. *Baylisascaris procyonis* infection in the frontal cerebral lobe white matter. A) Larval nematode seen in multiple transverse sections, surrounded by mild chronic inflammation and reactive changes. Hematoxylin and eosin stain; original magnification ×10. B) Morphologic features of the larvae included maximum diameter of 65 μm; thin, striated cuticle (Cu); single paired lateral alae (Al); and paired excretory columns (EC) that were smaller in diameter than the central intestine (In). Hematoxylin and eosin stain; original magnification ×40.

are characteristic of *B. procyonis* roundworms and distinguish it from other nematodes that are known to affect humans (*2*,*3*).

This case is unusual in several respects: the patient is the oldest known person with confirmed *B. procyonis* NLM; it is only the second case reported from Canada (*5*); and it is a pathologically proven example of cerebral *B. procyonis* infection in a human without major clinical manifestations. Although this patient's dementia could have masked subtle neurologic features, no changes were witnessed by caregivers or relatives, and the patient voiced no concerns. Her long-standing dementia was fully explained by Alzheimer-type pathology, and it is highly unlikely that the low-level and restricted anatomic distribution of parasitic infection would have contributed to her dementia. More likely, the combination of confusion and poor hygiene and ambulatory state in the patient may have predisposed her to acquiring *B. procyonis* roundworms through ingestion of contaminated soil. The existence of mild or subclinical *B. procyonis* brain infection is recognized in veterinary medicine (*1*,*2*). This case expands the currently recognized spectrum of human disease caused by *B. procyonis* roundworms to include mild or subclinical cerebral infection in elderly persons and suggests that dementia may increase the risk for exposure.

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DOI: http://dx.doi.org/10.3201/eid1802.111046

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Dengue Surveillance among French Military in Africa

To the Editor: In their recent article, Amarasinghe et al. (*1*) describe dengue virus distribution in Africa. Their data were based on published reports of dengue cases among local populations and travelers returning from Africa. To complement the description by Amarasinghe and colleagues of dengue serotypes found in Africa, we report results from dengue virologic testing during 1998–2010. The tests were performed at the Arbovirus National Reference Center (Tropical Medicine Institute of the Military Health Service, Marseille, France).

Each year, ≈14,000 French soldiers are stationed in dengue-endemic areas of Africa (mainly Cameroon, Central African Republic, Chad, Djibouti, Gabon, Côte d'Ivoire, Senegal, and Mayotte and Reunion islands), from which they travel throughout Africa. The population of soldiers is under constant epidemiologic surveillance. If symptoms of dengue fever develop in a soldier, a blood sample and a dengue-specific questionnaire from the patient are sent to the Tropical Medicine Institute of the Military Health Service. Virus culture and reverse transcription PCR, or both, were performed on early samples; otherwise, serologic testing was performed by using in-house assays (IgM antibody capture ELISA and direct IgG ELISA).

During the 12 years of surveillance, the laboratory received 2,423 samples from patients with suspected dengue within the French Armed Forces in Africa. Of these, 224 were probable acute dengue infections: 202 had positive IgM serologic results for dengue, and 22 were confirmed as dengue cases by RT-PCR or culture (Table). Serologic

Table. Countries in Africa with evidence of dengue virus transmission among French Armed Forces, 1998–2010

Country and year	No. cases	Testing method	Infection status	Dengue virus serotype
Cameroon, 2010	1	PCR	Confirmed	1
Cape Verde, 2010	5	Culture	Confirmed	3
Central African Republic, 1995	1	Serology	Probable	Unknown
Chad, 1998–2001, 2003, 2006, 2009–2010	28	Serology	Probable	Unknown
Comoros				
2010	1	PCR, culture	Confirmed	1
2010	2	PCR	Confirmed	3
Côte d'Ivoire				
1999	1	Culture	Confirmed	1
2000, 2004–2007	11	Serology	Probable	Unknown
2010	1	PCR	Confirmed	3
Djibouti				
1998	4	Culture	Confirmed	1
1998	24	Serology	Probable	Unknown
2000	2	Culture	Confirmed	1
2000	4	Serology	Probable	Unknown
2001–2005	123	Serology	Probable	Unknown
2005	1	PCR	Confirmed	ND
2006	4	Serology	Probable	Unknown
2008	2	Serology	Probable	Unknown
Gabon				
1998, 2006–2008	22	Serology	Probable	Unknown
2010	1	PCR	Confirmed	1
Mayotte, 2009	1	Culture	Confirmed	1
Senegal, 2009	1	PCR	confirmed	3
Somalia, 1999	1	culture	confirmed	2

data may be confusing because of potential cross-reactions with other flavivirus antibodies (in particular in Chad with West Nile virus).

Because of probable underreporting from the field, our reported number of confirmed dengue cases likely underestimates the actual number of cases among French troops stationed in Africa. Nonetheless, our data complement those reported by Amarasinghe et al. by demonstrating additional locations for circulation of serotype 1 (Cameroon, Djibouti, Gabon, Mayotte) and serotype 3 (Comoros). Military epidemiologic surveillance systems can detect dengue circulation where soldiers stay. Thus, these systems could serve to evaluate the risk for dengue infection in countries without local epidemiologic surveillance systems, thereby improving knowledge about dengue circulation in African countries.

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DOI: http://dx.doi.org/10.3201/eid1802.111333

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Clonal Spread of *Mycoplasma pneumoniae* in Primary School, Bordeaux, France

To the Editor: *Mycoplasma pneumoniae* is responsible for ≈20% of all cases of community-acquired pneumonia. The most common form of the infection is tracheobronchitis, for which an etiologic diagnosis is seldom reached (*1*). Although tracheobronchitis is often mild, the infection is disruptive, with the cough lasting several weeks, and consumes substantial resources (*2*). *M. pneumoniae* infections occur endemically and epidemically worldwide, especially in children and young adults (*1*). In 2010, an increased incidence was reported from Denmark (*3*), England and Wales (*4*), and Israel (*5*). Several outbreaks have been reported in closed or semiclosed settings, as indicated on the basis of similar clinical symptoms, chest radiograph results, and detection of the bacteria (*1*).

Previous *M. pneumoniae* typing methods were based on the analysis of the gene encoding the cytadhesin P1 (MPN141) or the gene *MPN528a* (*6*). These methods only enabled the separation of isolates into 2 types and a few variants; therefore, clinical isolates were previously poorly differentiated. We recently developed a multilocus variable-number tandem repeat analysis (MLVA), based on the study of the whole genome, that can differentiate >26 distinct variable-number tandem repeat types (*7*). We report the use of this MLVA typing method to show evidence of a clonal spread of a unique strain of *M. pneumoniae* among children in a French primary school and their household contacts.

In January 2011, 6 children (4–9 years of age), who attended the same primary public school in Bordeaux, France, reported fever, pharyngitis, rhinorrhea, and dry cough that later became mucoid. One of the children was admitted to the pediatric ward of the University Hospital of Bordeaux, and atypical pneumonia was confirmed by radiologic testing. A diagnosis of tracheobronchitis was confirmed by general practitioners for the 5 other children. Three of the children were administered β-lactam antimicrobial drugs that did not modify the course of the illness. An additional child (4 years of age), a first cousin of one of the 6 case-patients, also received a diagnosis of tracheobronchitis after repeated contact with his cousin.

Throat swab or blood samples were obtained from the 7 children, and throat swab samples were obtained from the household members of 4 of their families. DNA was extracted from throat specimens, and a TaqMan real-time PCR was performed to detect *M. pneumoniae* as described (*8*). MLVA typing was performed on the same DNA extracts, according to the method of Dégrange et al. (*7*). *M. pneumoniae*–specific IgM and IgG in serum specimens were assessed by ELISA. PCR was used to detect *Bordetella pertussis*, *B. parapertussis*, *Chlamydia pneumoniae*, *Streptococcus pneumoniae*, and viruses commonly responsible for respiratory tract infections. In France, 10% of *M. pneumoniae* isolates are resistant to macrolides (*9*); thus, we used real-time PCR and melting curve analysis to detect macrolide resistance–associated mutations in the 23S rRNA gene (*9*).

The 7 children were confirmed to be positive for *M. pneumoniae* infection by PCR or by the presence of *M. pneumoniae*–specific IgM (Figure). No other respiratory tract pathogens were found. In all cases, MLVA determined the strain type to be 34572, also called MLVA type J (*7*); this finding suggests clonal spread of a specific *M. pneumoniae* strain. No macrolide resistance–associated mutation was found in the 23S rRNA gene. All children were treated with roxithromycin or clarithromycin and rapidly recovered, although PCR results remained positive for up to 6 weeks in subsequent throat samples. This length of persistence is in accordance with a previous study showing that the median time for carriage of *M. pneumoniae* DNA was 7 weeks after disease onset and that adequate treatment did not shorten this period (*10*).

M. pneumoniae DNA was also found in throat swab specimens of 3 household contacts (2 adults and a 1-year-old child) in 3 separate

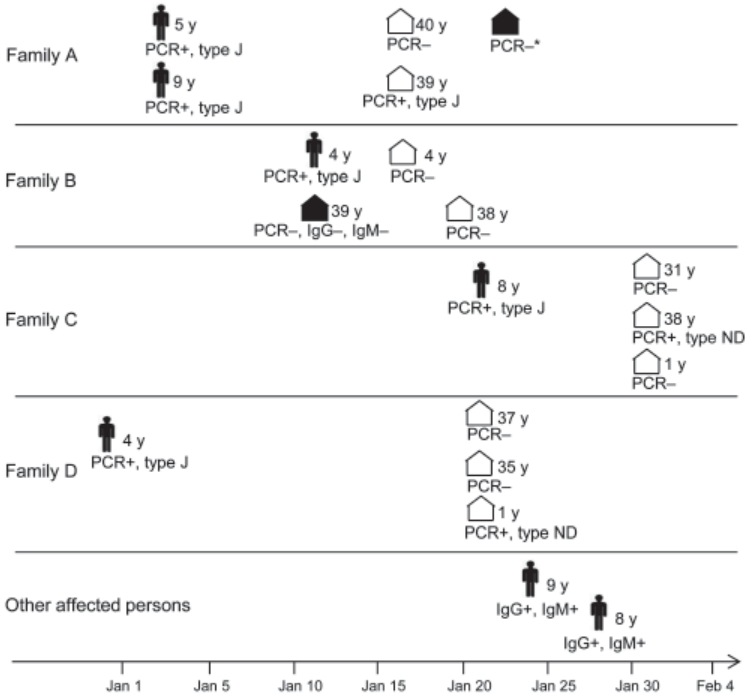

Figure. Timing and characteristics of patients and contacts in a study using the multilocus variable-number tandem repeat (MLVA) typing method to show evidence of clonal spread of a unique strain of *Mycoplasma pneumoniae* among children attending a French primary school and their household contacts. Dates correspond to the date of specimen collection during December 30, 2010–February 1, 2011. Figure shapes indicate affected children, by age in years; white house shapes indicate asymptomatic household contacts; black house shapes indicate household contacts with respiratory symptoms. PCR+, throat swab specimen positive by *M. pneumoniae*–specific real-time PCR; PCR–, throat swab specimen negative by *M. pneumoniae*–specific real-time PCR; type J, MLVA type J; type ND, MLVA type not determined; *PCR performed after an 8-day macrolide treatment; IgG+, IgM+, presence of specific *M. pneumoniae* IgG and IgM in serum; IgG–, IgM–, absence of specific *M. pneumoniae* IgG or IgM, respectively, in serum.

families (Figure). The MLVA type was determined in 1 contact; it also was MLVA type J, suggesting that carriage in this contact was related to spread of the same clone. Of interest, none of these 3 household members had respiratory symptoms. Nilsson et al. (*10*) also reported a high frequency of *M. pneumoniae* DNA carriage in household contacts; however, in contrast to contacts in our study, all of the household contacts in the study by Nilsson et al. had ongoing or recent respiratory tract symptoms.

