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Vaccine-Preventable Diseases

July 2018

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On the Cover

Pieter Brueghel the Elder (c.1525–1569), *The Wine of Saint Martin's Day* (1565–1568) (detail)**.** Tempera on linen. 58.2 in x 106.5 in/147.8 cm x 270.51 cm, Museo del Prado, Madrid, Spain, © Museo Nacional del Prado/Art Resource, NY.

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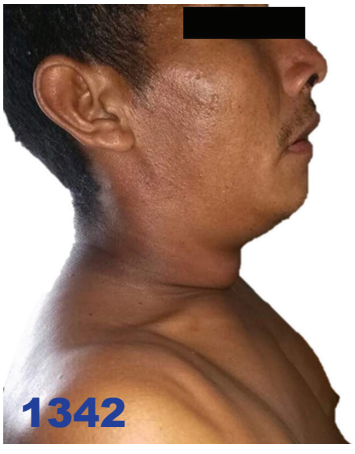

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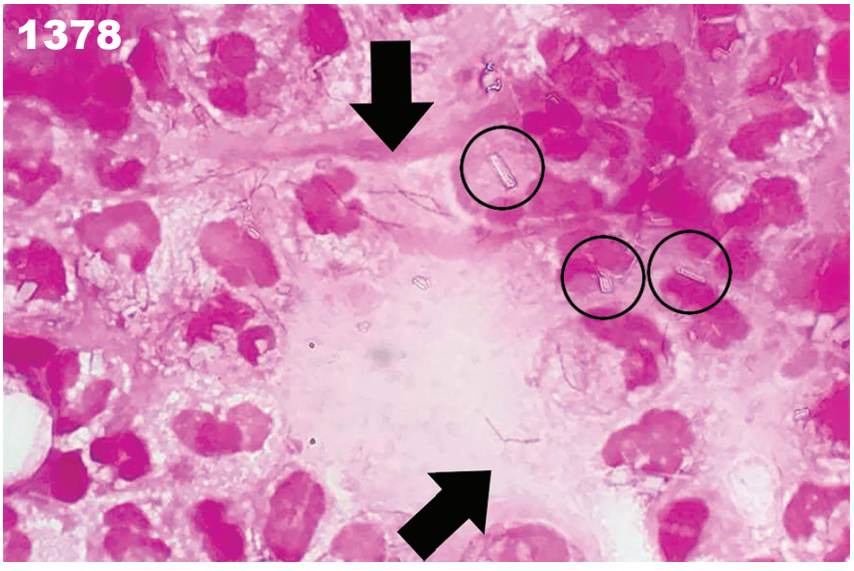

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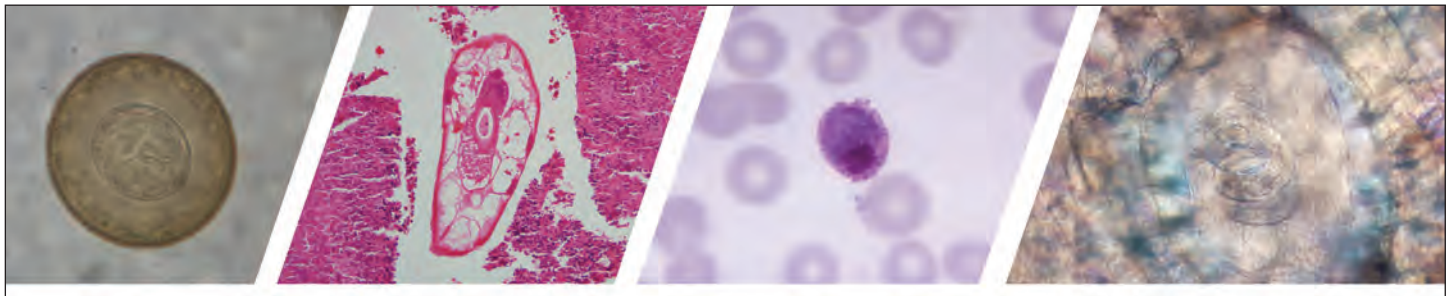

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Progress and Remaining Gaps in Estimating the Global Disease Burden of Influenza

Joseph Bresee, Julia Fitzner, Harry Campbell, Cheryl Cohen, Vanessa Cozza, Jorge Jara, Anand Krishnan, Vernon Lee, for the WHO Working Group on the Burden of Influenza Disease

Influenza has long been a global public health priority because of the threat of another global pandemic. Although data are available for the annual burden of seasonal influenza in many developed countries, fewer disease burden data are available for low-income and tropical countries. In recent years, however, the surveillance systems created as part of national pandemic preparedness efforts have produced substantial data on the epidemiology and impact of influenza in countries where data were sparse. These data are leading to greater interest in seasonal influenza, including implementation of vaccination programs. However, a lack of quality data on severe influenza, nonrespiratory outcomes, and high-risk groups, as well as a need for better mathematical models and economic evaluations, are some of the major gaps that remain. These gaps are the focus of multilateral research and surveillance efforts that will strengthen global efforts in influenza control in the future.

Influenza has long been a global public health priority because of the ever-present threat of another global pandemic. In addition, many countries (especially in more affluent, temperate areas) prioritize influenza prevention and control programs because of the annual effects of seasonal influenza. The 3 influenza pandemics that occurred during the 20th century clearly illustrated the major impact from the global spread of a new influenza A virus (*1*) and spurred early vaccine development (*2*,*3*). The reemergence of avian influenza A(H5N1) in Asia in 2004 served as a reminder of this threat and brought about an acceleration of national and international efforts to prepare for the next pandemic (*4*). These efforts, including the expansion of influenza surveillance and laboratory capacity, contributed to a more effective response during the 2009 influenza A(H1N1) pandemic (*5*).

Although the threat of novel influenza A viruses and pandemics has mobilized national preparedness efforts, in many parts of the world the importance of seasonal epidemics of influenza has been relatively underappreciated. On the basis of findings from recent influenza respiratory mortality studies, including estimates from a study conducted by Iuliano et al (*6*), the World Health Organization (WHO) has indicated that 290,000–650,000 respiratory deaths from seasonal influenza epidemics occur annually (*7*). Most high-income countries, where substantial work to document the disease burden from annual influenza epidemics has been conducted, have longstanding and robust influenza vaccination programs (*8*); in these settings, the use of influenza antiviral drugs and antibiotics to treat influenza-associated lower respiratory tract infections is relatively routine. These strategies have likely resulted in a reduction in the burden of disease as well as improved clinical outcomes for patients with influenza. In tropical and low- and middle-income countries (LMICs), seasonal influenza has often been viewed as a disease of relevance primarily to industrialized countries. The historical paucity of data on influenza from these settings has likely contributed to this view. More data on influenza burden in these locations are needed to make compelling arguments to policy makers for investments in seasonal influenza control and prevention. These data are particularly important in the light of challenges related to the variable effectiveness of current vaccines (*9*) and the programmatic and economic difficulties in conducting annual influenza vaccination programs or in implementing appropriate use of antivirals for treatment in LMICs. The resulting relative underuse of vaccines in many LMICs represents important missed opportunities for disease prevention (*10*). Furthermore, the lack of antiviral drug treatment and influenza vaccination programs for reduction of seasonal influenza burden in these settings also jeopardizes the capacity for effective responses when the next pandemic emerges because national pandemic response plans rely, in part, on the timely and efficient use of

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medical countermeasures, such as antiviral drugs and vaccines for pandemic viruses (*11*,*12*).

Progress, but Gaps Remain

The growth of influenza surveillance and research in the past 10 years has generated substantial new data on the epidemiology and risk from influenza around the world, notably in tropical countries and LMICs (*13*–*16*). These data have confirmed that influenza is a major cause of hospitalization and severe acute respiratory disease in all settings, whether rich or poor, tropical or temperate, urban or rural (*17*–*19*), and that the risk for severe influenza outcomes might be higher in LMICs than in high-income countries (*20*). In South Africa, for instance, rates of influenza-associated mortality among the elderly were 3–4 times higher than those among the elderly in the United States (*21*). One global estimate of childhood influenza deaths indicated that 99% of all influenza-associated deaths among children <5 years of age were in LMICs (*18*). That this finding is true for influenza, as it is for other infectious diseases, is not surprising, and similarly, is likely the result of differential access to medical care and preventive strategies, coupled with the prevalence of certain high-risk conditions and the underlying age structure of the populations. In some LMICs that have collected robust disease burden information in the past decade, vaccine programs have expanded (*22*–*24*). In addition, disease burden data from these settings were one driving factor for updating of the WHO influenza vaccine recommendations issued in 2012 by the WHO Strategic Advisory Group of Experts (*25*).

However, although data on influenza disease burden have expanded in recent years, considerable gaps persist. First, high-quality and up-to-date estimates of the extent of severe influenza at global and regional levels are needed to inform global policymakers and public health advocates as they set their priorities. Although new estimates of the global respiratory mortality rates attributable to influenza are available (*6*), additional models that take advantage of the expansion in influenza surveillance and laboratory confirmation, especially in tropical countries and LMICs, should yield more accurate country- and region-specific disease estimates. Second, at the country level, too many LMICs have yet to develop reliable national estimates of the full extent of influenza disease that would enable evidence-based decisions about local influenza prevention investments. Third, the ability to target vaccination campaigns to key populations within a country depends on having reliable data on the burden of disease and on the possible effect of vaccination among specific high-risk target groups. The value of risk group–specific estimates was evident during the 2009 pandemic, when data on the high risk for severe outcomes among pregnant women led to aggressive efforts to vaccinate and appropriately treat this group and convince obstetricians to recommend and offer vaccines (*26*,*27*); this effort provided data for the WHO Strategic Advisory Group of Experts' 2012 recommendations (*25*). Conversely, the scarcity of adequate data on severe disease among pregnant women during seasonal epidemics was one reason cited by the Global Alliance for Vaccines and Immunizations for their decision against opening an investment window to fund low-income countries to vaccinate pregnant women as part of their most recent vaccine investment strategy (*28*). Data have long indicated that persons with specific underlying diseases are at high risk for severe influenza, but without a better understanding of the burden of the disease in these groups in countries considering vaccination policies, expecting policymakers to invest in programs to target them is unrealistic. Few data have been collected outside of high-income countries on other components of the health burden, especially the contribution of influenza infections to illness and death from underlying diseases made worse by influenza, such as cardiac or chronic pulmonary diseases (*29*), and on non–health-related effects of influenza, such as the economic burden and effect on productivity (*30*). Data on each of these components will advance decisions on the rational use of resources to prevent influenza, and the need for these data was highlighted in the recent revision of the WHO Influenza Research Agenda (*31*).