In summary, we report an outbreak of *M. pneumoniae* infections confirmed by MLVA, a discriminatory typing method. MLVA typing revealed the clonal spread of a single *M. pneumoniae* type J strain in children attending the same primary school and in their household contacts. The cases we identified may represent only a small proportion of the actual cases, which were likely underestimated due to mild symptoms, poor knowledge of *M. pneumoniae* infections by general practitioners, and lack of PCR availability. We showed that MLVA typing of *M. pneumoniae* can be used to detect clonal spread and outbreaks. This approach might also be useful for studying the worldwide emergence of *M. pneumoniae* macrolide resistance and for finding resistant clones with the potential for spreading.

Acknowledgments

We thank Florence Bernard for technical assistance.

This study was supported by internal funding.

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DOI: http://dx.doi.org/10.3201/eid1802.111379

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Risk for Emergence of Dengue and Chikungunya Virus in Israel

To the Editor: In recent years, *Aedes albopictus*, a mosquito vector of dengue and chikungunya viruses, has rapidly expanded in Europe. Since 2007, the presence of viremic patients with imported cases of dengue and chikungunya virus infection has resulted in several incidences of autochthonous transmission of the viruses in Italy, France, and Croatia (*1–4*).

A. albopictus mosquitoes have invaded Israel since 2002. A recent national survey showed wide distribution of the mosquito in Israel (*5*), and dengue and chikungunya virus infection are increasingly reported in travelers from Israel who return home from trips to other countries (*6*,*7*). We looked for overlap between the distribution areas of *A. albopictus* mosquitoes in Israel and the living areas of travelers who have returned to Israel with acute dengue or chikungunya virus infections. We discuss the possibility of autochthonous transmission of these viruses in Israel.

All cases of imported, serologically proven acute dengue and chikungunya virus infection registered during 2008–2010 at the National Center for Zoonotic Viruses, Central Virological Laboratory, Israel Ministry of Health, were included in the study. For dengue diagnosis, IgM capture ELISA was run in parallel with dengue indirect IgG ELISA, according to the manufacturer's (PANBIO, Brisbane, Australia) instructions. For chikungunya diagnosis, microchip technology (Euroimmune, Gross-Groenau, Germany) was performed to detect specific IgM and IgG antibodies. Paired acute- and convalescent-phase samples with a ≥4-fold increase confirmed an acute case.

Laboratory diagnosis was matched to clinical observations; travel history, along with previous vaccination for yellow fever; and tickborne or Japanese encephalitis. Local infection was differentiated from imported infection by interpretation of a questionnaire that is required from treating physicians before serologic testing. Testing for West Nile virus and Sindbis virus were done to rule out cross-reactions with endemic flaviviruses and alphaviruses, respectively.

Geographic distribution of *A. albopictus* mosquitoes in Israel during 2008–2009 was observed by monitoring the presence of eggs, larvae, and adult mosquitoes in ovitraps and by monitoring reports from municipalities and pest management professionals (*5*). Patients' areas or municipalities of residence were plotted on a map describing the currently known distribution of *A. albopictus* mosquitoes in Israel. We evaluated the number and proportion of dengue and chikungunya patients in *A. albopictus*–endemic regions.

During the study years, 41 and 15 patients, respectively, received diagnoses of dengue and chikungunya virus infection at the National Center for Zoonotic Viruses (Figure). Of the 41 dengue and 15 chikungunya patients, 27 (66%) and 12 (80%), respectively, lived in areas where *A. albopictus* mosquitoes were endemic (Figure). No autochthonous cases were reported.

The establishment of *A. albopictus* mosquitoes in Israel provides suitable conditions for autochthonous transmission of dengue and chikungunya viruses. Although it was traditionally regarded a secondary vector for dengue virus, *A. albopictus* can spread the virus. The potential risk for local outbreaks of dengue or chikungunya virus disease is dependent on the presence of viremic patients and a suitable vector (*8*). Recent reports emphasized the risk for autochthonous transmission of dengue, yellow fever, and chikungunya viruses in Europe. This risk is strengthened by the history of yellow fever and dengue in temperate regions. Dengue virus transmission may follow 2 general patterns: epidemic and hyperendemic dengue. Epidemic dengue transmission may occur as an isolated event when virus is introduced into a region with susceptible hosts and an adequate vector. Such events involve a single virus strain and may manifest in explosive transmission.

Reported cases of autochthonous dengue in France and Croatia were presumed to be related to newly spread *A. albopictus* mosquitoes (*2*–*4*). An outbreak of chikungunya virus infections that occurred in Italy during the summer of 2007 involved >200 persons (*1*). *A. albopictus* mosquito spread in Hawaii was regarded as the cause of a dengue outbreak in 2001 (*9*), and in Florida, dengue fever was recently documented in Key West, where *A. aegypti* mosquitoes are established (*10*).

In this report, we document importation of dengue and chikungunya viruses by travelers to *A. albopictus*–endemic areas in Israel. We show that most patients in Israel with imported dengue and chikungunya virus infection reside

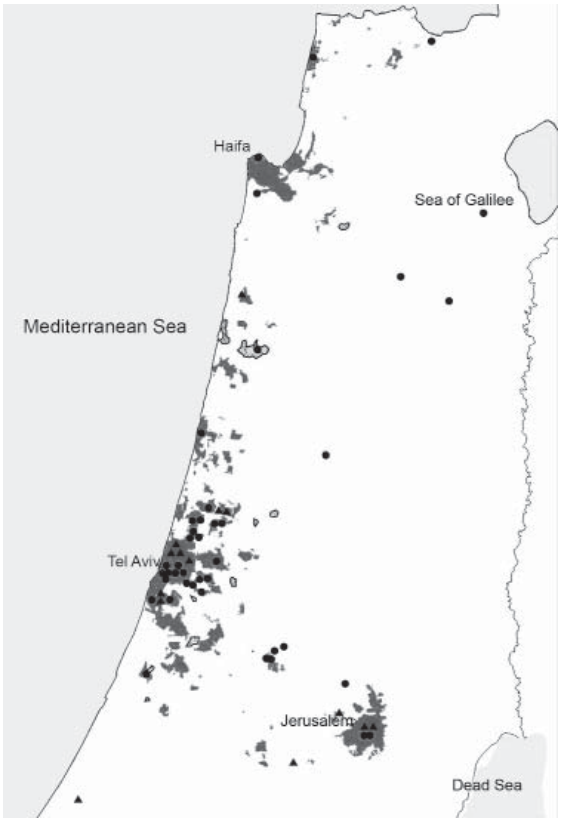

Figure. Patients with imported dengue (black circles) or chikungunya (black triangles) virus infection living in *Aedes albopictus*–endemic areas of Israel, 2008–2010. Gray shading indicates known and black outline suspected *A. albopictus*–endemic areas. Of the patients with dengue and chikungunya virus disease, 66% (27/41) and 80% (12/15), respectively, lived in *A. albopictus*–endemic areas.

in *A. albopictus*–endemic areas of the country. The reported number of serologically proven cases probably underestimates the true extent of the diseases in Israeli travelers because underdiagnosis and underreporting are common. Both dengue and chikungunya virus infection result in viremia that may last up to 5 days, and viremic patients living in *A. albopictus*–endemic areas put the area population at risk for infection.

In summary, we report conditions in Israel suitable for autochthonous transmission of dengue and chikungunya viruses. Although no autochthonous cases have been reported in Israel, they have been reported from other countries where *A. albopictus* mosquitoes are newly endemic. In Israel and other areas where this species is newly endemic, both dengue and chikungunya virus infection should be considered in the differential diagnosis of acute febrile illnesses, even when the patients do not report recent travel to tropical areas. Enhanced surveillance may be needed to prevent epidemic spread of these diseases. Consideration must be taken to isolating suspected (viremic) dengue and chikungunya patients to prevent the establishment of autochthonous transmission.

Acknowledgments

We thank the staff of the National Center for Zoonotic Viruses and the Division of Pest Surveillance and Control for their help in performing the study.

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DOI: http://dx.doi.org/10.3201/eid1802.111648

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Possible Pet-associated Baylisascariasis in Child, Canada

To the Editor: *Baylisascaris procyonis*, a roundworm parasite of raccoons (*Procyon lotor*), increasingly is being documented as a cause of severe human disease (*1*). Approximately 130 species of wild and domesticated animals have been affected with *B. procyonis* neural larva migrans, and the parasite is increasingly recognized as a cause of human encephalitis (*2*; K. Kazacos, unpub. data). The first recognized human case was reported in 1984 in a 10-month-old child in Pennsylvania, USA (*3*). Since then, ≈30 additional cases of severe or fatal *B. procyonis* encephalitis have been reported in the United States (*4*–*7*; K. Kazacos, pers. comm.). To our knowledge, only 1 account of human *B. procyonis* infection has been reported in Canada (in 2009) (*8*). We report another case of human *B. procyonis* infection in Canada, indicating its probable transmission from peridomestic raccoons.

In 2008, a 14-month-old previously healthy boy in Hamilton, Ontario, Canada, sought care for fever, regression in speech for 5 days, and failure to bear weight for 2 days. His parents also noticed that he was not tracking with his eyes. Caregivers recalled a macular rash on the face and trunk that had faded over time. The child was hospitalized, and a workup for encephalitis was initiated. He was hemodynamically stable and had flaccid tone, with inability to bear weight. No visible rashes were found. A fundoscopic examination indicated no evidence of unilateral chorioretinitis. The child was unable to track objects, which suggested vision loss in both eyes. Blood cultures, urine cultures, and lumbar puncture were performed. Results of blood analyses showed the

following: lymphocytes 24% (45%–76%), monocytes 41% (3%–6%), eosinophils 32% (0%–3%), protein 34 g/L (42–74 g/L), and glucose 3.0 mmol/L (3.3–5.8 mmol/L). Magnetic resonance imaging of the brain showed diffuse white matter lesions scattered in the subcortical and deep white matter over both cerebral hemispheres and periventricular region, most prominent in the left parietal lobe and frontoparietal regions, and subtle hyperintense lesions in bilateral dentate nucleus (Figure, panel A). No meningeal enhancement was noted.

Because of eosinophilic meningoencephalitis, thorough analyses were conducted for immunologic and infectious etiologies. The family confirmed the presence of numerous raccoons in their backyard, which raised a concern for *Baylisascaris* encephalitis, and samples of cerebrospinal fluid and serum were sent to Purdue University (West Lafayette, IN, USA) for serologic testing. On the basis of clinical findings, the child was given albendazole 200 mg orally 3×/day and prednisone 25 mg orally for 4 weeks. Results of ELISA were positive for *B. procyonis* from serum (optical density = 0.744; cutoff 0.250) but negative from cerebrospinal fluid. *B. procyonis*–specific protein bands were seen on Western blotting (*9*). The parents reported that the child had no access to the backyard, but they and their dog often moved between the backyard and the house. Environmental sampling was conducted in conjunction with the public health department. Thirty samples were taken from the patient's home and yard. A sample of raccoon feces taken from a garbage bag from the porch of the house contained embryonated *B. procyonis* eggs (Figure, panel B). No eggs were identified in the dog. Nine months after the initial hospitalization, the child had substantial physical and motor delays, was legally blind in both eyes, and had epilepsy.

To our knowledge, this is the second case of *Baylisascaris* encephalitis identified in Canada. Both cases are noteworthy for profound neurologic impairment. Similar to cases reported from the United States, the case reported here highlights the dangers of peridomestic raccoons, which are becoming increasingly common in both countries. Although the classical risk factors for pica/geophagia or developmental delay were not reported by the patient's parents, he could have become infected only through ingestion of infective eggs, from an as-yet-undetermined location, object, or source. The case reported here illustrates the need for a collaborative approach in unusual cases; we included clinicians and public health and laboratory specialists in the workup of this case. We found a strong correlation between the serologic findings, the child's clinical signs, other clinical information (e.g., eosinophilia, magnetic resonance imaging findings), the age of the child, and the recovery of embryonated *B. procyonis* eggs from his environment. We postulate that he became infected by ingesting raccoon feces/infective eggs unintentionally brought into the home or through exposure in adjacent structures, such as the porch. Although no eggs were identified in the dog, several reports have documented intestinal infection of domestic dogs with *B. procyonis*, albeit at a prevalence thousands of times lower than that in raccoons (*3*; J. Yang, unpub. data). That the dog served as a vector after being exposed to raccoon feces and infective eggs is far less likely. A recent report from the Centers for Disease Control and Prevention suggests possible transmission from pet kinkajous (*10*). We speculate that more common domestic pets also might serve as possible reservoirs for and sources of infection.

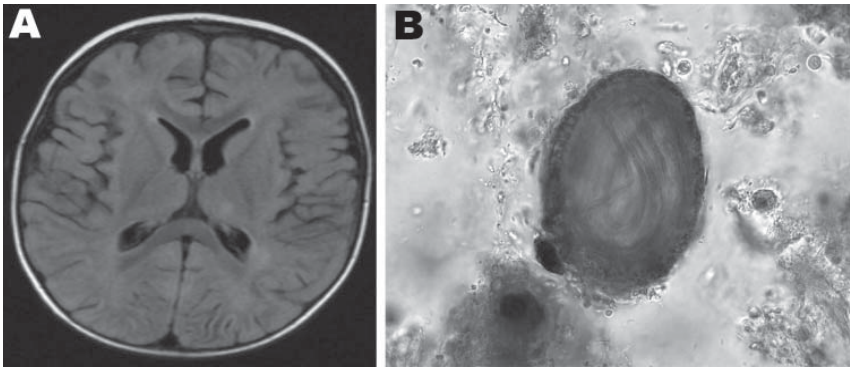

Figure. A) Magnetic resonance imaging of the brain of a 14-month-old child with baylisascariasis encephalitis. B) *Baylisascarasis procyonis* embryonated egg found in wet preparation of raccoon feces; original magnification ×100. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/2/11-0674-F.htm).

Acknowledgments

We thank the clinical laboratory staff and the public health departments for expert assistance and Sriveny Dangoudoubiyam for performing the serology.

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DOI: http://dx.doi.org/10.3201/eid1802.110674

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Zika Virus Infection, Cambodia, 2010

To the Editor: *Zika virus* (ZIKV), a member of the family *Flaviviridae,* genus *Flavivirus*, was first isolated from the blood of a sentinel rhesus monkey from the Zika Forest of Uganda in 1948 (*1*). Since that time, serologic studies and virus isolations have demonstrated that the virus has a wide geographic distribution, including eastern and western Africa; the Indian subcontinent; Southeast Asia; and most recently, Micronesia (*2*–*5*). The virus is transmitted primarily through the bite of infected mosquitoes and most likely is maintained in a zoonotic cycle involving nonhuman primates (*1*), although recent evidence suggests the possibility of occasional sexual transmission in humans (*4*). Few case reports have described the clinical characteristics of ZIKV infection in humans. Most reports describe a self-limiting febrile illness that could easily be mistaken for another arboviral infection, such as dengue or chikungunya fever. We report a confirmed case of ZIKV infection in Cambodia.

Since 2006, the US Naval Medical Research Unit No. 2 (NAMRU-2) has conducted surveillance for acute fever to determine causes of the infection among patients who seek health care at local clinics in Cambodia. Patients were enrolled by the health clinic physician after they gave informed consent in accordance with an institutional review board protocol approved by NAMRU-2 and the National Ethics Committee for Human Research of Cambodia. At enrollment, the physician administered a questionnaire and collected specimens (blood and throat swabs). All items were transported to the NAMRU-2 laboratory in Phnom Penh, where testing was conducted for a variety of viral, bacterial, and parasitic pathogens. In August 2010, a blood specimen was collected from a 3-year-old boy at a health clinic in Kampong Speu Province, Cambodia. The child's reported clinical symptoms included 4 days of fever and sore throat and cough and a headache for 3 days. A maculopapular rash was not observed, and the boy was not hospitalized. The clinic staff conducted a follow-up interview and reported that the patient recovered fully.

ZIKV infection was confirmed in this patient by using PCR, sequencing, and serology and through virus isolation. ELISA for chikungunya and dengue virus IgM and IgG antibodies on acute- and convalescent-phase serum was negative. A universal flavivirus real-time PCR screen that targets the nonstructural (NS) 5 gene (*6*) determined that the patient's serum was positive for flavivirus RNA, but subsequent species-specific PCR ruled out 2 other flaviviruses that are highly endemic to the region (dengue and Japanese encephalitis viruses) (*7*–*9*). This result was the first nondengue, non–Japanese encephalitis virus flavivirus detected after samples from ≈10,000 enrolled patients were tested. Nucleic acid sequencing of the amplicon isolated by gel purification produced a 100-bp fragment with 100% sequence identity to ZIKV (nucleotide position 8,969 of the NS5 gene of the isolate GenBank accession no. EU545988). ZIKV infection subsequently was serologically confirmed by hemagglutination-inhibition tests on paired serum samples. The patient's

Table. Reported or observed clinical signs and symptoms in persons with Zika virus infection, 1962–2010

Sign or symptom	Country, year of infection origin,* no. (%) patients					
	Uganda, 1962, n = 1	Laboratory acquired, 1973, n = 1	Indonesia, 1977–1978, n = 7	Micronesia, 2007, n = 28	Senegal/United States, 2009, n = 3	Cambodia, 2010, n = 1
Fever	1 (100)	1 (100)	7 (100)	20 (65)		1 (100)
Headache	1 (100)			14 (45)	3 (100)	1 (100)
Malaise	1 (100)		5 (71)		3 (100)	
Maculopapular rash	1 (100)			28 (100)	3 (100)	
Fatigue or myalgia	1 (100)	1 (100)	1 (14)	14 (45)	1 (33)	
Arthritis and arthralgia			1 (14)	20 (65)	3 (100)	
Chills		1 (100)	2 (29)		2 (67)	
Dizziness			5 (71)			
Joint swelling or edema				6 (19)	2 (67)	
Stomachache			6 (86)			
Retro-orbital pain		1 (100)		12 (39)		
Conjunctivitis			1 (14)	17 (55)	1 (33)	
Anorexia			4 (57)			
Photophobia					1 (33)	
Vomiting			1 (14)	3 (10)		
Diarrhea			3 (43)			
Constipation			3 (43)			
Sore throat						1 (100)
Cough						1 (100)
Aphthous ulcer					2 (67)	
Hypotension			2 (29)			
Hematuria			1 (14)			
Prostatitis					1 (33)	
Hematospermia					1 (33)	
Sweating		1 (100)				
Lightheadedness					1 (33)	

*References: Uganda (*2*), laboratory-acquired (*10*), Indonesia (*5*), Micronesia (*9*), Senegal/United States (*4*). Blank cells indicate no reported information.

acute-phase sample was negative, but a convalescent-phase sample gave a positive reaction with ZIKV antigen to a serum dilution of 1:320 and was negative to antigens for the 4 dengue serotypes and yellow fever and West Nile viruses. These results demonstrate that the patient had a clear monotypic flavivirus immune response with seroconversion against ZIKV, indicating a recent primary infection.

The most common signs and symptoms reported in confirmed ZIKV infections are fever, headache, malaise, maculopapular rash, fatigue or myalgia, and arthritis and arthralgia (Table). In addition to fever and headache, the patient in this study had a sore throat and cough. Because of the patient's age, additional information about symptoms was difficult to obtain.

The clinical characteristics exhibited by this case-patient are similar to those of shown in a small cluster of ZIKV infections described in Indonesia during 1977–1978 in which maculopapular rash was not observed (*5*). Maculopapular rash was reported as a common sign in case-patients from the recent Yap Island outbreak (*3*), as well as in case reports from Uganda (*2*), Senegal, and the United States (*4*), A case report of laboratory-acquired ZIKV infection also noted the lack of maculopapular rash (*10*).

The clinical features of ZIKV infection are similar to those of dengue virus and chikungunya virus infections, and both arboviruses are found in Southeast Asia. In this region, laboratory-based confirmation is essential. The extent of ZIKV infections in Cambodia is unknown; further studies are needed to clarify the prevalence and geographic distribution of ZIKV infection in the country.

Acknowledgments

We thank the enrolling health center staff at Kampong Speu Province and the NAMRU-2 staff who contributed to the execution of this study and the follow-up investigation.

This study was funded by the US Department of Defense Global Emerging Infections Surveillance and Response System, a division of the Armed Forces Health Surveillance Center.

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DOI: http://dx.doi.org/10.3201/eid1802.111224

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Recombination of Human Coxsackievirus B5 in Hand, Foot, and Mouth Disease Patients, China

To the Editor: Hand, foot, and mouth disease (HFMD) is an acute viral infectious disease in infants and young children. However, since 2008, HFMD has emerged as a major public health problem in the People's Republic of China, resulting in millions of infections with hundreds of deaths (*1*). Human enteroviruses (HEVs), including HEV71, echoviruses, and coxsackie viruses A and B (CAV and CBV), are the major pathogens of HFMD (*2*). In mainland China, HEV71 and CAV16 have been recognized as the dominant causative agents for HFMD.