Ongoing Work to Address the Gaps and Future Needs

Substantial work is under way to fill these gaps. WHO has created a robust program to collect data on global and national influenza burden and to better determine the burden among risk groups. Two manuals have been developed to guide member states' efforts to measure influenza disease (*32*) and economic burden (*33*) from data collected through ongoing influenza surveillance. Both manuals are being used by countries, in part, because of the Pandemic Influenza Preparedness Implementation Plan that has facilitated country-level disease burden estimation in many countries around the world (*34*). These efforts have led to recent publications from LMICs, supported by WHO, that provide important influenza disease burden data (*34*). WHO has also sponsored reviews of influenza-associated disease burden among pregnant women and their infants (*35*,*36*). Additional multinational collaborations are under way that will continue to develop more credible global influenza mortality and hospitalization estimates based on recent work to develop national estimates, as well as information on influenza burden among key high-risk groups (Figure) (*6*). These efforts take advantage of the recent increase in local- or country-level studies that have extended data beyond temperate, high-income settings. WHO is also

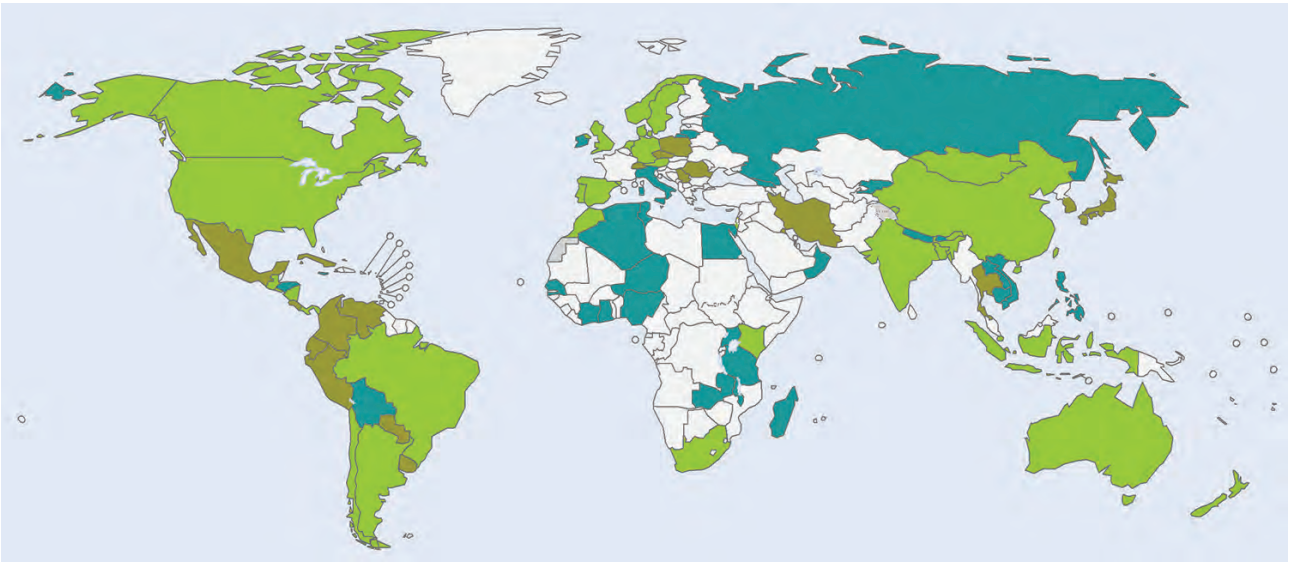

Figure. Countries with burden of disease estimates that have agreed to share data for the global estimate of influenza mortality and influenza-associated hospitalizations, as of April 2017: blue, morbidity estimates; brown, mortality estimates; green, morbidity and mortality estimates; white, data not available.

mapping existing knowledge from published literature to enable easier access to available data and identify key remaining gaps. Finally, WHO is developing a collection of economic tools to support the use of disease burden data to estimate the overall costs, the cost of vaccination programs, and the cost-effectiveness of vaccination.

In addition, other global partners are working in this area. The US Centers for Disease Control and Prevention has established collaborations with >50 countries around the world to strengthen surveillance and laboratory testing capacities, including expanding global capacity for genetic sequencing, that have produced data on influenza epidemiology and disease burden. The Multinational Influenza Seasonal Mortality Study (*37*), coordinated by the Fogarty Center at the US National Institutes of Health, has been working with countries to estimate influenza mortality from diverse settings since 2001. Institute Pasteur and Agence de Médecine Préventive have worked in low-income countries in Africa to build surveillance capacity. More recently, the Global Health Security Agenda has increased resources to many developing countries to strengthen surveillance and response capabilities that will also lead to new data on the relative burden of influenza compared with other infectious diseases. The European Center for Disease Control has developed free software that supports countries in the region to estimate the burden of influenza and other infectious diseases (*38*). These efforts have led to a substantial increase in country-specific estimates of burden in the past 5 years and promise to lead to many more in the next 1–2 years.

Much has been accomplished, and in the next few years a more complete picture of the burden of influenza will be available. However, more work will still be needed if we are to measure the full burden of influenza and, more important, the preventable burden. This knowledge will enable decision-makers to weigh the value of vaccination and encourage the use of antiviral drugs against myriad other health needs in their countries, as well as providing additional impetus for the development of newer, more effective treatments and vaccines. Vaccine probe studies have been proposed as a method to measure the preventable fraction of disease burden, focusing the studies on outcomes of greatest public health interest (e.g., pneumonia and death rates). Whether vaccine probe studies could be designed sufficiently to account for the variable and relatively modest vaccine efficacy and variability in annual disease burden that is characteristic of influenza is uncertain but should be further discussed (*39*). Finally, the year-to-year variability in disease burden requires that data be collected over multiple years and that new methodologic approaches be developed and validated to measure burden in settings with year-round circulation of influenza (*40*)

Conclusions

Influenza has long been a compelling example of a global pandemic threat, but the annual disease burden has been relatively underappreciated, leading to missed opportunities for disease reduction and prevention. Convincing evidence of seasonal burden of disease in more settings and for a wider array of influenza outcomes will be the foundation of arguments for strengthening programs to control annual influenza and to reduce the threat of future pandemics (*41*,*42*).

About the Author

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Progress in Vaccine-Preventable and Respiratory Infectious Diseases—First 10 Years of the CDC National Center for Immunization and Respiratory Diseases, 2006–2015

Anne Schuchat, Larry J. Anderson,[1] Lance E. Rodewald, Nancy J. Cox, Rana Hajjeh,[2] Mark A. Pallansch, Nancy E. Messonnier, Daniel B. Jernigan, Melinda Wharton

The need for closer linkages between scientific and programmatic areas focused on addressing vaccine-preventable and acute respiratory infections led to establishment of the National Center for Immunization and Respiratory Diseases (NCIRD) at the Centers for Disease Control and Prevention. During its first 10 years (2006–2015), NCIRD worked with partners to improve preparedness and response to pandemic influenza and other emergent respiratory infections, provide an evidence base for addition of 7 newly recommended vaccines, and modernize vaccine distribution. Clinical tools were developed for improved conversations with parents, which helped sustain childhood immunization as a social norm. Coverage increased for vaccines to protect adolescents against pertussis, meningococcal meningitis, and human papillomavirus–associated cancers. NCIRD programs supported outbreak response for new respiratory pathogens and oversaw response of the Centers for Disease Control and Prevention to the 2009 influenza A(H1N1) pandemic. Other national public health institutes might also find closer linkages between epidemiology, laboratory, and immunization programs useful.

By 2005, global spread of highly pathogenic avian influenza A(H5N1) (*1*), major disruption of US vaccine supplies (*2*), and anticipated introduction of multiple new vaccines, including those targeting emerging drug-resistant respiratory infections, provided a rationale for the Centers for Disease Control and Prevention (CDC) to establish the National Center for Immunization and Respiratory Diseases (NCIRD). A closer linkage between science and program was an explicit goal of the center's formation, and NCIRD has been on the cutting edge of applying advances in technology to public health. In April 2006, the new center brought scientific units responsible for epidemiologic and laboratory aspects of most vaccine-preventable and other acute respiratory infectious diseases together with programs supporting public sector immunization. The center's activities aligned to address 8 initial strategic priorities (Table 1).

NCIRD concentrated the expertise of the agency on the viral and bacterial agents that cause pneumonia, influenza, and other acute respiratory syndromes; responsibilities for tuberculosis remained elsewhere (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/7/17-1699-Techapp1.pdf). The expanded list of newly vaccine-preventable diseases meant response in areas traditionally managed by communicable disease units relied on immunization expertise, and the newer vaccines in turn required enhanced laboratory-based surveillance for accurate postlicensure evaluations. The mission of NCIRD was to prevent disease, disability, and death through immunization and control of respiratory and related infectious diseases. The center embedded field staff within and provided funding and technical assistance to state, local, and territorial health departments to strengthen detection, prevention, and control of these conditions, with particular emphasis on childhood immunization, influenza, and emerging respiratory infectious disease threats. The center also housed several World Health Organization (WHO) International Collaborating Centers and provided leadership for global laboratory networks for influenza, polio, measles, rotavirus, and bacterial meningitis, among others.

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Table 1. Strategic priorities for the National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention

Strategic priorities	Implementation examples
Improve immunization programs	Implemented central vaccine distribution and Vaccine Tracking System (VtrkS); supported development of adolescent platform for vaccination
Strengthen systems to evaluate policy effectiveness	Initiated annual estimation of influenza vaccine impact based on influenza surveillance, vaccine effectiveness studies, and immunization coverage surveys; introduced National Immunization Survey-Teen and quality standards for systems monitoring school-based immunization coverage
Accelerate vaccine-preventable disease reduction worldwide	Implementing partner for the Hib Initiative (2005–2009), which facilitated decisions to introduce *Haemophilus influenzae* b (Hib)–containing vaccine in all Global Alliance for Vaccines and Immunization–eligible countries and provided model framework for subsequent new vaccine introduction efforts
Reduce complications of pneumonia and influenza	Issued evidence-based guidance for influenza antiviral use to reduce severity of influenza illness
Improve pandemic preparedness	Enhanced laboratory detection of novel influenza viruses and led response to first influenza pandemic of the 21st century
Strengthen response to respiratory outbreaks	Developed Unexplained Respiratory Disease Outbreak tool kit for state, local, and international partners (https://www.cdc.gov/urdo/index.html)
Develop and promote strategies to reduce respiratory infections, vaccine-preventable diseases, and control antimicrobial resistance	Expanded the Advisory Committee on Immunization Practices recommendations for annual influenza vaccination and age groups recommended for pneumococcal conjugate vaccination; incorporated Get Smart: "know when antibiotics work" activities into national strategy to reduce antimicrobial resistance
Improve identification of causes of respiratory infections	Validated TaqMan technology for multiple pathogen diagnosis of respiratory syndromes; completed multicenter studies of etiology of pneumonia in the community in adults and children

During the subsequent decade, NCIRD established strong evaluation systems to measure policy and program impacts. NCIRD also spearheaded modernization of the nation's immunization activities and collaborated with other infectious disease programs to invest in advanced molecular detection technology to accelerate prevention, detection, and control of influenza and other respiratory threats. The center aimed to sustain the public's acceptance of vaccination while providing technical assistance and on-the-ground support for outbreak responses to previously rare vaccine-preventable diseases. In 2009, the staff of NCIRD led the response of CDC to the first influenza pandemic of the 21st century. In subsequent years, response of NCIRD to other novel respiratory threats, such as Middle East respiratory syndrome coronavirus, severe respiratory illness associated with enterovirus D68, and increases in Legionnaires' disease, involved collaboration with clinicians, state, and local health departments and, when appropriate, international organizations and ministries of health.

Immunization System Change at CDC and Health Departments

During 2005–2015, the childhood immunization schedule expanded substantially, with corresponding increased costs (Figure 1), sustained high immunization coverage of traditional vaccines, and increased coverage of newer vaccines (Figure 2). Licensure of several vaccines and shifting epidemiology in selected vaccine-preventable diseases placed a premium on strong surveillance and evaluation systems to provide evidence needed for policy. The Advisory Committee on Immunization Practices (ACIP) released numerous recommendations during this period (Table 2). Clinicians developed an adolescent platform to permit delivery of vaccines to children 11–12 years of age. The program introduced the National Immunization Survey-Teen (a large telephone survey of parents, with healthcare provider verification of records) in 2006 to monitor coverage of immunizations in persons 13–17 years of age. Expanded monitoring of influenza vaccine coverage relied on several new techniques, including leveraging hospital reporting to the Center for Medicare and Medicaid Services, which incentivized reporting of influenza vaccination of healthcare workers.

Monitoring of vaccine performance resulted in updates to vaccine recommendations. Continued varicella outbreaks led to recommendation of a second dose of varicella-containing vaccine. Emergence of waning immunity following meningococcal conjugate vaccine (Men ACWY) prompted recommendation for a booster dose. Resurgence of pertussis prompted investigations of vaccine performance and characterization of circulating strains. Surveillance detected emergence of pertactin-deficient *Bordetella pertussis* and epidemiologic studies documented the limited duration of protection afforded by acellular vaccines (*3*). Accordingly, updated tetanus, diphtheria, and acellular

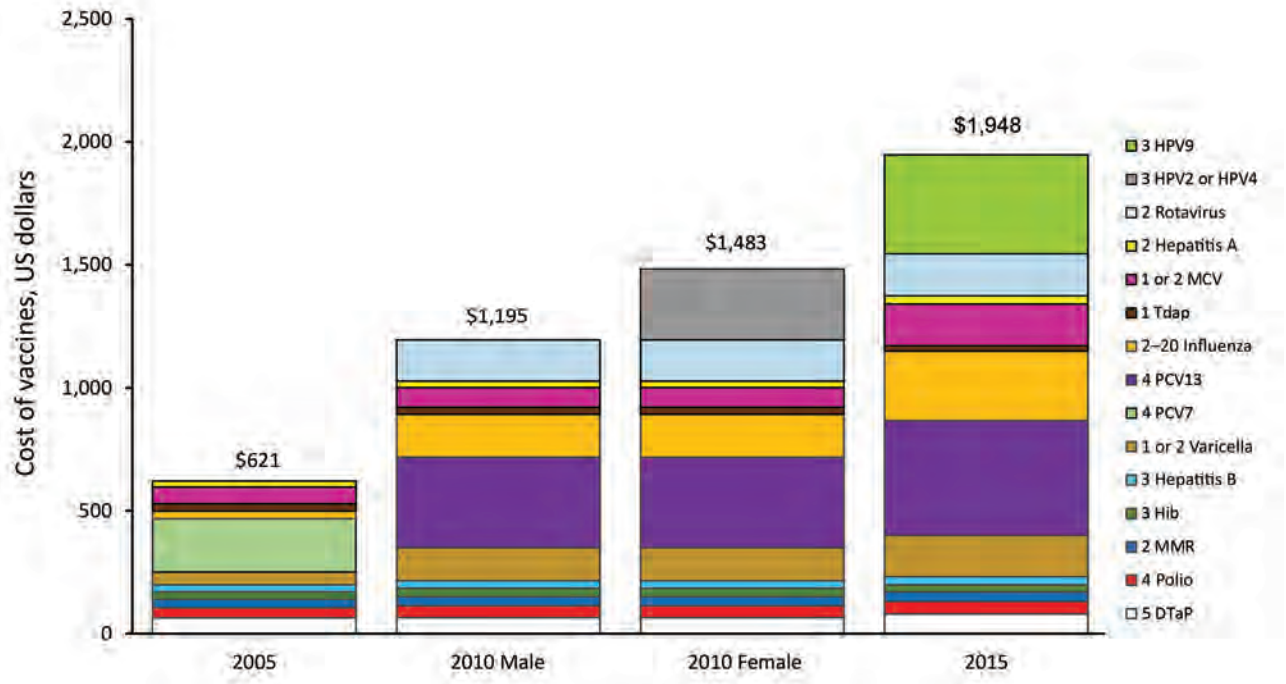

Figure 1. Cost of vaccines routinely recommended from birth through 18 years of age, according to public sector contract prices, United States, 2005, 2010 (by sex), and 2015. Values in key indicate no. vaccinations/series. Data were based on federal contract prices as of September 1, 2005; April 6, 2010; and April 1, 2015. DTaP, diphtheria, tetanus, and acellular pertussis vaccine; Hib, *Haemophilus influenzae* type b vaccine; HPV, human papillomavirus vaccine; MCV, meningococcal conjugate vaccine; MMR, measles, mumps, and rubella vaccine, PCV, pneumococcal conjugate vaccine; Tdap, tetanus, diphtheria, and acellular pertussis vaccine.

pertussis vaccine recommendations advised women to receive this vaccine during each pregnancy to prevent severe disease in early infancy.

NCIRD implemented changes that modernized the nation's immunization infrastructure and strengthened the program's efficiency. Establishment of a centralized vaccine distribution system in 2008 eliminated 99% of the 430 vaccine depots that states previously relied on to store vaccines, resulting in direct shipping to >40,000 provider sites, with reduced inventory costs and waste. NCIRD replaced a DOS-based vaccine ordering and management system with the vaccine tracking system VtrkS (https://www.cdc.gov/vaccines/programs/vtrcks/index.html), an SAP-enabled data management and analytic system (https://www.sap.com/products/data-services.html), thereby improving the accountability of the CDC multibillion-dollar vaccine supply chain. Improvements in immunization information systems (i.e., registries) have been slowed by diversity of registries in states; barriers to interstate data sharing; and incomplete records, particularly for adult vaccinations. Investments focused on increasing their interoperability with electronic medical records, expanding physician use, developing clinical decision support tools (e.g., prompts for which vaccine doses are due or overdue), and improving efficiency and accuracy of data entry.

Provisions of the Affordable Care Act of 2010 regarding prevention services required updated insurance plans to include all vaccines routinely recommended by the ACIP without copayments when administered by an in-network provider. The Vaccines for Children Program, implemented in 1994, provides free vaccines for children who are either uninsured or Medicaid eligible, among others (http://www.cdc.gov/vaccines/programs/vfc/index.html). By requiring first dollar coverage for ACIP-recommended vaccines, the Affordable Care Act provision eliminated most out-of-pocket costs for persons with either public or private insurance. A notable exception occurs for vaccines covered under Medicare part D (e.g., shingles), for which senior citizens might still have large out-of-pocket expenditures. NCIRD helped health departments establish billing systems so that insurance companies paid for vaccines given to their covered members, saving limited public sector resources (https://www.cdc.gov/vaccines/programs/billables-project/success-stories.html).

Protecting the infrastructure supporting immunization was essential during the decade, even once most insurance plans fully covered vaccination (*4*). State, local, and federal immunization programs responded to resurgent pertussis; outbreaks of measles, mumps, and meningococcal meningitis; extended shortages of *Haemophilus influenzae*

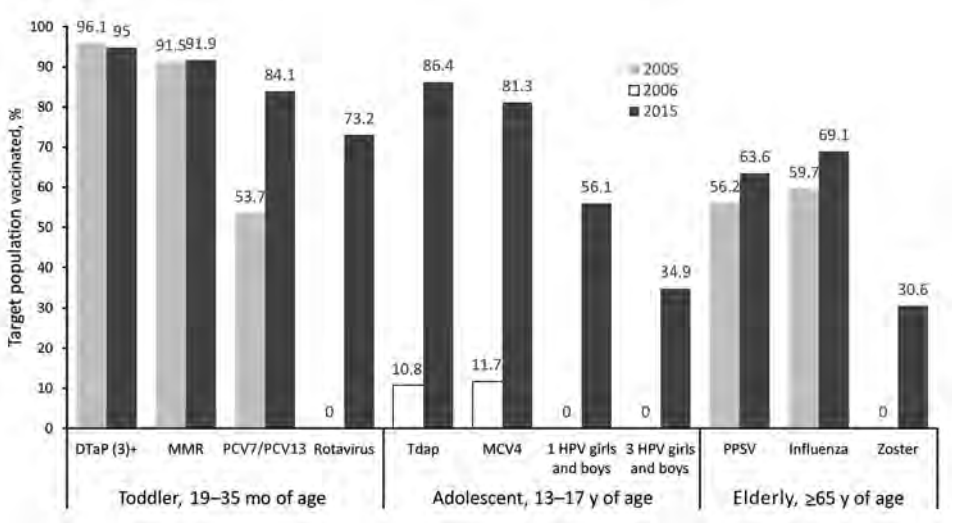

Figure 2. Selected immunization coverage by vaccine and target group, United States, 2005–2006 and 2015. PCV7 and PCV13 are ≥4 doses. Rotavirus coverage is 2 or 3 doses depending on product. PCV7 is a pneumococcal conjugate vaccine with 7 serotypes, and PCV13 is a pneumococcal conjugate vaccine with 13 serotypes. Data were obtained from the National Immunization Survey for toddlers, the National Immunization Survey-Teen (NIS-Teen) for adolescents, the National Immunization Survey-Flu for influenza, and the National Health Interview Survey for PPSV and zoster. DTaP, diphtheria, tetanus, and acellular pertussis vaccine; HPV, human papillomavirus vaccine; MCV, meningococcal conjugate vaccine; MMR, measles, mumps, and rubella vaccine; PCV, pneumococcal conjugate vaccine; PPSV, pneumococcal polysaccharide vaccine; Tdap, tetanus, diphtheria, and acellular pertussis vaccine.

type b (Hib) conjugate, and 5-in-1 infant combination vaccines. Enhancements made at the national level included randomly sampling cellular telephones and land lines to access participants for the National Immunization Survey (*5*), and strengthening the link between pediatric immunization caregivers, oncologists, and cancer survivor advocates in an effort to improve human papillomavirus vaccination of children 11 and 12 years of age (https://www.cancer.org/health-care-professionals/national-hpv-vaccination-roundtable.html).

Sustaining high levels of vaccine acceptance was also a focus. Based on formative research with parents and clinicians, and in partnership with the American Academy of Pediatrics, NCIRD released a communication tool kit aimed at promoting effective vaccine conversations between providers and parents. CDC enhanced transparency of the ACIP process by introducing Grading of Recommendations Assessment, Development and Evaluation for systematic review of the evidence (*6*), and webcast the committee's public meetings (*7*). Although vaccinating children remained a social norm during this period, with <1% of toddlers receiving no vaccine doses (*8*), outbreaks of measles identified geographic areas where personal belief exemptions were becoming more common (*9*,*10*).

On the occasion of the 20th anniversary of implementation of the Vaccines for Children program, CDC staff

Table 2. Selected recommendations of the Advisory Committee on Immunization Practices, Centers for Disease Control and Prevention, 2005–2015*

New vaccine (indication)	Date of ACIP recommendation
Meningococcal ACWY conjugate (adolescents)	2005 Feb
Tdap (adolescents)	2005 Jun
Measles, mumps, rubella, and varicella (MMRV)	2005 Oct
Influenza (24–59-month-old children)	2006 Feb
Rotavirus (infants)	2006 Feb
Human papillomavirus (adolescent girls)	2006 Jun
Second-dose varicella	2006 Jun
Zoster (shingles)	2006 Oct
Influenza (persons 6 mo–18 y of age)	2009 Feb
13-valent pneumococcal conjugate vaccine (children)	2010 Feb
Influenza (universal in persons >6 mo of age)	2010 Feb
Second dose of meningococcal ACWY conjugate (adolescents)	2010 Oct
Human papillomavirus (adolescent boys)	2011 Oct
Tdap (in every pregnant woman)	2012 Oct
13-valent pneumococcal conjugate vaccine (persons ≥65 years of age)	2014 Aug
Meningococcal B vaccine (during outbreaks and in high-risk persons)	2015 Feb
HPV-9 (adolescents)	2015 Feb
Meningococcal B (adolescents, category B)	2015 Jun

*ACIP, Advisory Committee on Immunization Practices; HPV, human papillomavirus; Tdap, tetanus, diphtheria, and acellular pertussis.

completed analyses of the impact of childhood immunization. Twenty years of childhood immunization, at actual coverage levels, averted 322 million illnesses and 732,000 premature deaths, at a net savings of $295 billion in direct costs and $1.38 trillion in total societal costs (*11*). The childhood immunization series was estimated to save $3 in direct medical costs for each dollar invested (*12*).

Improving Prevention and Control of Respiratory Infections

Substantial improvements in control of respiratory infections in the United States occurred during the decade. Scientists in industry and academia have advanced molecular detection for respiratory pathogens during this period, and NCIRD staff capitalized on advancements to apply tools to the public health need for detection of respiratory infections (*13*), thereby extending their value beyond commercial or clinically relevant applications. NCIRD applied new multipathogen diagnostic tests to characterize the burden of community-acquired pneumonia in the United States (*13–15*) and collaborated on similar efforts in developing country settings (*16*,*17*).