During a recent HFMD outbreak in Changchun during 2010, three of 16 throat swab samples tested positive for HEV but negative for HEV71 and CAV16 by reverse transcription PCR. All 3 samples were then placed into human rhabdomyosarcoma cells, and typical cytopathic effects were observed 3–4 days later. All the isolates were finally characterized as CBV5 by using serologic and molecular technology and designated as CBV5/CC10/10, CBV5/CC10/16, and CBV5/CC10/17, respectively. The complete genome of these Changchun isolates was determined as described (*3*) and submitted to GenBank (accession nos. JN580070, JN695050, and JN695051, respectively. The genome RNA of CBV5/CC10/10 is 7,402 bp long, and the 5′- and 3′-untranslated regions are 743 and 101 bp, respectively. The coding regions of these Changchun isolates are highly homologous, with amino acid identity of 100% and only a 3-nt difference exists among them. The complete genome of 4 CBV5 strains were indexed previously in GenBank, and the nucleotide sequence identities of the newly isolated CBV5/CC10/10 with the other 4 CBV5 strains were among 80.6%–88.1%. Results of homology and phylogenetic analyses based on the complete viral protein 1 sequence (849 bp) showed that the nucleotide identity of viral protein 1 among 17 different CBV5 strains was 78.9%–95.6% and the amino acid identity was 92.9%–98.9%. The neighbor-joining tree indicated that the new isolated CBV5 Changchun strains were most closely related to the strains isolated from mainland China and that they divided into a distinct lineage from other CBV5 strains outside China (Figure). CBV5 infections were reported in mainland China during 2002–2010 in Zhejiang, Shandong, and Henan Provinces (*4*). These Changchun isolates were highly homologous with the recent Henan isolate, COXB5/Henan/2010, with a nucleotide identity of 88.1%. These results indicated that CBV5 might have been circulating in China for nany years and represented an independent evolution tendency.

Homology and BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) based on the complete genome sequence showed that these newly CBV5 isolates have 85% identity with some human CBV3 strains. Because RNA recombination is a well-known phenomenon for HEVs during viral evolution and reemergence (*5*–*8*), recombination analysis between newly isolated CBV5 and other HEVs was performed by using SimPlot software. Similarity scanning analysis (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/pdfs/11-1524-Techapp.pdf) by using CBV5/CC10/10 as query sequence showed that the 5′ half (nt 1–4481) of the genome had high similarity (>93%) to CBV5 strain COXB5/Henan/2010, and the 3′ half (nt 4661–7402) showed high similarity (>97%) to CBV3 strain Beijing0811. Then, bootscanning

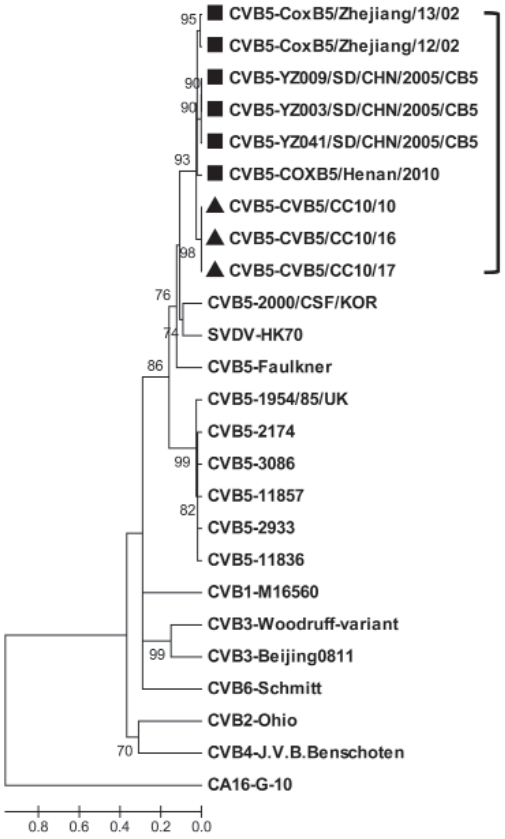

Figure. Phylogenetic analysis of selected human coxsackievirus B (CBV) strains from different origins based on the viral protein 1 gene sequences. The neighbor-joining tree was generated by using MEGA4 software (www.megasoftware.net), and the prototype strain of coxsackievirus A (CAV) 16 was used as outgroup. The Changchun strains isolated in this study are indicated by triangles and other Chinese CBV5 strains are indicated by squares. Scale bars indicates nucleotide substitutions per site.

analysis (online Technical Appendix Figure 2) showed that CBV5/CC10/10 was most closely related to COXB5/Henan/2010 in the 5′ half of the genome, and to CBV3 Beijing0811 in the 3′ half of the genome. Both analyses showed that the small fragment within 2C domains are highly similar (>84%) to a CBV5 strain from South Korea, CBV5/2000/CSF/KOR. Genetic algorithms for recombination detection analysis also indicated that the putative breakpoints were located within the 2C domain. We found no recombinant evidence between CBV5 and other HEVs during the analysis. Together, these accordant results demonstrated that recombination has possibly occurred within the 2C domain, and these Changchun isolates are possibly progeny of intertypic and intratypic recombination of CBV strains circulating in China (COXB5/Henan/2010 and Beijing0811) and South Korea (2000/CSF/KOR).

HEVs can be divided into ≈100 serotypes, and intertypic or intratypic recombination among different viruses occurs frequently, which is information necessary for disease control and surveillance. In China, dozens of HEVs, including HEV71, CAV, CBV, and echoviruses, have been isolated from HFMD patients and identified as the pathogens causing HFMD (*1*,*2*,*9*,*10*). Recombination events among HEVs have been shown to play roles in HFMD outbreaks. A recombinant HEV71 with CAV16 was suggested to be responsible for the HFMD outbreak in Fuyang, China, in 2008 (*9*). Recombination also occurred among the CAV (types 2, 4, 5, and 10) isolates during a HFMD outbreak in China during 2009 (*10*). However, most recombinant studies have focused on CAV and poliovirus. Recently, Oberste et al. (*6*) proposed the possible role of recombination within CBV strains based on phylogeneic analysis of individual genes. Our current study identified and characterized the newly CBV5 isolates from HFMD patient as intratypic and intertypic recombinants, and further surveillance is warranted that focuses on the emerging recombinant viruses among HFMD causative agents and investigations on the pathogenic role and disease associations of the recombinant CBVs.

This study was supported in part by the Major Special Program of National Science and Technology of China (no. 2009ZX10004-204), the National Natural Science Foundation of China (no. 81000721), and Beijing Natural Science Foundation (no. 7112108). C.F.Q. was supported by Beijing Nova Program of Science and Technology (no. 2010B041).

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DOI: http://dx.doi.org/10.3201/eid1802.111524

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Nontuberculous Mycobacteria in Tap Water

To the Editor: A recently published study by Falkinham (*1*) showed that 17 (46%) of 37 households were contaminated with nontuberculous mycobacteria (NTM) of the same species as those found in patients with lung disease and that 7 (41%) of 17 had the same DNA fingerprint as the patient. One patient's isolate from sputum matched the isolate found in the shower water. Therefore, the patient's lung disease was likely acquired by inhalation of aerosols while showering. An isolate from another patient matched the isolate found in tap water. If the patient drank the contaminated water, *Mycobacteria avium* may have reached the lungs by aspiration because 26% of patients with NTM lung disease have been found to experience gastroesophageal reflux disease (GERD) (*2*). Even if none of these scenarios was present, however, NTM patient contamination of samples is still likely. Six of the 7 matching households had water heater temperatures ≤125°C, indicating a negative correlation between NTM growth and temperature. Most *M. avium* and *M. intracellulare* are killed in <5 seconds (*3*) when exposed to 70°C; thus, all NTM species would likely be killed a few seconds after water reached the boiling point.

In a recent study, we have shown that Canadian-born persons from ethnic groups from eastern and Southeast Asia were less likely to be colonized with *M. avium* complex than were other ethnic groups (*4*). We hypothesized that boiling water before consumption, a common practice in persons from Asia, may have partially protected them against pulmonary colonization. Another protective factor is the low prevalence of GERD in persons from Asia (<7%) (*5*), compared with 19.8% in white persons from Olmstead County, Minnesota, USA. Future studies like that of Falkinham are needed to determine routes of transmission. Factors to investigate in such studies include the ethnicity of participants and associated predisposing disorders, particularly GERD; culturing of gastric washings; handwashing frequency; and water consumption habits (whether drinking from the bottle, from the tap, or after boiling).

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DOI: http://dx.doi.org/10.3201/eid1802.110455

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Leishmania infantum and Human Visceral Leishmaniasis, Argentina

To the Editor: In Argentina, 14 autochthonous human cases of visceral leishmaniasis (VL) were reported during 1925–1989. These cases occurred in different localities in Salta, Jujuy, Santiago del Estero, and Chaco Provinces of northwestern Argentina (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/2/11-0924-FA1.htm), where cutaneous leishmaniasis (CL) caused principally by *Leishmania* (*Viannia*) *braziliensis* is endemic.

It had been postulated that scattered/sporadic VL cases could be caused by visceralization of dermatrophic *Leishmania* spp. because of 1) absence of already recognized *L.* (*Leishmania*) *infantum* vector species; 2) geographic overlap with the region where CL is endemic; 3) simultaneous symptoms of CL; or 4) lack of detailed parasitic characterization at the molecular level for cases of suspected VL (*1*). However, during recent decades, urban outbreaks of VL have spread to southern regions of South America (Mato Grosso do Sul, Brazil, and Asunción, Paraguay) near the northern border with Argentina.

In May 2006, an autochthonous human case of VL was reported in Posadas (northeastern Argentina); it was associated with the canine visceral form of the disease. In addition, the presence of *Lutzomyia longipalpis* sandflies was also reported (*2*). Currently, 58 human VL cases have been reported in Posadas (*3*), and >7,000 infected dogs, *Lu. longipalpis* sandflies, and canine VL have been found 350 km south of Posadas (*4*).

During 2007–2008, new VL cases in 4 children and 7 dogs were reported clustering in time and space in La Banda-Santiago del Estero in the dry Chaco region of Argentina. This focus showed a different pattern from that found in the only urban outbreak of VL reported (nearly the same number of cases in humans and dogs, and the suspected vector was *Lu. migonei* sandflies instead of *Lu. longipalpis* sandflies) (*5*).

We report a case of autochthonous human VL in Salta Argentina that was caused by *L.* (*L.*) *infantum*. This parasite was characterized by cytochrome *b* (*cytb*) gene sequencing. Sequencing of this gene has been validated for precise characterization of *Leishmania* spp. (*6*,*7*).

On September 9, 2009, a 44-year-old man from Salta, Argentina (online Appendix Figure), was admitted to the Infectious Disease Service at Hospital Señor del Milagro in Salta. The patient had fever, weight loss, dyspepsia, and splenomegaly that evolved over 3 weeks. Physical examination showed cutaneous and mucosal paleness**.** His general condition was feverish and rapidly deteriorating.

Laboratory tests at the time of final diagnosis showed anemia, leukocytopenia, thrombocytopenia, and increased levels of lactate dehydrogenase. Results of urinalysis and coproculture were negative for parasites. Electrophoresis of serum proteins showed increased levels of gamma globulins. The differential diagnosis was negative for malaria, mycosis, autoimmune hepatitis, and lymphoma. A bone marrow smear showed abundant amastigotes by Giemsa staining (Figure, panel A). The patient was treated with liposomal amphotericin B, 3 mg/day for 7 days, and recovered (*8*).