Hospitalizations and deaths from respiratory infectious disease in the United States continued to decrease during this period. Pneumonia and influenza decreased from the sixth to eighth leading cause of death in the United States during 2006–2015, and age-adjusted mortality rates decreased by 17.4%, from 18.4 deaths/100,000 persons in 2006 to 15.2 deaths/100,000 persons in 2015 (*18*). Severe influenza seasons contributed to variability in influenza hospitalizations, but pneumonia hospitalizations decreased by 11.7%, from 1,781,137 in 2006 to 1,571,428 in 2014 (Figure 3).

Several advances probably contributed to the decrease in all-cause pneumonia hospitalizations. Pediatric use of pneumococcal conjugate vaccine (PCV) averted an estimated 47,000 (43%) hospitalizations in children <2 years of age and 168,000 pneumonia hospitalizations in all age groups by 7–9 years after introduction of 7-valent PCV (PCV7) vaccine (*19*). Although increases in drug-resistant nonvaccine serotype invasive pneumococcal disease occurred, an expanded serotype formulation, a 13-valent pneumococcal conjugate vaccine (PCV13), replaced PCV7 in 2010. The Active Bacterial Core surveillance of the Emerging Infections Program Network conducted studies of serotype-specific changes in invasive pneumococcal disease during the decade, which suggest that much of the decrease in adult pneumococcal illness was caused by reduced transmission of vaccine-type pneumococci from vaccinated children to adults (*20*). In addition, highly active antiretroviral therapy reduced the extremely high risk for pneumonia among persons infected with HIV and likely lowered all-cause pneumonia burden in this population (*21*).

Given the higher risk for invasive pneumococcal disease associated with active and passive cigarette smoke, reductions in cigarette smoking among adults and in secondhand smoke exposure during this period might also have reduced pneumonia (*22–24*). Annual vaccination rates against seasonal influenza among adults ≥65 years of age increased from 59.7% in 2005 to 69.1% in 2015 (*25*), and vaccination rates among other age groups, especially children, increased substantially. In August 2014, PCV13 was recommended for persons ≥65 years of age, in addition to the longstanding recommendation for 23-valent pneumococcal polysaccharide vaccine (*26*). Quantifying the independent role any of these factors played in the decrease in hospitalizations and deaths would be difficult.

During this period, program monitoring focused on strengthening the link between data and action. NCIRD collaborations sped up the availability of data on influenza vaccine coverage and effectiveness. Interim estimates of early vaccine coverage were released each December for

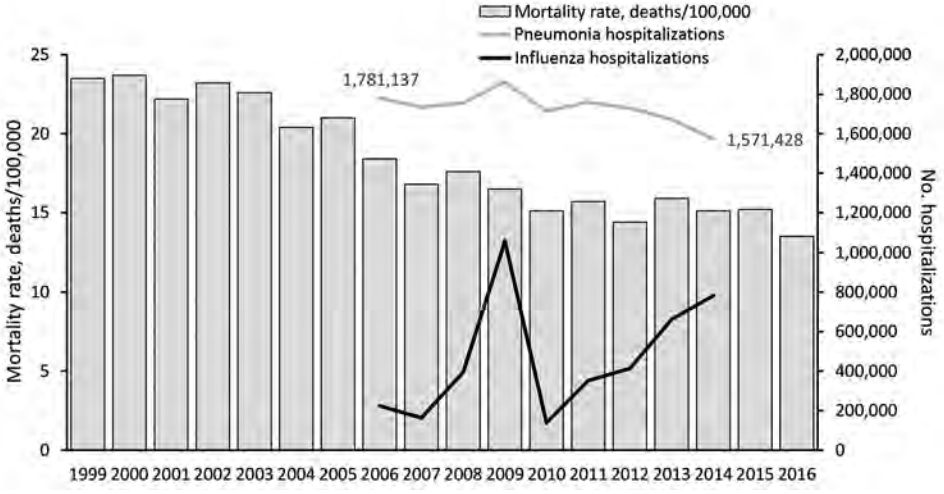

Figure 3. Pneumonia and influenza mortality rates and hospitalization counts by year, United States, 1999–2016. Age-adjusted mortality rates were obtained from the National Center for Health Statistics and based on underlying cause of death; nationwide hospitalization estimates were obtained from the Agency for Healthcare Research and Quality Healthcare Cost and Utilization Project.

the ongoing influenza season, and preliminary estimates of the effectiveness of the influenza vaccine for that season were disseminated during the first quarter of each calendar year (*27*). Final data from each season on influenza illness, vaccine effectiveness, and vaccine coverage were incorporated into a model that estimated the annual impact of vaccination and the incremental benefit that would be achieved with increased coverage of current vaccines or with future vaccines with higher efficacy (*28*,*29*). The model estimates that, from 2010–11 through 2015–16, a total of 26,808,150 illnesses and 415,115 hospitalizations were prevented through seasonal influenza vaccination (*30*). Influenza vaccination was associated with reduced risk for laboratory-confirmed influenza-associated pediatric death (*31*).

In 2004–05, state and local public health and clinicians faced substantial challenges from a sudden decrease in expected influenza vaccine doses for the US market (*2*). Investments of the US government and vaccine manufacturers led to a diversified influenza vaccine supply and substantially more doses produced in more recent years. New formulations include quadrivalent, cell-based, recombinant, high-dose, and adjuvanted products (*32*). Universal recommendations for annual vaccination and more predictable supply resulted in more persons in the United States immunized against influenza. Data from the National Health Interview Survey suggest the proportion of all US adults receiving influenza vaccine increased from 27.4% in 2005–06 (*33*) to 44.8% in 2014–15 (*34*).

Annual measurements of vaccine effectiveness show major limitations in protection by current vaccines. New questions concern possible lower effectiveness after sequential vaccination and lower effectiveness against influenza A(H3N2) virus. Public health practitioners and clinicians thus face new challenges, despite ample supply, promoting the imperfect vaccines currently available. Although researchers focus on development of universal influenza vaccines, incremental improvements, such as cell-derived vaccine candidate viruses, which can avoid egg-adapted vaccine virus changes, offer promise in the interim.

Strengthening Health Protection and Response

In conjunction with national strategies for pandemic preparedness issued by the US government (*35*,*36*), CDC worked with state and local health departments, clinicians, nongovernmental organizations, and global partners to strengthen readiness for avian and pandemic influenza. Exercises helped ready public health authorities for pandemics and other biologic threats, including assessment of community mitigation strategies. Public engagement efforts conducted in 2005–2009 permitted planners to incorporate values of citizens into prioritization for scarce vaccine supplies and led to development of strategies to reach workers in critical infrastructure fields (*37*). CDC supported strengthened detection (e.g., enhanced diagnostic and surveillance systems) and improved response capabilities for influenza (*38*,*39*). Investments and technical assistance also focused on upgrading capacities within state and local public health agencies in the United States and ministries of health in other countries to address other emerging or severe respiratory pathogens.

On April 21, 2009, CDC reported 2 human cases of respiratory illness in southern California caused by a novel influenza virus that had a previously unseen combination of genes that originated from viruses circulating previously in pigs, birds, and humans (*40*,*41*). Additional cases in California, Texas, New York, and Mexico led to recognition of the 2009 influenza A(H1N1) pandemic. Elderly populations had some cross-protection against the virus, but children and younger adults were disproportionately affected (*42*). Facilitated by preparedness investments, the influenza laboratory of NCIRD was able to adapt a PCR test for detection of the pandemic H1N1 virus, obtain Emergency Use Authorization from the US Food and Drug Administration, and rapidly ship new diagnostic test kits to states and 153 countries (*38*). CDC and its partners rapidly prepared a candidate vaccine virus so that the multiple steps of vaccine manufacturing could get under way quickly. The pandemic response effort of CDC supported epidemiologic and virologic investigations, adapted the centralized vaccine distribution system used for the Vaccines for Children Program, and made 330,000 shipments of monovalent influenza vaccine. CDC worked with state and local health departments to support vaccination at high-throughput public clinics, private provider offices, occupational clinics and, eventually, pharmacies. During October–December 2009, more than 80 million persons in the United States received the monovalent H1N1 vaccine. The pandemic response incorporated risk communication strategies, frequent media briefs, and messages shared through trusted community-based partners to reach diverse populations. The initial response included large numbers of school closures, which were highly disruptive. Updated policy advised more limited use of school dismissals.

Unfortunately, large amounts of vaccine were not available until several weeks after the peak of the fall pandemic wave. The 2009 H1N1 pandemic virus caused lower overall severe illness in the fall of 2009 than had been expected. However, in 2014–15, circulation of a drifted influenza A(H3N2) virus caused substantially more severe disease, resulting in an estimated 707,155 hospitalizations (*28*,*30*).

During its first decade, NCIRD also addressed several novel respiratory pathogens, including enterovirus D68 and concurrent increases in acute flaccid myelitis in children. Emergence of virulent avian influenza A(H7N9) virus in China in 2013 prompted intensified surveillance and pre-pandemic vaccine investments. Evolution of viruses and geographic spread has further increased concern (*43*). During 2012–2015, sporadic cases and outbreaks of severe respiratory illness caused by the newly recognized Middle East respiratory syndrome coronavirus were detected in Saudi Arabia and elsewhere on the Arabian Peninsula. As occurred for severe acute respiratory syndrome coronavirus and Ebola virus, transmission in hospital settings affected patients and healthcare workers (*44*). NCIRD coordinated agency preparedness and response to Middle East respiratory syndrome. When the virus was imported to Indiana and Florida in the United States by 2 healthcare workers who had been exposed in hospitals in Saudi Arabia, prompt detection and response included effective infection control and contact tracing. No further spread from these importations occurred.

Global Vaccine-Preventable Disease Progress

Vaccine-preventable disease control has benefited from longstanding efforts by the United Nations Children's Fund, the WHO Expanded Program on Immunization, and the Global Polio Eradication Initiative, among others. NCIRD collaborated with these and newer organizations, such as the Global Alliance for Vaccines and Immunization (Gavi), which supports new and underused vaccination in low-resource countries.

The first decade of NCIRD coincided with major progress in accelerated uptake of new or underused vaccines in developing countries. NCIRD epidemiologic and laboratory studies contributed to the evidence base in support of broader use of pneumococcal conjugate and rotavirus vaccines, and NCIRD staff played key leadership or technical roles in public–private partnerships, such as the Hib Initiative (*45*) and pneumococcal and rotavirus Accelerated Development and Introduction Plans (*46*), funded by Gavi, and provided laboratory and statistical support to the Meningitis Vaccine Project led by PATH (Seattle, WA, USA) and WHO. Use of Hib vaccine increased from 19 Gavi-supported countries in 2005 to all 73 by 2014. Use of pneumococcal conjugate increased to 54 countries, and use of rotavirus vaccines increased to 37 countries (Figure 4). MenAfriVac, developed by Serum Institute of India with support from the Meningitis Vaccine Project, was administered in campaigns during 2010–2015 that reached 235 million persons within the African meningitis belt. These efforts have eliminated epidemics of group A meningococcal meningitis (*47*), although more recent outbreaks of group C meningococcal disease have occurred. During this period, NCIRD assisted ministries of health in assessing benefits of the newer vaccines (*46*,*48*,*49*), as well as the occurrence and favorable benefit-risk ratio associated with intussusception after rotavirus vaccination (*46*).