After a comprehensive interview, we verified that this patient had not been in the VL-endemic area in Argentina. However, he had worked (deforestation activities) during January–February 2009 on a farm in

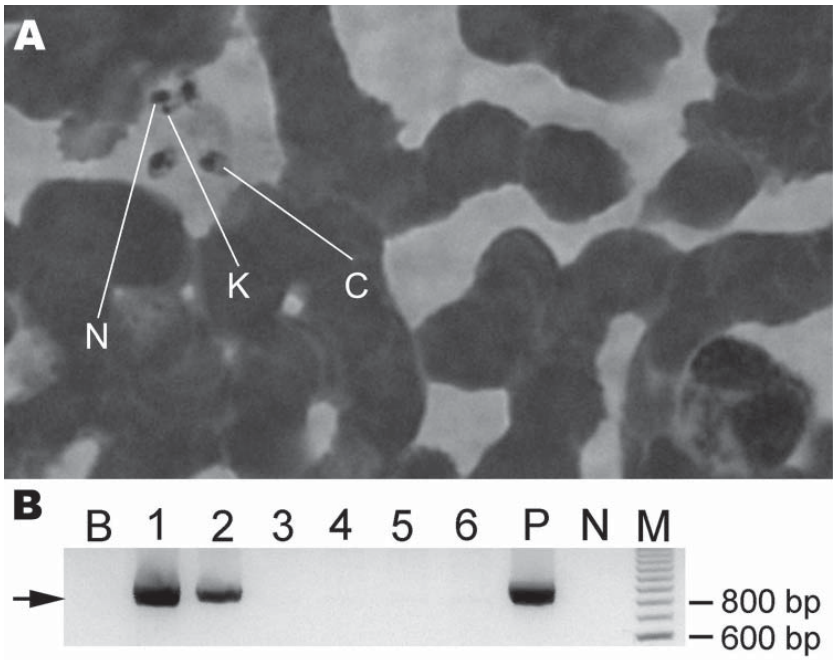

Figure. Case of autochthonous human visceral leishmaniasis in a 44-year-old man, identified by parasitologic diagnosis and molecular detection of the causative species, Salta, Argentina. A) *Leishmania* amastigotes in a bone marrow smear. N, nucleus; K, kinetoplast; C, cytoplasm (Giemsa stained, original magnification ×1,000). B) Amplification by nested PCR of cytochrome *b* gene of *Leishmania infantum*. Arrow indicates amplified fragment of ≈850 bp. Lane B, blank control; lanes 1 and 2, patient bone marrow aspirate samples; lanes 3–6, samples from *Leishmaniasis* spp.–negative persons; lane P, positive control; lane N, negative control; lane M, 100-bp molecular mass marker.

Finca Las Maravillas (22º3′29.30″S, 63º14′28.17″W), where he had been bitten by phlebotomines and acquired the disease. This farm was situated in the dry Chaco region near the border with Bolivia and Paraguay (zones with VL) (*9*), a region with intensive deforestation and agricultural activities.

For species identification, DNA was extracted from a bone marrow aspirate and peripheral blood. We amplified by nested PCR and sequenced the *cytb* gene (Figure, panel B) (*6*). The aligned 817-bp sequence obtained showed 100% homology with the *cytb* gene of the MHOM/TN/80/IPT1 *L.* (*L.*) *infantum* World Health Organization reference strain (Tunisian strain) and 99.3% homology with the MHOM/BR/74/PP75 *L.* (*L.*) *chagasi* strain (Brazilian strain) (*7*).

L. (*L.*) *infantum* was identified as the causative agent of this VL case in Salta, Argentina, where VL cases had not been seen for 50 years. Our findings indicate that this case was not caused by visceralization or a dermatropic *Leishmania* spp. We suggest that the scattered pattern of VL incidence in the dry Chaco region is caused by an enzootic cycle with accidental human transmission (*5*).

There are no reports of *Lu. longipalpis* sandflies in the study area or surrounding areas (*10*). Nevertheless, studies on natural infections of vector sandflies and reservoir-host animals (especially dogs) are needed. Therefore, the search for naturally infected sandflies and reservoirs of this infection should be intensified. Epidemiologic surveys of dogs are needed to identify spread of VL foci in areas of deforestation. Deforestation could alter vector and reservoir range and parasite density in the enzootic cycle and increase human exposure to infected vectors.

Acknowledgments

We thank Servicio de Enfermedades Infecciosas-Hospital Señor del Milagro, Salta, Argentina, and Noemí Dávalo for support during the clinical phase of the study; Jesús Sajama for drawing the map; and Rubén Cardozo for conducting field research.

This study was supported in part by the El Consejo de Investigación de la Universidad Nacional de Salta and Comisión Nacional Salud Investiga-Ministerio de Salud de la Nación, Argentina.

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DOI: http://dx.doi.org/10.3201/eid1802.110924

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Plasmodium falciparum in Asymptomatic Immigrants from Sub-Saharan Africa, Spain

To the Editor: A range of infectious diseases have been described in asymptomatic immigrants (*1*), which may justify the implementation of screening after obtaining consent. In particular, asymptomatic malaria caused by *Plasmodium falciparum* parasitemia among recently arrived immigrants may be a major public health problem outside malaria-endemic areas because these patients may be involved in autochthonous transmission cycles and may act as reservoirs capable of reintroducing malaria into areas where it had been previously eradicated.

In 2010, we reviewed the medical records of 314 asymptomatic (defined as patients with no symptoms at the time of consultation) immigrants from sub-Saharan Africa who had settled in Spain, had not traveled to their countries of origin since arrival, and had been examined at the Tropical Medicine Unit (TMU) of the Ramon y Cajal Hospital in Madrid during the previous 5 years. Systematic screening included a blood count; serum biochemistry; basic urine analysis; serologic tests for HIV infection, hepatitis B or C infection, syphilis, and schistosomiasis (if epidemiologic risk factors were present); tuberculin skin test; analysis of fecal samples for parasites; and PCR to identify *Plasmodium* spp. (*2*). Date of arrival in Spain was obtained from the patient and corroborated by the nongovernmental organizations caring for them.

PCR for *Plasmodium* spp. was performed for 216 patients, and 10 (4.6%) had positive test results for *P. falciparum*. Nine were men; median age was 27 years (interquartile range [IQR] 20–31 years). The median period from arrival in Spain to malaria diagnosis was 4.5 months (IQR 1.75–12.5 months). Three men received a diagnosis of *P. falciparum* malaria >1 year after arrival.

Patient 1 was a 32-year-old man from Angola who came to the TMU for screening 13 months after arriving in Spain. He was treated with a standard regimen of artesunate plus sulfadoxine/pyrimethamine; latent tuberculosis (TB) infection and schistosomiasis were also diagnosed. Patient 2 was a 17-year-old man from Senegal, seen at the TMU 14 months after arrival. Malaria treatment was not prescribed because he was lost to follow-up. He was also diagnosed with latent TB infection. Patient 3 was a 28-year-old man from Guinea who visited the TMU 28 months after arrival in Spain. He was treated with a standard regimen of atovaquone/proguanil and also received diagnoses of tuberculosis (TB) infection, schistosomiasis, and strongyloidiasis. No statistically significant association was observed between positive or negative PCR for *P. falciparum* and a diagnosis of tuberculosis (TB) infection, hepatitis B or C virus infection, HIV infection, syphilis, intestinal parasite infection, or schistosomiasis. None of the 3 patients had received a blood transfusion since arriving in Spain.

Reported prevalence of imported malaria among immigrants may be >10%, according to some studies (*1*), with higher rates among persons from sub-Saharan Africa (malaria caused by *P. falciparum* occurring mainly 3 months after arrival). Clinical symptoms of malaria in immigrants are typically mild, with low levels of parasitemia. Many immigrants may be asymptomatic (*1*,*3*), which has been explained by partial immunity acquired gradually after prolonged exposure in areas with stable malaria transmission. Because infected persons may initially have no symptoms, implementation of malaria screening for recently arrived immigrants from disease-endemic areas would seem advisable (*4*).

How long a low level of *P. falciparum* parasitemia may persist once exposure to malaria has been discontinued is not known. Mathematical models have estimated the maximum duration of *P. falciparum* infection after interruption of transmission at ≈4 years (*5*), although delayed clinical presentations of *P. falciparum* malaria have been described as long as 2 (*6*), 4 (*7*), or even 8 years (*8*) after patients have left malaria-endemic areas. These data highlight that low asymptomatic parasitemia may persist long after migration.

Determining in which patients with asymptomatic parasitemia clinical malaria will develop and when, or if, any external factors may act as triggers would also be useful. A study in France found that 2.3% of malaria cases among immigrants developed >1 year after their arrival and that pregnancy and co-infection with HIV were factors associated with late presentation of malaria caused by *P. falciparum* (*9*).

Asymptomatic malaria cases may affect public health in non–disease-endemic areas because persons with low-grade parasitemia are capable of infecting mosquitoes (*10*). These persons could act as unidentified reservoirs and contribute to transmission in areas where malaria has been eliminated. In addition, congenital transmission or transmission by blood transfusion or organ transplantation may occur even when the donor has lived for years outside the malaria-endemic area.

Our cases highlight how malaria parasites may persist in asymptomatic immigrants long after their arrival in the host country (up to 28 months). On the basis of published reports of symptomatic delayed cases, we believe that the prevalence of

persistently low-level parasitemia among asymptomatic immigrants is probably higher than previous estimates. Screening for malaria among immigrants long after arrival would help determine if there are any factors that influence the development of clinical malaria. Delayed screening could also be particularly relevant in certain risk groups, such as pregnant women and persons who are HIV positive. As a public health measure, such delayed screening could play a role in preventing outbreaks or reintroducing malaria in countries where it has been eradicated.

Acknowledgments

We thank L. Velásquez for technical assistance and help with the database.

Support was provided by the Red de Investigación de Centros de Enfermedades Tropicales (RICET). RD 06/0021/0020.

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DOI: http://dx.doi.org/10.3201/eid1801.111283

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Pandemic (H1N1) 2009 Virus Circulating in Pigs, Guangxi, China

To the Editor: A novel swine-originated influenza A virus known as pandemic (H1N1) 2009 was first isolated from humans in Mexico in April 2009 (*1*), and a worldwide pandemic followed, which affected >214 countries and resulted in >18,000 deaths (*2*). In August 2010, the World Health Organization stated that the pandemic caused by this virus had ended. As this virus emerged, animals, including swine, turkeys, ferrets, cats, and cheetahs, were found to have been infected (*3*). In addition, transmission from humans to pigs in porcine herds has been reported (*4*).