Global eradication of polio has been a priority since 1988. During 2006–2015, polio transmission was interrupted in Egypt and India, and rapid response efforts halted outbreaks of imported polio in many counties in Africa, as well as in Tajikistan and the Middle East. By 2015, wild poliovirus continued to circulate in Afghanistan, Pakistan, and Nigeria, although annual case counts had decreased from 1,997 wild poliovirus cases in 2006 to 74 in 2015. NCIRD scientists led the Global Polio Eradication Initiative laboratory network, enhanced approaches to environmental monitoring, and assessed use of bivalent and monovalent oral polio vaccine and fractional doses of inactivated polio

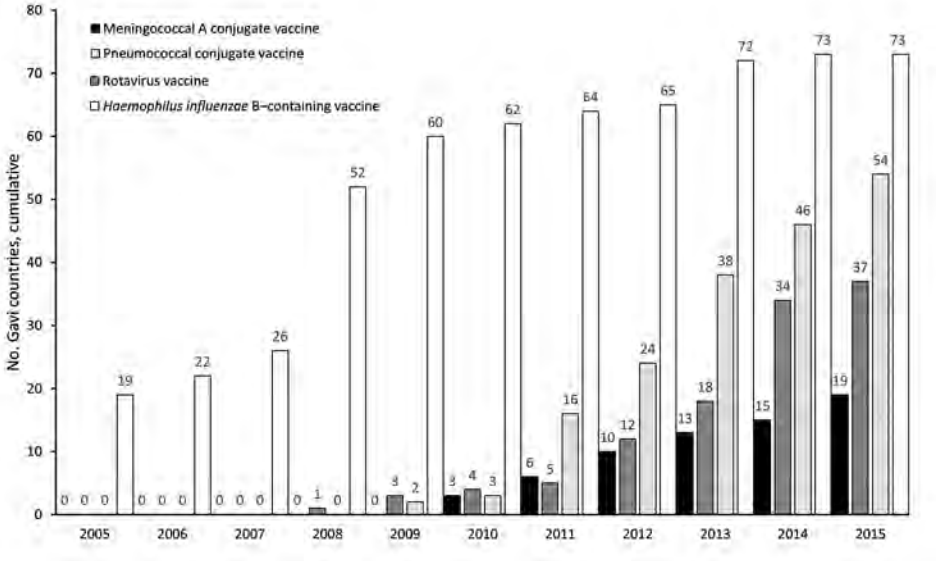

Figure 4. Cumulative number of Gavi-eligible countries using selected new or underused vaccines in routine programs, 2005–2015. Data were obtained from Gavi. Gavi, Global Alliance for Vaccines and Immunization.

vaccine as tools for achieving eradication. In 2011, the Global Immunization Division moved from NCIRD to the newly formed Center for Global Health to continue programmatic aspects of eradication and vaccine-preventable disease control. Laboratories at CDC for vaccine-preventable diseases, including polio and measles, remained in NCIRD. Strong collaboration between the Global Immunization Division and NCIRD has continued, as has sharing of the US experience with vaccine hesitancy with partners facing similar challenges in other regions.

Ongoing Challenges

Globalization and microbial adaptation continue to threaten disease control progress. Sustaining community acceptance is needed even once effective and safe interventions are available. Although technological improvements to laboratory detection, vaccine development and production, and information systems have been marked during the first decade of NCIRD, application to public health, including immunization and disease reporting, is slow. Business drivers to improvements in electronic health records and fragmentation of the public health enterprise (e.g., different immunization registries in each state) have limited progress. Another challenge has been lower than expected vaccine performance (e.g., acellular pertussis, live attenuated influenza vaccine). Development of influenza vaccines with broader protection is a priority, but their availability remains years away. Decreasing survey response rates threaten continuity of health monitoring, while the potential of big data for conditions requiring precise laboratory confirmation, such as respiratory pathogens, might be limited. The Global Health Security Agenda includes roadmaps and accountability metrics to strengthen response to emerging threats, but sustained governmental and private sector commitments to this effort will be essential.

Conclusions

Establishment of a center housing epidemiologic, laboratory, and program units related to vaccine-preventable and acute respiratory infectious disease provided a platform for strategic improvements to disease prevention and control that was timely given the influenza pandemic in 2009, the changing epidemiology of vaccine-preventable diseases, and increased demands on the immunization system in the United States. NCIRD worked in concert with state and local public health, as well as with numerous global partners, to optimize use of available prevention tools, strengthen detection and control measures, and build the evidence base for the next generation of interventions, such as improved influenza vaccines and vaccines in development against respiratory syncytial virus. Pandemic influenza and Ebola showed that implementing vaccination or testing new countermeasures in the midst of epidemics is difficult. Global initiatives suggest that partnerships between public and private sectors, engaging scientific and programmatic perspectives, might achieve more impact than single-agency efforts.

As ministries of health in industrialized and resource-poor countries increase attention to pandemic-prone respiratory infections and expand their immunization programs to address common endemic infectious diseases, organizational change similar to ours might be beneficial. However, organizational design cannot address or anticipate every issue, and will not eliminate the interdependence of programs or the value of collaboration across programs, sectors, and nations.

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About the Author

Dr. Schuchat is Principal Deputy Director of CDC and was director of NCIRD during 2006–2015. She has served in other leadership posts, including acting CDC director (January–July 2017 and January–March 2018) and chief of the Respiratory Diseases Branch (1998–2005). She has also played key roles in CDC emergency responses, including the influenza A(H1N1) pandemic response in 2009, the severe acute respiratory syndrome outbreak in Beijing in 2003, and the bioterrorist anthrax response in 2001. Her research interests include prevention effectiveness, vaccine evaluations, and global health.

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Integrated Serologic Surveillance of Population Immunity and Disease Transmission

Benjamin F. Arnold, Heather M. Scobie, Jeffrey W. Priest, Patrick J. Lammie

Antibodies are unique among biomarkers in their ability to identify persons with protective immunity to vaccine-preventable diseases and to measure past exposure to diverse pathogens. Most infectious disease surveillance maintains a single-disease focus, but broader testing of existing serologic surveys with multiplex antibody assays would create new opportunities for integrated surveillance. In this perspective, we highlight multiple areas for potential synergy where integrated surveillance could add more value to public health efforts than the current trend of independent disease monitoring through vertical programs. We describe innovations in laboratory and data science that should accelerate integration and identify remaining challenges with respect to specimen collection, testing, and analysis. Throughout, we illustrate how information generated through integrated surveillance platforms can create new opportunities to more quickly and precisely identify global health program gaps that range from undervaccination to emerging pathogens to multilayered health disparities that span diverse communicable diseases.

The potential to combine public health and environmental surveillance data with innovations in machine learning, statistical modeling, and data visualization has contributed to an emerging vision of precision public health, the idea that global health programs should use high-resolution data to guide interventions and direct scarce resources to those who would benefit most (*1*). Robust disease surveillance is a cornerstone of global health efforts that range from detecting emerging pathogens and epidemics to the control or elimination of vaccine-preventable diseases, HIV, malaria, and neglected tropical diseases (NTDs) (http://www.who.int/neglected_diseases/9789241564540/en/) (*2*–*4*). Most infectious disease surveillance maintains a single-disease focus. In this perspective, we encourage an integrated approach to surveillance of population immunity and infectious disease transmission. First, we argue that antibody-based methods provide a unique opportunity to augment and integrate surveillance across diverse global health initiatives. Second, we highlight multiple areas for synergy through integration, where the combined result will add more value to public health efforts than independent disease monitoring through vertical programs. Finally, we draw on innovations in laboratory and data science to suggest key ingredients for an integrated serologic surveillance (serosurveillance) platform. Throughout, we show how information generated through an integrated platform can create new opportunities to more quickly and precisely identify public health program gaps, and we draw on examples from many global health programs that use serosurveillance to target and monitor their efforts.

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Serology for Integrated Surveillance

Antibodies are unique among biomarkers in their ability to identify persons with protective immunity to vaccine-preventable diseases and to measure past exposure to diverse pathogens that range from viruses to bacteria, parasitic protozoa, and nematodes. All pathogens leave behind immunologic footprints in the form of antibodies that last for months to years and can be detected by testing dried blood spots or serum samples against panels of well-defined antigens. Antibody response provides an objective and sensitive way to uncover immunization coverage gaps or waning immunity to vaccine-preventable diseases (*5*–*7*) and monitor a population's exposure to malaria (*8*), enteric pathogens (*9*–*12*), and many NTDs (*13*–*17*). Antibody response can also be a key tool to monitor epidemics, such as HIV (*18*) and emerging pathogens (*16*,*19*). Antibody levels reflect past exposure over a period of months to years, so cross-sectional surveys contain an immense amount of information about past vaccination and pathogen exposure (*8*,*20*).

Enteric pathogen incidence estimated from serosurveillance in European countries is 2–6 orders of magnitude higher than the rates estimated from standard case-based surveillance (*9*,*10*). This surprising statistic illustrates a key advantage of serosurveillance: most pathogen infections have asymptomatic presentation or mild symptoms, so reported clinical cases typically represent the tip of the iceberg in terms of actual infection. When measured in young

children, antibody levels reveal recent often subclinical infections and, when measured on the population level, reflect the degree of disease endemicity, a characteristic that makes serosurveillance extremely useful for monitoring transmission interruption or recrudescence in malaria and NTD elimination settings (*8*,*21*). Measuring antibodies to vaccine-preventable diseases in young children can track improvements in infant vaccination through routine health services and complement program coverage data (*5*,*7*).

The development of multiplex serologic assays and the geographic overlap of many communicable diseases creates an opportunity to extend scarce global health resources beyond single-disease testing. For example, in the past 10 years Uganda completed ≥20 population-based serosurveys, including the Malaria Indicator Survey (2009, 2014); the AIDS Indicator Survey (2011); the Demographic and Health Surveys (2011, 2016); and NTD transmission assessment surveys in subsets of districts for lymphatic filariasis, onchocerciasis, schistosomiasis, soil-transmitted helminths, and trachoma (annually since 2008). Uganda's highly monitored population illustrates how integrated serologic testing could potentially reduce the number of surveys required to monitor diverse global health programs. Alternatively, if disease-specific serosurveys remained in place but the number of antigens included in the tests were expanded, this strategy would increase the spatiotemporal resolution of information available across programs. In principle, integrated serosurveillance could include separate laboratory assays run on the same specimens, but multiplexed assays enable the highest efficiency in terms of cost and sample volume requirements. For example, multiplex bead assays on the Luminex platform (Luminex Corporation, Austin, TX, USA) enable the measurement of antibody responses to as many as 100 different antigens with just 1 μL of serum (*15*). When samples from a serosurvey in Cambodia were analyzed at the US Centers for Disease Control and Prevention (Atlanta, GA, USA), the cost of adding a tetanus toxoid–coupled bead to a multiplex assay with 19 other antigens was US $0.30/sample, and the total cost of the 20-plex assay was less than that of a double-antigen ELISA developed for tetanus (US $30/sample; P.J. Lammie, unpub. data) (*21*,*22*). In other surveys with the laboratory work performed in-country, our team at the Centers for Disease Control and Prevention estimated that the marginal cost of a 20-plex bead assay was US $20/sample (P.J. Lammie, unpub. data), similar to the cost of the 2 separate ELISAs for measles and rubella.

Synergy through Integrated Serosurveillance

Uncovering Public Health Program Gaps and Overlap in Disease Exposure

Age-structured serosurveys can provide information about age-specific immunity gaps for vaccine-preventable diseases that result from missed vaccination or waning immunity (*7*,*20*). For example, immunizing reproductive-age women through antenatal care visits is needed for boosting tetanus seroprotection and preventing neonatal tetanus in lower-income countries. Serologic data from Cambodia revealed the success of that country's maternal and neonatal tetanus elimination program and identified that additional effort is still needed to reach young, first-time mothers (Figure 1, panels A, B) (*25*). This example illustrates how analyses of single antibodies can reveal public health program gaps, but the most novel and synergistic opportunities will most likely emerge from concurrent measurement of antibody responses to a broad pathogen panel.