Swine influenza A virus (SIV) belongs to the family *Orthomyxoviridae* and is a causative agent of respiratory disease in pigs (*5*). Currently, 3 subtypes of influenza viruses are circulating in the swine population globally: H1N1, H3N2, and H1N2 (*6*,*7*). Pigs can be simultaneously infected with avian influenza viruses and human influenza viruses, and the viruses can exchange genes and produce new variants, which suggests that pigs have become mixing vessels for influenza viruses (*8*). Pandemic (H1N1) 2009, caused by a virus usually circulating in pigs in Europe and Asia, is a triple hybrid that contains swine, human, and avian virus gene segments, which further emphasizes that SIVs pose a serious threat to public health. We describe an outbreak of pandemic (H1N1) 2009 virus, which was isolated from a pig farm in Guangxi Province, People's Republic of China, and report the consequences of subsequent epidemiologic studies.

In January 2011, an outbreak of severe respiratory problems occurred in pigs on a pig farm. Nine hundred growing and fattening pigs exhibited

clinical signs of influenza, including fever, cough, runny nose, loss of appetite, lethargy, edema, watery eyes, conjunctivitis, diarrhea, and vomiting. The incidence rate was ≈80%, and the death rate was 22%. The outbreak lasted ≈2 weeks. However, no outbreak of respiratory disease occurred in other pig farms in the same area simultaneously, and no evidence of human-to-pig transmission was found.

We collected lung samples from 3 dead pigs with underlying illness for reverse transcription PCR and virus isolation in 10-day-old specific pathogen–free embryonated chicken eggs. Viral RNA was extracted from the tissue suspension and allantoic fluids. Virus isolation was assessed by hemagglutination inhibition (HI) assay and neuraminidase inhibition assay by using a panel of reference serum samples (National Reference Laboratory for Avian Influenza, Harbin Veterinary Research Institute, Harbin City, China). Later, 3 viruses were isolated. All of them had hemagglutination (HA) activity, and the HA titers ranged from 128–256. The HA-positive isolates were further identified as subtype H1N1. Subsequently, nucleotide sequences of the 8 viral genes were amplified and sequenced (GenBank accession nos. JN222372–JN222379). This analysis showed high identities to a pandemic strains A/California/04/2009 (H1N1), hemagglutinin (99.2%), neuraminidase (99.1%), matrix (99.3%), nucleoprotein (99.5%), nonstructural protein (98.5%), polymerase acidic protein (98.5%), polymerase basic protein 1 (99.7%), and polymerase basic protein 2 (99.6%) genes. Bacteria were cultured from spleen, liver, and heart-blood samples from 5 pigs. Four pigs were infected with porcine streptococci, and 1 pig with mild symptoms was negative for the bacteria.

We sought to gain more insight into the epidemiology of pandemic (H1N1) 2009 virus in Guangxi Province and collected 600 bronchial swab samples and 200 blood serum samples when pigs were slaughtered every month from February through June 2011. The samples were used to isolate virus in 10-day-old specific pathogen–free embryonated chicken eggs, and then the virus isolates were subjected to sequencing of a partial genome of the HA gene. Overall, we obtained 10 strains of subtype H1N1 influenza virus, including 5 strains of classic swine H1N1, 3 strains of Eurasian avianlike H1N1, and 2 strains of pandemic (H1N1) 2009 virus, which were derived from 3,000 bronchial swab samples.

In addition, a serologic survey was implemented by using HI testing with pandemic (H1N1) 2009 virus and SIV (H1N1) antigens. Serologic studies showed that 251 of the 1,000 samples tested had positive HI titers for pandemic (H1N1) 2009 virus, and 248 of these samples had positive HI titers for SIV (H1N1). Notably, cross-reactivity of pandemic (H1N1) 2009 virus between H1 subtype viruses has been reported recently in pigs (*9*). However, the higher rate of positive test results indicated that swine serum samples contained antibodies against pandemic (H1N1) 2009 virus.

Our findings strengthened previous data by showing that growing and fattening pigs are susceptible to infection of pandemic (H1N1) 2009 virus (*4*). Analysis of the complete genome sequence of the subtype H1N1 isolates suggests that no gene reassortment occurred. The results of serologic studies demonstrated that uninfected pig farms are also susceptible to pandemic (H1N1) 2009 virus infection. Our results suggest that the pandemic virus is currently circulating in swine populations and posing a challenge to pigs in southern China. Increasing serologic surveillance of pigs for prevention and better control of pandemic influenza is urgently needed in China.

Acknowledgments

We are grateful to Guangxi Center for Animal Disease Control and Prevention for their cooperation in carrying out the study and for their valuable assistance with sample collection. We also thank Yuliang Liu and Lei Yao for editing the manuscript and Huanliang Yang for technical assistance.

This study was supported by Guangxi Science and Technology Department Program (2010GXNSFD013033).

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DOI: http://dx.doi.org/10.3201/eid1802.111346

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Cutaneous Melioidosis in Adolescent Returning from Guadeloupe

To the Editor: Melioidosis is an emerging zoonosis caused by a highly invasive and drug-resistant bacterium, *Burkholderia pseudomallei*, that is found in soil and is endemic to Southeast Asia and the Pacific region. Few cases occur in other regions (most imported by travelers) (*1*–*5*), but sporadic cases have originated from the Caribbean (*6*–*8*).

Melioidosis can manifest many years after exposure to *B. pseudomallei* and can cause severe, systemic infection, including multiple abscesses of internal organs and skin. A less severe manifestation, primary cutaneous melioidosis, causes skin lesions and milder clinical illness. We describe an adolescent patient who had a benign, cutaneous form of melioidosis; she had recently returned to France from Guadeloupe, a Caribbean archipelago.

A 15-year-old girl without a medical history, except for asthma, was evaluated in September 2010 for muscle weakness, weight loss of 15%, cough, and fever ≥40°C. During a trip to Guadeloupe 3 weeks before, she had been infected by dengue virus, along with her brother and father, who recovered rapidly. Treatment with amoxicillin and clavulanic acid was started after her return to France, despite the lack of a clear diagnosis, and induced a slight decrease in fever.

Clinical examination showed a body mass index <15, multiple small adenopathies (<10 mm), small papulous skin eruptions, and an inflammatory 15-mm–wide tumefaction on the upper arm, evoking an adenopathy on ultrasound investigation. Biological screening 2 weeks later showed persistence of inflammation. Results of serologic tests for cytomegalovirus, Epstein-Barr virus, parvovirus B19, chikungunya virus, *Rickettsia*, *Coxiella*, *Chlamydia*, *Brucella*, and *Borrelia* spp. did not show acute infectious disease; results were positive for recent mycoplasma infection, despite absence of typical signs and symptoms. A 2-week treatment regimen with spiramycin was started; general improvement followed, and the cough resolved.

The tumefaction of the upper arm persisted, and a biopsy was performed. Histologic results were nonspecific; culture on sheep's blood Columbia agar and chocolate agar produced small colonies of gram-negative bacilli after 24 hours' incubation at 35°C in an atmosphere of 5% CO_2. This bacillus was later identified as *B. pseudomallei* by using the Vitek2 test card (bioMérieux, Marcy l'Etoile, France). Identification was confirmed by sequencing of 16S rRNA.

The patient was treated with intravenous ceftazidime (150 mg/kg for 10 d), followed by oral cotrimoxazole (800 mg of trimethoprim and 160 mg of sulfamethoxazole, 2×/d), with a total treatment duration of 12 weeks. Eleven weeks after treatment ended, the patient had recovered, and the tumefaction of the arm had disappeared.

The differential diagnosis for primary cutaneous melioidosis includes pyogenic abscesses, insect bites, mycobacterial and other granulomatous lesions, and adenopathies, but melioidosis is usually not suspected in these conditions, particularly in patients from non–disease-endemic regions such as the Caribbean. Clinical phenotypes of melioidosis range from asymptomatic carriage to fulminant shock syndrome (*1*–*5*); death rates for the latter are ≈100% (*3*). Subacute melioidosis may be associated with pulmonary and general signs; chronic variants could give rise to abscesses or septicemia in cases of concomitant immunodeficiency (*1*–*5*), even decades after exposure. Signs and symptoms of melioidosis can mimic those of tuberculosis, even though there is no link between the infectious agents (*2*,*4*).

Cutaneous melioidosis may be primary (a single, nonspecific, sometimes ulcerated lesion, measuring from several millimeters to several centimeters) or secondary (multiple lesions associated with visceral infection). In a study of 486 melioidosis patients in Australia, 58 (12%) had the primary cutaneous form (*9*). These cases were characterized by younger patient age (more common among children <16 years of age), higher frequency during the dry season, better prognosis in spite of a possible chronic evolution, and absence of classic risk factors (*9*) such as diabetes, alcoholism, chronic renal or pulmonary infections, surgery, pregnancy, or cystic fibrosis (*1*–*5*).

The case we describe is consistent with the cutaneous variant of melioidosis. However, the patient's initial general symptoms (probably attenuated by early treatment with antimicrobial drugs) could have indicated a transitory, disseminated phase of disease such as that experienced by 4 (all adults) of the 58 cases of primary cutaneous melioidosis in the Australian study (*9*). It is not known whether *B. pseudomallei* was transmitted to the patient by an airborne route or percutaneously as in most cases (i.e., wounds infected by contaminated water or mud); other transmission modes are anecdotal (*1*–*5*). Moreover, our patient had none of the classic risk factors, although dengue fever as an underlying co-infection has been described (*10*).

The patient was treated with intravenous ceftazidime and oral cotrimoxazole at the minimum treatment duration recommended for melioidosis (*1*–*5*). Purely cutaneous variants of melioidosis may be treated exclusively by oral cotrimoxazole over 12 weeks (*9*), but we opted to prescribe initial intravenous treatment because of her general symptoms. We stopped follow-up 11 weeks after the treatment period ended because of persisting illness remission, but lifelong monitoring is recommended for adult patients (*1*,*4*) because relapses occur in ≈10% of adult patients despite well-conducted antimicrobial drug treatment (*3*,*4*).

In conclusion, melioidosis as a potential emerging infectious disease should be considered in cases of isolated skin lesions as well as in cases of unexplained fever with nonspecific symptoms. Furthermore, the disease should be considered not only among travelers returning from known disease-endemic regions but also in those coming from the Caribbean.

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DOI: http://dx.doi.org/10.3201/eid1802111603

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Geographic Distribution of Endemic Fungal Infections among Older Persons, United States

To the Editor: We read with interest the article by Baddley et al. (*1*) and appreciate their efforts to characterize incidence rates of mycoses. We agree that histoplasmosis, blastomycosis, and coccidioidomycosis are differential diagnoses for patients with consistent symptoms but who reside outside mycosis-endemic areas.

However, we believe that the methods of Baddley et al. probably do not determine the true incidence of these mycoses in sparsely populated states such as Arkansas. Their estimates contrast markedly with surveillance data from the Arkansas Department of Health (Table) and with our clinical experience as infectious disease physicians. We characterize Arkansas as a state in which histoplasmosis and blastomycosis incidence is high and coccidioidomycosis incidence is low; however, Baddley et al. indicate that in Arkansas, incidence of blastomycosis is relatively low and incidence of coccidioidomycosis is high.