The simultaneous measurement of antibody responses to multiple pathogens could uncover populations with high exposure to multiple infectious diseases and potentially multilayered health disparities. This, in turn, should create opportunities to integrate program delivery rather than relying on multiple vertical programs separately delivering

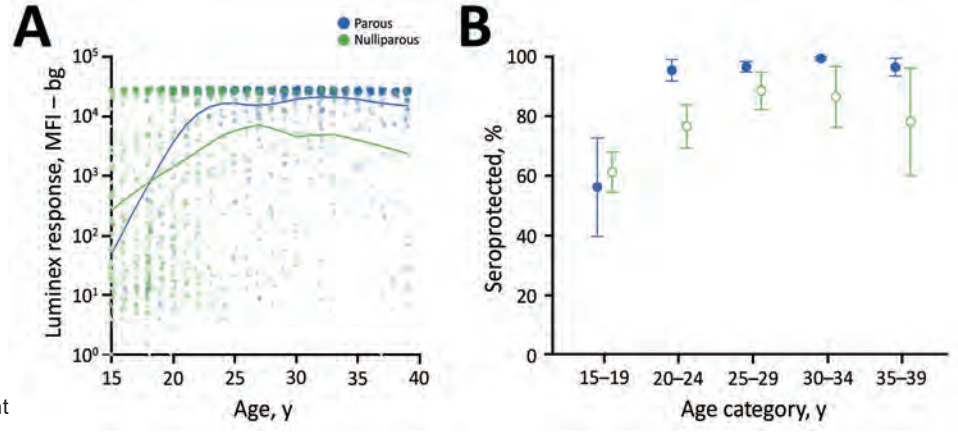

Figure 1. Tetanus toxoid antibody response of 2,150 women, by age and reproductive status, Cambodia, 2012. Specimens were collected from women who had (parous) and had not (nulliparous) previously given birth in a nationally representative immunization coverage survey (*23*) and measured by using the Luminex platform (Luminex Corporation, Austin, TX, USA). A) Mean antibody response. B) Percentage and 95% CIs of women seroprotected (>100 MFI) (*23*). We estimated age-dependent means and seroprevalence using previously described methods (*24*). Data set and computational notebook are available through the Open Science Framework (https://osf.io/2kr8b). MFI – bg, mean fluorescence intensity minus background.

interventions, a paradigm shift that aligns with calls for integrated global health systems (*26*). High pairwise correlation (r>0.5) of mean antibody responses to different antigens across geographic clusters in the Cambodia serosurvey suggests overlap in disease exposure and transmission (Figure 2, panel A). Cluster-level mean antibody responses reveal regional differences in exposure but also provide multilayered information that could help identify opportunities for coordinated response. For example, clusters in the western and northern regions of Cambodia with the highest *Plasmodium falciparum* antibody levels also have high antibody levels to the causative agent of lymphatic filariasis (*Wuchereria bancrofti*) and *Strongyloides stercoralis* but low antibody levels to tetanus toxoid (Figure 2, panel B, far right columns under West and North).

The Cambodia survey illustrates how a relatively small panel of antigens in a multiplex assay can extend the value of blood collection, and including a broader panel (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/7/17-1928-Techapp1.pdf) could provide even more information in future surveys. Similar measurements among young children would more accurately reflect recent exposure than measurement in adults, which would reflect both short-term and long-lived antibody responses (*27*). For pathogens with exposure that begins at birth, measurements in young children capture the key period of age-dependent antibody acquisition that differentiates transmission across populations (*24*). High-resolution maps of overlapping serologic responses estimated through geostatistical predictive algorithms could contribute information to integrated surveillance-and-response systems (discussed later) (*27*,*28*). If extended in this way, multiplex serologic testing could create new opportunities for coordinated and appropriate response across diverse diseases.

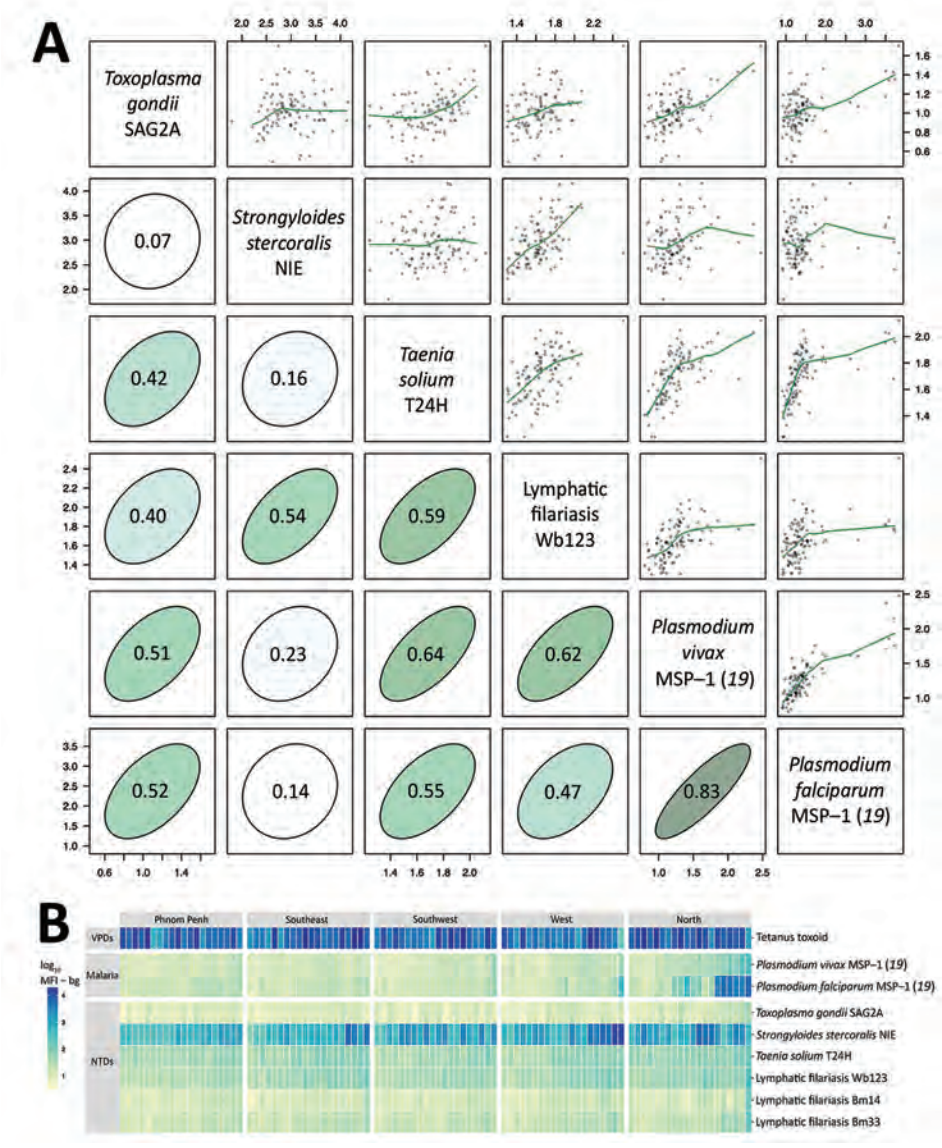

Figure 2. Antibody response to tetanus toxoid and causative agents of malaria and NTDs measured by multiplex bead assay among 2,150 women, Cambodia, 2012. Specimens were measured by using the Luminex platform (Luminex Corporation, Austin, TX, USA) (*25*). A) Relationship between pairs of antibodies measured by mean antibody response (log_{10} MFI – bg) in each of the 100 sampling clusters. Scatter plots include nonparametric locally weighted regression fits trimmed to reduce edge effects. Correlation ellipses depict the strength of the association on the basis of the Pearson correlation (r estimates). Both axes indicate mean antibody response. B) Heatmap of mean antibody response to tetanus toxoid and pathogens that cause malaria and NTDs in 100 sampling clusters stratified by region and then sorted by mean antibody response. Data set and computational notebook are available through the Open Science Framework (https://osf.io/2kr8b). MFI – bg, mean fluorescence intensity minus background; MSP, merozoite surface protein; NTDs, neglected tropical diseases; SAG2A, surface antigen 2A; VPDs, vaccine-preventable diseases.

New Opportunities to Measure Health Burden and Progress toward United Nations Sustainable Development Goals

Integrated serosurveillance would dramatically expand our knowledge of pathogens that are not currently part of routine surveillance in low- and middle-income countries. Serologic data combined with mathematical models were critical to developing global burden of disease estimates for rubella and hepatitis (*7*). Similar efforts could augment burden of disease estimates for enteric pathogens, NTDs, and other pathogens that have well-defined antigen targets but lack population-based information about incidence. For example, including antibody responses to diverse enteric pathogens in population-based surveys could improve burden of disease estimates and provide an objective indicator through which we could view progress toward achieving related United Nations sustainable development goals 3.3 (improving health and well-being target 3, which includes reducing waterborne diseases) and 6 (universal access to clean water and sanitation) (*11*,*29*).

New Opportunities for Immunoepidemiology

Integrating multiplex assays into geolocated, population-based surveys would enable an unprecedented characterization of population-level, multipathogen immunologic profiles. Large-scale studies drawing on this resource could lead to new biological insights about how disease occurrence is interrelated and provide clues for future experimental studies to examine mechanisms of immune modulation (*19*,*30*). For example, helminths infect >800 million persons worldwide, and growing evidence suggests these pathogens influence malaria infection, HIV infection, vaccine effectiveness, and even fecundity through complex immunoregulatory pathways (*31*–*34*). As another example, studies have linked seasonal El Niño atmospheric conditions to profound shifts in environmentally mediated infections, such as regional-scale cholera epidemics and mosquitoborne pathogen transmission (e.g., malaria, arboviruses) (*35*,*36*). Antibody titers in young children are a sensitive measure of recent pathogen exposure and have even been shown to fluctuate with seasonal changes in malaria transmission (*24*). Because antibody levels reflect symptomatic and asymptomatic infections, measuring antibody responses could reveal a more complete picture of seasonally driven changes in transmission compared with case-based surveillance.

Path toward Integrated Serosurveillance

Specimen Collection

Serum samples and dried blood spots are already collected for programs such as the Demographic and Health Surveys, Malaria and AIDS Indicator Survey, and NTD transmission assessment surveys. With small changes in protocol, samples from these platforms could be used for routine multiplex testing. In most cases, protocol changes will amount to ensuring adequate consent to enable broad-based serologic testing and sending specimens to regional or national laboratories with the capacity for multiplex testing. Broader multiplex testing of routine clinical samples could supplement the information available through these population-based surveys, and expanded testing within other focused serologic surveys could contribute as well (*16*,*25*,*37*). In all cases, adequate safeguards for participant privacy and biosafety will be essential and should follow similar protocols already in place for existing periodic serosurveys (e.g., Malaria and AIDS Indicator Surveys) and global laboratory testing networks (*38*). Dried blood spots collected on filter paper from finger or heel pricks are easy to collect, transport, and preserve, and antibody results from dried blood spot eluates are comparable with those obtained directly from serum samples for malaria, some helminths, and a broad range of viral pathogens, including vaccine-preventable diseases and dengue (*39*–*42*). Additional validation studies would help ensure that field protocols are adequate for the broad set of antigens envisioned in an integrated platform (online Technical Appendix Table).

A challenge to integrated serosurveillance is that different diseases have different ideal populations for monitoring, governed by disease-specific transmission dynamics. For example, children are a focus of most NTD and vaccine-preventable disease monitoring, but teens and adults are most relevant in surveillance of HIV, tetanus protection, and in some cases malaria (e.g., forest workers). Enteric pathogen transmission is often so intense in low-income settings that most population-level heterogeneity in antibody response occurs in the first few years of life (*24*), an age range that is only partly represented in population-based samples. Moreover, HIV surveillance programs have demonstrated that sampling methods that extend beyond population-based surveys and passive clinical surveillance are needed to capture the highest risk groups, and similar methods will probably be necessary for malaria and other pathogens in elimination settings (*43*). Aligning optimal sampling methods across diseases is a substantive challenge, but adaptive geostatistical design methods (*44*) could potentially start with the information collected during the initial integrated, population-based serosurvey and then augment the information needed for disease-specific control priorities through follow-on, enrichment sampling of persons in ideal age ranges and with high-risk characteristics.

Specimen Testing

From a large set of potential antigens (online Technical Appendix Table), each survey could include a subset tailored to specific disease monitoring objectives, while new antigens could be added in response to emerging threats. For

example, including a recombinant chikungunya virus antigen in multiplex testing of an existing filariasis surveillance cohort tracked the virus's introduction to Haiti during 2013–2014 (*16*). Technologic advances on bead-based platforms, such as Luminex, will further extend the number of antigens included in a single assay, and new antigen discovery through high-throughput screening studies will further enrich integrated platforms (*45*). A current limitation of the technology is that individual antigen-coupled beads are generally not yet commercially available; future commercial development of these reagents would enable better standardization across laboratories. Bead-antigen coupling and bead degradation can introduce variability into multiplex assays, so the use of standardized operating procedures and a consistent bead preparation for each survey are essential. Another limitation is that global reference serum standards to translate arbitrary units in serologic assays into international unit values are mainly limited to vaccine-preventable diseases. Reference standards for other pathogens, such as non–*P. falciparum* malarias and NTDs, would add immense value because they would enable direct comparison of quantitative results across surveys and laboratories. Finally, antibody measurements will be most useful if they are integrated into a coordinated repository and testing platform, such as the recently proposed World Serology Bank (*19*), which could further streamline laboratory protocols, accessibility to reagents, and funding. Surveillance laboratory networks for vaccine-preventable diseases provide a model for how globally standardized testing can work in practice (*38*).

Analysis Pipelines to Provide Actionable Information

Integrated serosurveillance will only reduce infectious disease transmission if it translates into actionable information and triggers a response by effective programs. In this context, information must be timely, accurate, and high resolution to be actionable from a programmatic perspective. Generating actionable information at spatial scales much smaller than national or district levels is commonplace in high-income countries and should be a near-term, attainable goal for the rest of the world (*1*). Efforts in precision global health exemplify how information could be integrated across serosurveys in space and time; high-resolution estimates of child growth failure, measles immunization gaps, and malaria mortality rates show how advances in computation, modeling, and data science have accelerated the development of new pipelines for processing, analysis, and visualization to support precision public health that spans from village to continental scales (*46*–*48*). Integrated serosurveillance will be best positioned to contribute to this endeavor if serology measurements flow into efficient data pipelines and analysis methods are general enough to accommodate diverse pathogens. The breadth of antigens incorporated into multiplex assays (online Technical Appendix Table) means that a single integrated serosurveillance platform could potentially generate spatially explicit estimates of vaccine immunity, malaria transmission, NTD transmission, and HIV incidence.

For antigenically stable pathogens, force of infection can be estimated from cross-sectional surveys with general methods that range in approach from mathematical modeling to nonparametric survival analyses (*20*). For infections that lead to partial or transient immunity, it might be possible to extend existing approaches to estimate force of infection among young children, provided that antibody levels remain sufficiently elevated for multiple years. The distribution of infectious disease transmission in populations is often highly heterogeneous in space, and for this reason, malaria and NTD elimination efforts have led to the development of sophisticated data pipelines that aggregate, analyze, and map surveillance data with rapid updates (*49*,*50*). Mapping antibody response is a relatively underexploited opportunity, and existing platforms could be extended to include multiplex serologic data. Combining antibody levels, seroprevalence, or force of infection estimates with geospatial prediction algorithms could lead to high-resolution, richly layered maps of infectious disease exposure and immunity that would be an immense resource for precision guidance of global public health programs.

Financing

Integrated serosurveillance will generate information that is a global public good (*26*), and international financing will be essential to support coordination across programs for specimen storage, testing, analysis, and reporting. Coordinated financing would also help ensure harmonization across each step in the collection, testing, and analysis pipeline. As the global community prepares for a world after polio eradication, extending the polio surveillance infrastructure and integrating surveillance across vaccine-preventable diseases has been proposed (*38*). World Health Organization reference laboratory networks for vaccine-preventable diseases already support serologic testing for measles, rubella, yellow fever, and Japanese encephalitis and have the technical capacity to support high-throughput serologic assays. Additional financial support that builds from this existing laboratory infrastructure could reinforce investments that are already in place and extend the serologic testing platform beyond vaccine-preventable diseases. In an analogous example, the World Health Organization's Global Rotavirus Laboratory Network tests fecal specimens for the presence of >20 enteric pathogens other than rotavirus using multiplex molecular assays (*38*). For data analysis and synthesis, the Institute for Health Metrics and Evaluation's Local Burden of Disease Project provides an example of how coordinated financing can be used to

aggregate, analyze, and disseminate information through sophisticated data pipelines to support diverse precision public health efforts (http://www.healthdata.org/lbd).

Conclusions

Integrated serologic surveillance is a concept with enormous potential. If deployed at scale through existing surveys and integrated into existing data pipelines, multiplex antibody testing will enable the global community to more quickly identify and respond to public health gaps, including undervaccination, emerging infectious diseases, persistent areas of high transmission, and recrudescence of NTDs or malaria in elimination settings. The key elements for building an integrated serosurveillance platform are within reach, and in our view, it is time to move beyond proof-of-concept studies toward a systematic, integrated platform.

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Effects of Sexual Network Connectivity and Antimicrobial Drug Use on Antimicrobial Resistance in *Neisseria gonorrhoeae*

Chris R. Kenyon, Ilan S. Schwartz

Contemporary strategies to curtail the emergence of antimicrobial resistance in *Neisseria gonorrhoeae* include screening for and treating asymptomatic infections in high-prevalence populations in whom antimicrobial drug–resistant infections have typically emerged. We argue that antimicrobial resistance in these groups is driven by a combination of dense sexual network connectivity and antimicrobial drug exposure (for example, through screen-and-treat strategies for asymptomatic *N. gonorrhoeae* infection). Sexual network connectivity sustains a high-equilibrium prevalence of *N. gonorrhoeae* and increases likelihood of reinfection, whereas antimicrobial drug exposure results in selection pressure for reinfecting *N. gonorrhoeae* strains to acquire antimicrobial resistance genes from commensal pharyngeal or rectal flora. We propose study designs to test this hypothesis.

The rapid emergence of antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* has led to fears that gonorrhea may soon become untreatable (*1*). An incompletely explained feature of the emergence of AMR in *N. gonorrhoeae* is its repeated emergence in core groups (*2*). As Lewis noted, AMR first emerged in core groups of sex workers in East Asia and elsewhere from the 1960s onward (*2*). In the past 3 decades, however, AMR has repeatedly emerged in men who have sex with men (MSM) (*2*,*3*). In both the United States and the United Kingdom, *N. gonorrhoeae* resistant to several antimicrobial drugs emerged in MSM years ahead of men who have sex with women (MSW) (Figure 1).

Several explanations have been proposed for this observation (*4*). Excess use of antimicrobial drugs is a possibility. One study found that MSM with a diagnosis of gonorrhea were more likely to report recent antimicrobial drug use than were MSW. After controlling for antimicrobial drug use, however, MSM remained at significantly higher risk for *N. gonorrhoeae* with resistance to all classes of antimicrobial drugs tested ($p<0.001$ for all) (*3*). Higher prevalence of HIV, which has been associated with various types of AMR in some studies, could also cause elevated resistance rates in MSM groups (*5*).

We focus on the emergence of *N. gonorrhoeae* AMR in MSM and hypothesize that the combination of high sexual network connectivity and excess antimicrobial drug use plays an important role in AMR genesis. This connectivity–AMR hypothesis proceeds in 2 steps: first, that high-equilibrium prevalence of *N. gonorrhoeae* in contemporary MSM populations is a function of densely connected sexual networks; and second, that extensive antimicrobial drug use (such as with STI screening and treatment) may temporarily reduce *N. gonorrhoeae* prevalence in this setting but produce selection pressure for *N. gonorrhoeae* to acquire AMR.

STI Prevalence as a Function of Network Connectivity

STIs are transmitted along sexual networks, and as a result, the equilibrium prevalences of these STIs are determined by structural characteristics of these networks (*6*,*7*). These characteristics include the number of partners per unit time, prevalence of concurrent partnering, size of core groups, type of sex, size of sexual network, length of gaps between partnerships, degree and type of homophily (preference for partners with similarities to oneself), and relationships between core and noncore groups (*7*). Combinations of these attributes should result in higher network connectivity in some populations than in others (*6*,*7*). Studies have found a correlation between markers of network connectivity and the prevalence of various major STIs (*7*), including *N. gonorrhoeae* (*8*,*9*). STI prevalence can also be influenced by other risk factors that can affect the probability of transmission per contact (such as male circumcision, condom use, and presence of other STIs) or the duration of infectivity (such as STI early detection and

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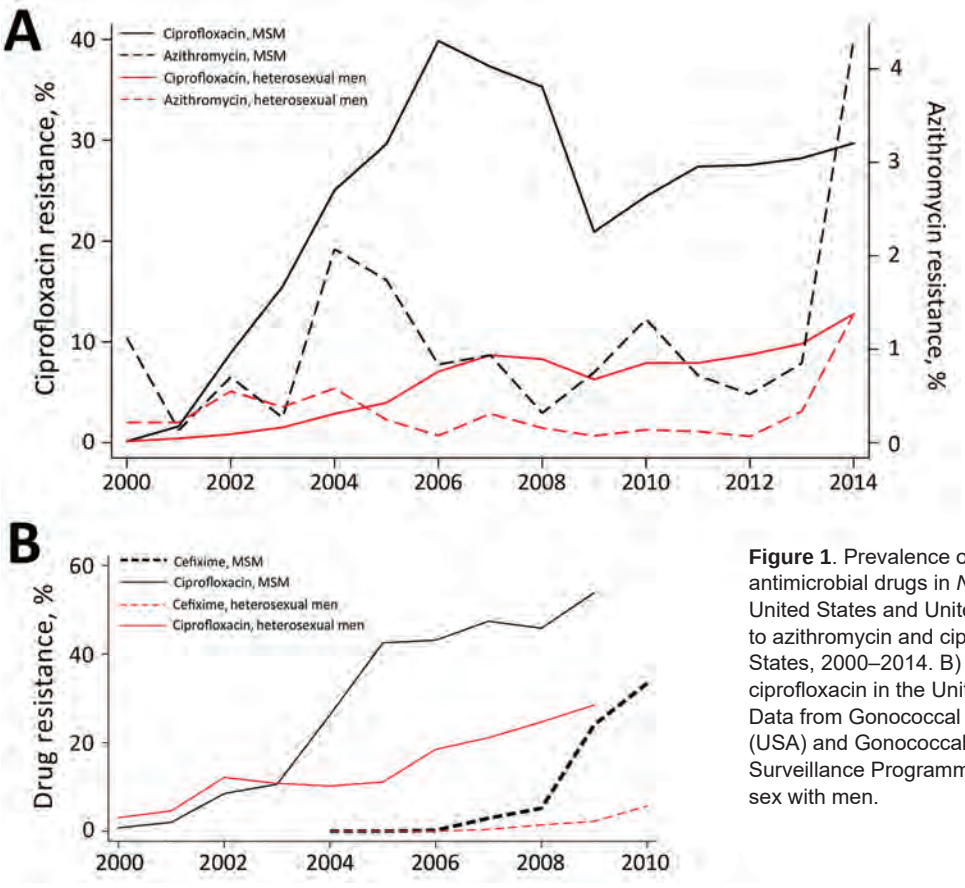

Figure 1. Prevalence of resistance to major antimicrobial drugs in *Neisseria gonorrhoeae*, United States and United Kingdom. A) Resistance to azithromycin and ciprofloxacin in the United States, 2000–2014. B) Resistance to cefixime and ciprofloxacin in the United Kingdom, 2000–2010. Data from Gonococcal Isolate Surveillance Program (USA) and Gonococcal Resistance to Antimicrobials Surveillance Programme (UK). MSM, men who have sex with men.

treatment efficacy) (*10*). Although the relative contributions of these risk factors to STI prevalence vary considerably among populations, a consistent feature of contemporary STI epidemics in MSM populations in numerous countries is their association with dense sexual networks (*11*,*12*). For instance, nationally representative data from the United Kingdom, United States, and Australia reveal that MSM report considerably more sexual partners per unit of time than do heterosexual men (Table 1). An example of the prevalence of multiple partnering in MSM is given by >180,000 participants in the European Men Who Have Sex with Men Internet Survey (*13*); 67% of respondents reported a nonsteady partner in the past year, and 37.7% of respondents reported ≥10 partners in the past year. These high rates of partner change, combined with high rates of partner concurrency (*14*) and other determinants of network connectivity, translate into dense networks (*7*). Network connectivity is particularly dense in preexposure prophylaxis (PrEP) cohorts, in which the median number of sex partners typically exceeds 10 per 90 days. In the iPrEx study, for example, participants reported a mean of 18 partners (SD ± 35) in the preceding 90 days (*15*). The resulting dense network typically sustains equilibrium prevalences of both *N. gonorrhoeae* and *Chlamydia trachomatis* at >10% (*15*). In comparison, the prevalence of *N. gonorrhoeae* in the general heterosexual population in the United Kingdom is estimated at <0.1% and of *C. trachomatis* at 1.3% (*16*).