To investigate whether this finding might be associated with their small 5% sample of Medicare beneficiaries, we used data from the Arkansas census to determine that in 2008 the population of adults

Table. Reported cases of fungal diseases in Arkansas, by year*

Disease	No. cases/no. cases in persons ≥65 y of age										Incidence rate (95% CI)†	
	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	Overall	Persons ≥65 y
Blastomycosis, n = 166/43	20/5	15/3	20/9	15/3	19/6	17/4	16/6	13/3	15/1	16/3	4.3 (2.9–5.7)	1.1 (0–2.3)
Coccidioidomycosis, n = 3/0	2/0	0	0	0	0	1/0	0	0	0	0	0.08 (0–0.4)	0
Histoplasmosis, n = 372/65	15/3	13/3	23/6	22/2	16/4	42/9	51/9	66/4	78/13	46/12	9.6 (0–20)	1.7 (0–3.5)

*Data from Arkansas Department of Health.
†No. cases/100,000 person-years, 1999–2008.

≥65 years of age was ≈407,014, and during 1999–2008, there were ≈3,840,896 person-years for persons in this age group. A 5% sample would account for ≈192,045 person-years. Using their rate ranges (7.84–12.3 cases/100,000 person-years for histoplasmosis, 3.97–6.71 for coccidioidomycosis, and 0.39–0.86 for blastomycosis), we calculated the approximate numbers of cases in their sample: 15–23 histoplasmosis cases, 7–12 coccidioidomycosis cases, and only 1 blastomycosis case. Compared with rates from surveillance averaged over the 10 years, the midpoints of the Baddley et al. estimates are ≈6-fold higher for histoplasmosis, ≈60-fold higher for coccidioidomycosis, and ≈0.4-fold lower for blastomycosis. Only their estimate for blastomycosis incidence falls within the 10-year 95% CIs from surveillance data. We believe that the small cell sizes require that the rate estimates of Baddley et al. be interpreted with care, especially with respect to less populous states.

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DOI: http://dx.doi.org/10.3201/eid1802.111537

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In Response: We thank Haselow et al. (*1*) for their careful review of our article (*2*). They raise the relevant concern about potential instability of incidence rates from our data because of small cell sizes. We agree that use of administrative data has major limitations. As such, our intent was not to compare infection incidences of individual states; but rather, our intent was to focus on geographic distribution of endemic mycoses and whether infections occurred in non–mycosis-endemic areas.

Specifically, for blastomycosis, our study showed incidence in Arkansas to be 0.8 (0.12–5.8) cases per 100,000 person-years, comparable to the rate provided by Haselow et al. of 1.1 case per 100,000 person-years (*1*). For coccidioidomycosis, our study found the rate to be much higher than that calculated from the Arkansas surveillance data. Potential reasons for this discrepancy might be lack of case capture with surveillance data, because mandatory reporting for coccidioidomycosis is not required in Arkansas, or misclassification of incident cases in the administrative data. Finally, for histoplasmosis, the incidence rate calculated from administrative data was much higher than that reported by Haselow et al. By using administrative data, we identified a large number (15) of cases and doubt that rate instability is present. We agree that surveillance that uses administrative data has inherent limitations, which require that care be taken when interpreting epidemiologic measures, especially when sample sizes are small.

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Africa in the Time of Cholera: A History of Pandemics from 1817 to the Present

Myron Echenberg

Cambridge University Press, New York, NY, USA, 2011
ISBN: 978-0521188203
Pages: 232; Price: US $27.99, UK £17.99

In contrast to the setting of Love in the Time of Cholera by Gabriel Garcia Márquez, we live in a time when no one should have to contract or die of cholera. Nonetheless, ≈100,000 persons in Africa contracted cholera in 2011 alone, and >2,500 died in what Mintz and Guerrant have referred to as an "unconscionable tragedy" (*1*). In Africa in the Time of Cholera, Echenberg chronicles how, within a century, cholera has been transformed from an imported scourge to an African disease.

Echenberg sets the stage with a concise historical overview, depicting the eventual triumph over cholera in most of the world as a technological conquest over the original technological advances that created pandemic cholera. The first part of the book, devoted to the first 6 pandemics, is a lucid account of cholera in Africa before the 1950s. It includes some lesser known facets of cholera history, such as James Christie's excellent mid-19th century epidemiologic work in Zanzibar and the political consequences of that period's cholera outbreaks in Tunisia.

The second part, which covers the current and 7th cholera pandemic, begins with an overview of medical advances that have yielded today's intervention tools. The section unfortunately includes a smattering of scientific inaccuracies, mostly related to the cell biology of *Vibrio cholerae*, in an otherwise well-researched and accessible book. Drawing from biomedical as well as historical sources, Echenberg demonstrates how the failure to provide clean water and sanitation to most of Africa's inhabitants has led to explosive human and financial costs from cholera. He sketches portraits of these failures in countries with different histories and governance problems, illustrating infrastructural setbacks that have enabled cholera to erupt in present day Angola, South Africa, Senegal, and Zimbabwe.

The book highlights the success of oral rehydration therapy for cholera case management, and the futility of antimicrobial drug strategies because of drug resistance. Echenberg touches on the potential for vaccines, with appropriate caution, emphasizing that vaccination is no substitute for plumbing. Unfortunately, vaccines are often presented in a manner that entangles their weaknesses with those of antimicrobial drugs, underplaying the potential advantages that vaccines may have over drugs in dealing with outbreaks once they occur. It has taken cholera experts decades to advocate for vaccine use in high risk settings, culminating in World Health Assembly resolution WHA64.15 in May 2011, which urges that all states "give consideration to the administration of vaccines, where appropriate, in conjunction with other recommended prevention and control methods and not as a substitute for such methods."

The most compelling arguments for vaccine use in conjunction with preventive interventions were published just as the book was being completed (*2*), and it is unfortunate that they were not included in this otherwise commendable analysis of intervention possibilities. However, Echenberg is to be commended for the strength of the key message of the book: that lack of potable water and sanitation, the factors that eliminated cholera from much of the world, is the principal reason why today's cholera crisis (excluding complex emergencies, perhaps typified by the ongoing epidemic in Haiti) is largely African.

The story of cholera in Africa is long overdue and timely. In his usual engaging and accessible style, Echenberg has written another book that infectious disease experts should read for historical and social perspectives on the diseases they investigate and treat.

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DOI: http://dx.doi.org/10.3201/eid1802.111535

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Radcliffe Bailey (b. 1968) *En Route* (2005) Photograph on Plexiglas, coconut palms, felt, acrylic, and wood (172.7 cm × 172.7 cm × 14 cm) Collection of High Museum of Art, Atlanta, Georgia, USA

Memory as Medicine[1]

Polyxeni Potter

"The true art teacher I had was my mother," Radcliffe Bailey admits, "She led me to the well." With the family's encouragement, the artist started his creative journey early in life, "doodling and drawing," at first in New Jersey, where he was born, and after the family moved to Georgia, in the Atlanta College of Art, next door to the High Museum of Art, where he ran into the likes of Jacob Lawrence and Romare Bearden. "I've learned a lot from this museum, learned a lot about all different types of art." And "When you have the opportunity to see an artist like Jacob Lawrence… you can foresee it. You have the opportunity to think… this could actually happen [to me]."

Family and community remain a powerful force in Bailey's life and work. Layers of childhood memories blend with historical events to form the rich imagery of his large mixed media paintings and installations. These works are filled with tintypes of relatives, everyday objects, fanciful figurines, and Georgia red clay. "I use Georgia clay because it's in my backyard. My backyard is on Civil War grounds—boom. Then I trace that to family members that were in the Civil War… and to my father being a railroad engineer." "Atlanta has this interesting past that makes you want to dig deeper and understand what was once there, even though it may be covered…. Sherman burnt down the city. They say when you want to get rid of something, you burn it, but you don't really get rid of it. I can look out my back door and see a lot." As for style, "For me, it's always been about having conversations with everyone. I don't want to make work that's above, that speaks a certain way that a common person couldn't understand. I'm more concerned with having that conversation."

"I always come to painting as a sculptor," says Bailey, whose early art training was in sculpture and inspiration from such modern practitioners as Martin Puryear. "Everything is based on materials." "Painting came to me at the last minute…. That's why my paintings seem constructed. I am interested in questioning. What is painting? What is sculpture? I can build up paint so it feels like sculpture."

The language of music is of interest. "I've been listening to the sound of the wind and I've been listening

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DOI: http://dx.doi.org/10.3201/eid1802.AC1802

[1]Radcliffe Bailey exhibit, High Museum of Art, Atlanta, Georgia, http://www.high.org.

to my dogs bark at night on a full moon. Of course there are certain jazz musicians and female singers I listen to." "I love Sun Ra's music…. [He] brings together a mixture of time periods, and he fuses them together into something that sounds futuristic. I like to compare my work to that in terms of the sounds and the riffs of different times." And old objects are of great interest. He collects these found objects and adapts them to his work. "It's very much like hip-hop, patching and putting things together quiltlike, using old things to make new things."

"I've always felt like the only way I can heal myself throughout certain things is to go back through my memory, learn from memory." This travel through memory seems pertinent to *En Route,* on this month's cover. This work is one in a cluster with a historical theme: slavery's Middle Passage or the middle leg in the journey from Africa to a final destination, during which for 50 days or more, slaves were caged in suffocating quarters under inhumane conditions on the way to the West Indies, North America, South America, and Europe. The work contains several symbolic elements shared in the cluster: Water; the boat; blue color; tropical imagery; navigational tools (the sun, the moon, the planets).

"I started making these pieces that were very boxlike. When people look at them they think, 'Oh, those are some real big frames,' but they're actually constructed as medicine cabinets. The idea was… you go into your medicine cabinet to find something to heal you. And I always felt like my memory was my medicine."

With its Plexiglas surface and thick framelike perimeter, *En Route* has the feel of peering through a window into a luscious tropical scene made in eerie cobalt blue. The glasslike transparency of the water surface and the haunting array of African masks suspended in it add to the dreamlike unreality. Yet behind the water pool is a real figure in a boat ready to depart. And above the vegetation on the horizon, Bailey scatters clues—travel by sea, a musician, whose work transcends the sadness of historical fact; strings of dressing lines alluding to Marcus Garvey's 1920s Black Star Line, an enterprise intended to provide a better way to travel and trade around the Atlantic; letters; and architectural blocks with blank openings, perhaps suggesting "the door of no return" on Gorée Island in Senegal. "Think about a dream. How could I articulate a dream and make it make sense? I think the dream has so many places where you can enter and break away. My work is like a never-ending dream. And it is very cryptic."

"My desire is just to make art, to make things," Bailey says when asked to explain his work. "I like to think of my artworks as similar to the music of Thelonius Monk…. They're like rituals that just happen." Yet, just as you can scarcely separate the author of a novel from the story or the scientist from the experiment, you can hardly appreciate art apart from its source. The painter's life and times, knowledge of the world and outlook on life are an integral part of the artwork. And in Bailey's case, the choice of artifacts and the connections made between them create not an archive but a progression of history, which is why a work that recounts a sad chapter can actually be uplifting.

Layers of cryptic historical data feature too in science and are as essential to appreciating it as those of any painting in any era. Just as the specter of slavery, which has been exhaustively examined, could never be entirely resolved without continued historical scrutiny, emerging disease problems would be far more difficult to decipher outside their past lives and times. Not much has changed, for example, in the medicine cabinet as we look for ways to heal cholera. Lack of potable water and sanitation remain the principal reasons why cholera exists today and largely in Africa.

But much has been learned about pandemic influenza from examining the 1918 pandemic. And not just by revisiting it. Like the artist, public health practitioners are constantly reevaluating the pandemic, not quite knowing where new outlooks might take them. They resemble Radcliffe Bailey. "I'm like free jazz. I'm not concerned about where I'm going, just as long as I'm moving forward and documenting life." We benefit from reviewing old scourges: slavery, cholera, influenza. Each time we revisit them is like revisiting the art museum to see the old masters. We see something new. Not because the old masters have changed so much but because with new knowledge and outlook, we have.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Occurrence, Transmission, and Zoonotic Potential of Chronic Wasting Disease

Using Genotyping and Geospatial Scanning to Estimate *Mycobacterium tuberculosis* Transmission, United States

A Systematic Approach for Discovering Novel, Clinically Relevant Bacteria

Foodborne and Waterborne Infections in Elderly Community and Long-Term Care Facility Residents, Victoria, Australia

Chickens as Reservoir for Human Extraintestinal Pathogenic *Escherichia coli*

Pathogenic Potential to Humans of Bovine *Escherichia coli* O26, Scotland

Antibodies against *Taenia solium* Cysticerci among Refugees Resettled in United States

Lineage-specific Virulence Determinants of *Haemophilus influenzae* Biogroup Aegyptius

Nonpasteurized Dairy Products, Disease Outbreaks, and State Laws, United States

Causes of Bronchopneumonia Epizootics among Bighorn Sheep, Western United States

Community-associated *Clostridium difficile* Infections, Monroe County, New York

Ocozocoautla de Espinosa Virus and Hemorrhagic Fever, Mexico

Adenovirus-associated Deaths in the US Military, 1999–2010

Culturing Stool Specimens for *Campylobacter* spp., Pennsylvania, 2010

Escherichia coli O104:H4 Infections and International Travel

Laboratory Practices and Incidence of Non-O157 Shiga Toxin–producing *Escherichia coli* Infections

Highly Pathogenic Avian Influenza (H5N1) Outbreaks in Wild Birds and Poultry, South Korea

Epsilonproteobacteria in Humans, New Zealand

Novel Orthobunyavirus in Cattle, Europe

Complete list of articles in the March issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 7–24, 2012
16th Public Health Summer School
University of Otago, Wellington, New Zealand
http://www.uow.otago.ac.nz/summerschool.html

March 5–8, 2012
19th Conference on Retroviruses and Opportunistic Infections (CROI 2012)
Washington State Convention Center
Seattle, WA, USA
http://www.retroconference.org

March 11–14, 2012
ICEID 2012
Atlanta, GA, USA

May 6–9, 2012
8th International Symposium on Shiga Toxin (Verocytotoxin)–Producing *Escherichia coli* Infections
Amsterdam, the Netherlands
www.vtec2012.org

May 9–13, 2012
8th International Congress on Autoimmunity 2012
Granada, Spain

July 22–27, 2012
XIX International AIDS Conference (AIDS 2012)
Washington, DC, USA
http://www.aids2012.org/Default.aspx

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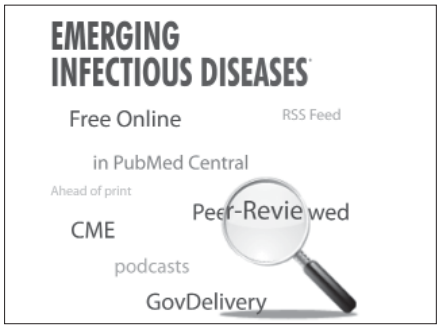

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Article Title

Invasive Pneumococcal Disease and Pandemic (H1N1) 2009, Denver, Colorado, USA

CME Questions

1. You are admitting a 55-year-old woman with a history of chronic obstructive pulmonary disease, type 2 diabetes mellitus, and steatohepatitis. She has had fever and cough for 8 days, and it has grown worse in the last 2 days.

You consider whether this patient has influenza, invasive pneumococcal disease (IPD), or both. Which of the following statements regarding the relationship between pandemic (H1N1) 2009 and IPD in the current study is most accurate?

A. Fewer than 20% of cases of IPD featured positive testing for pandemic (H1N1) 2009
B. Most cases of IPD did not feature influenza-like illness (ILI)
C. More patients had positive testing for pandemic (H1N1) 2009 than for ILI
D. The sensitivity of rapid influenza testing approached 100%

2. Which of the following variables most characterized pandemic (H1N1) 2009–associated cases of IPD vs IPD cases during the nonpandemic period?

A. Younger age and black race
B. Younger age and higher rates of chronic obstructive pulmonary disease
C. Female sex and higher rates of diabetes
D. Older age and higher rates of liver disease

3. As you take this patient's history, what information from the current study should you consider regarding the use of pneumococcal vaccine and treatment of influenza?

A. Nearly all eligible patients had received the pneumococcal polysaccharide vaccine (PPV23) vaccine
B. All children had received the 7-valent pneumococcal conjugate (PCV7) vaccine
C. Fewer than 10% of patients received antiviral medications
D. The most common reason for not prescribing antiviral medications was fear of side effects

4. The patient is diagnosed with influenza (H1N1) and IPD. What was the difference in the severity of cases of IPD in the pandemic vs the nonpandemic periods in the current study?

A. Pandemic (H1N1) 2009 was associated with higher rates of hospitalization and mortality
B. Pandemic (H1N1) 2009 was associated with higher rates of hospitalization only
C. Pandemic (H1N1) 2009 was associated with higher rates of mortality only
D. Pandemic (H1N1) 2009 was not associated with higher rates of hospitalization or mortality

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to **www.medscape.org/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Declining Guillain-Barré Syndrome after Campylobacteriosis Control, New Zealand, 1988–2010

CME Questions

1. Which of the following infections is most common prior to the development of Guillain-Barré syndrome (GBS)?

A. *Listeria monocytogenes*
B. *Campylobacter jejuni*
C. *Neisseria meningitidis*
D. *Neisseria gonorrhoeae*

2. Which of the following statements best describes the temporal relationship between campylobacteriosis and GBS in the current study?

A. Most patients had concurrent campylobacteriosis and GBS
B. GBS followed infection with *C. jejuni* by an average of 2 months
C. GBS followed infection with *C. jejuni* by an average of 6 months
D. There was no temporal relationship between campylobacteriosis and GBS

3. Which of the following age groups in the current study featured the strongest association between campylobacteriosis and GBS?

A. <5 years old
B. 5–9 years old
C. 10–19 years old
D. 60–69 years old

4. How did new food safety measures affect risks for campylobacteriosis and GBS in the current study?

A. Food safety measures failed to significantly alter the prevalence of campylobacteriosis or GBS
B. Food safety measures reduced the prevalence of campylobacteriosis only
C. Food safety measures reduced the prevalence of GBS only
D. Food safety measures reduced the prevalence of both campylobacteriosis and GBS

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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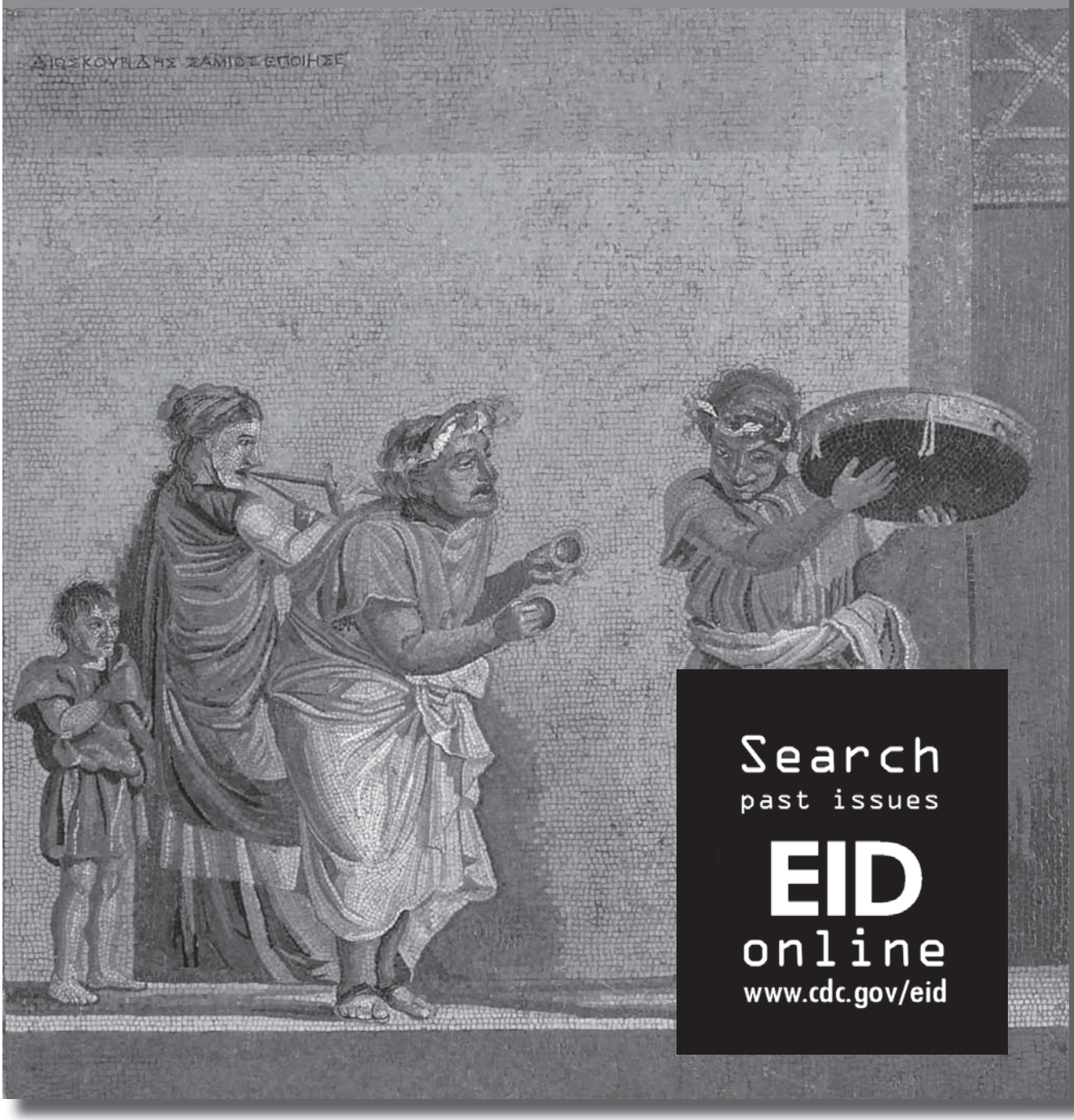
January 2012

Historical Connections



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Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures as separate files, in the native format when possible (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpg or .tif files. Other file formats may be acceptable; contact fue7@cdc.gov for guidance. Figures should not be embedded in the manuscript file. Use color only as needed. Use Arial font for figure lettering. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced to print size. Large figures may be made available online only. Place figure keys within the figure; figure legends should be provided at the end of the manuscript file.

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.