Connectivity–AMR Thesis—Combination of High Prevalence of Antimicrobial Use and Network Connectivity as Cause of Resistance

In the absence of an antimicrobial selection pressure, we would not expect a high prevalence of *N. gonorrhoeae* to lead to AMR (*17*,*18*). Under such pressure, however, *N. gonorrhoeae* has developed AMR to each antimicrobial therapy introduced to treat infections, often within as few as 3 years (*1*,*19*,*20*). This effect is similar to the rapid development of AMR observed in a range of other bacteria (*21*). Individual-level studies have also found recent antimicrobial drug use to be a risk factor for AMR in *N. gonorrhoeae* (*3*,*22*,*23*). We would thus expect higher rates of antimicrobial drug use to be a risk factor for the emergence of AMR. Four mechanisms have been proposed to explain this antimicrobial drug–induced selection of AMR in bacteria for which, as for *N. gonorrhoeae*, horizontal gene transfer is a major mechanism of AMR acquisition (Table 2) (*1*,*24*). We argue that high network connectivity coupled

Table 1. Number of partners of MSM and heterosexual men from Australia, the United States, and the United Kingdom*

Survey description	Sexual orientation of participants	Mean no. lifetime sex partners (95% CI or SD)	Median no. lifetime sex partners (IQR)	Mean (95% CI) or median no. recent sex partners†	Median no. recent sex partners (IQR)†
ASHR II‡	MSM	143.1 (95.7–190.6)	22 (7–100)	6.8 (5.1–8.5)	1 (1–10)
	Heterosexual men	17.9 (17.1–18.7)	8	1.4 (1.3–1.4)	1
NHANES§	MSM	26.9 (7.8)	22 (4–100)	NA	NA
	Heterosexual men	14.8 (1.6)	8 (3–20)	NA	NA
NATSAL II¶	MSM	NA	NA	24.1	4
	Heterosexual men	NA	NA	3.8	1

*ASHR II, Australian Study of Health and Relationships II; IQR, interquartile range; NA, not available; NATSAL, National Surveys of Sexual Attitudes and Lifestyles (United Kingdom); NHANES, National Health and Nutrition Examination Survey (United States).
†For ASHR and NHANES, recent refers to the previous 12 months; for NATSAL II, recent refers to the previous 5 years.
‡ASHR II is a nationally representative sample of adults 16–59 y in Australia. Data were collected during 2012–2013 (n = 20,094).
§NHANES is a nationally representative sample of civilian, noninstitutionalized adults 18–69 y in the United States. Data were collected during 2009–2012 (n = 13,374).
¶NATSAL is a national probability sample of adults 16–44 y in the United Kingdom. Data were collected during 2000 (n = 11,161).

with antimicrobial exposure constitutes an emergent fifth pathway to AMR in *N. gonorrhoeae*.

Figure 2 is a schematic representation of a PrEP MSM cohort with a dense sexual network and quarterly *N. gonorrhoeae* and *C. trachomatis* screening. In the absence of a global screen-and-treat strategy that leads to *N. gonorrhoeae* extinction, a typical local screen-and-treat approach induces a temporary decline in *N. gonorrhoeae* prevalence. Without altering the underlying determinant of high *N. gonorrhoeae* prevalence (network connectivity), *N. gonorrhoeae* tends to return to its high-equilibrium prevalence. Moreover, this strategy also increases the prevalence of the genes that encode AMR in *N. gonorrhoeae*. On the basis of studies using macrolides for other indications, ≈90% of patients treated with ceftriaxone and azithromycin, the currently recommended therapy for *N. gonorrhoeae* infection, would be expected to acquire macrolide resistance that can persist for up to 4 years in commensal pharyngeal and colonic bacteria (*25*,*26*). Recently treated patients are also at high risk for early reinfection because it is unlikely that their whole local sexual network has been effectively screened via partner tracing (*27*).

N. gonorrhoeae has a highly developed system of transformation to take up DNA from its environment, particularly from other *Neisseria* spp. (*28*), which, along with other mechanisms, may lead to AMR acquisition (*1*). Studies have established that transformation is a method by which *N. gonorrhoeae* acquired resistance to cefixime from commensal pharyngeal *Neisseria* species (*19*,*24*).

Further examples of the connectivity–AMR mechanism come from various branches of high-density animal husbandry in which antimicrobial drug–based strategies used alone to combat epidemics have led to the induction of AMR (*29*). Norwegian salmon farms, for example, contain roughly 200,000 salmon per pen (with population densities of $\leq$25 kg/m^3) and consequently are prone to outbreaks of various bacterial, viral, and parasitic diseases (*29*,*30*). Initially, these epizootic infections were controlled predominantly with prophylactic and therapeutic antimicrobial drugs, but the bacterial and ectodermal pathogens rapidly developed resistance (*29*). Consequently, zoo sanitation (increased separation and fallowing of the fish) and vaccination were introduced, which allowed a decrease of antimicrobials used from 48 tons to 1 ton annually while reducing the number and severity of outbreaks and increasing the total salmon harvest (*29*). Studies from other sites have linked declines in antimicrobial drug use to declines in AMR in salmon-associated infections (*31*).

Table 2. Four mechanisms whereby antimicrobial usage might select for antimicrobial resistance in *Neisseria gonorrhoeae* in a population

Mechanism	Description
Emergence of resistance during treatment	A large proportion of *N. gonorrhoeae* infections, particularly in MSM, are asymptomatic colonization of the pharynx, where the penetration of many antimicrobials is relatively poor. Because of this or other reasons for suboptimal therapy, a subpopulation of antimicrobial-resistant *N. gonorrhoeae* may emerge from treatment and may subsequently be transmitted to others.
Reduced transmission of susceptible strains	Treating patients with antimicrobial-sensitive *N. gonorrhoeae* reduces the probability of transmission to others, which in turn increases the probability that others will become infected with resistant *N. gonorrhoeae* strains.
Increased susceptibility to colonization	Eradicating a susceptible *N. gonorrhoeae* strain with treatment may enable infection by a new, resistant *N. gonorrhoeae* strain previously excluded through bacterial competition. This is possible mainly in high-transmission settings.
Increased density of resistant bacteria following treatment	If a person is infected with an antimicrobial-resistant *N. gonorrhoeae* strain, treatment may eradicate susceptible competing commensal microbes. Relieved of competition, the resistant *N. gonorrhoeae* strain could expand in the vacated niche.

*Based on Lipsitch et al. (*18*).

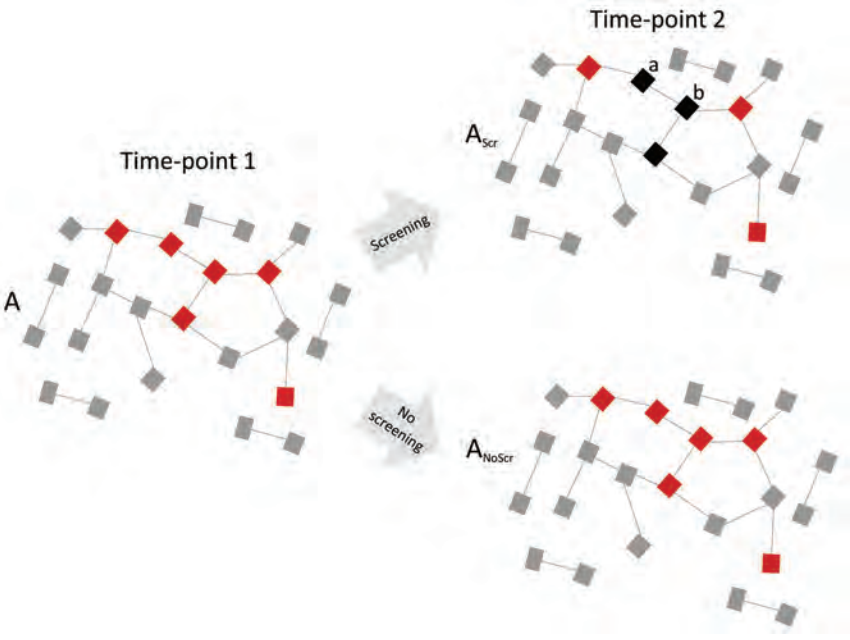

Figure 2. Diagram showing how high network connectivity combined with excess antimicrobial drug exposure from *Neisseria gonorrhoeae* preexposure prophylaxis could produce antimicrobial resistance (AMR). A dense sexual network translates into a high-equilibrium prevalence of *N. gonorrhoeae* (red squares) at time point 1. Active *N. gonorrhoeae* screening of 50% of this population every 3 months results in 50% lower *N. gonorrhoeae* prevalence at time-point 2 (3 months later) but at the expense of an altered resistome (A_{Scr}; black squares represent 3 patients with *N. gonorrhoeae* cleared by screening and treatment). The unchanged underlying network connectivity means that the prevalence of antimicrobial-sensitive *N. gonorrhoeae* is now 50% of its equilibrium prevalence, but if it acquired AMR it could return to its equilibrium prevalence. Furthermore, recently cured patients (a and b) are at high risk for reinfection from their partners at a time when their resistomes are enriched with resistance genes. Early reinfecting *N. gonorrhoeae* can acquire AMR by taking up these resistance genes by transformation. The screening program has thus both placed a selection pressure for the emergence of AMR and provided the resistance genes needed for AMR. In the absence of screening and excess antimicrobial drug use (A_{NoScr}), *N. gonorrhoeae* prevalence would not decline, but there would be no pressure to select for antimicrobial resistance. Gray squares indicate uninfected persons; lines represent sexual relationships.

We acknowledge that there is conflicting evidence of whether an excess use of antimicrobial drugs results in AMR. A recent ecologic analysis from the United States, for example, found no association between antimicrobial prescribing and gonococcal AMR in 23 STI clinics (*32*).

Effects of *N. gonorrhoeae* Screening on AMR

Theoretically, a sufficiently intense and synchronized global screen-and-treat program could lead to the extinction of *N. gonorrhoeae*. However, if the screening program falls short of complete eradication, and if a combination of high network connectivity and antimicrobial drug exposure is responsible for AMR, then paradoxically the more effective the screening program is at decreasing prevalence, the greater this AMR selection pressure would be. This conclusion is at odds with current initiatives to enhance *N. gonorrhoeae* screening in MSM and other high–*N. gonorrhoeae* prevalence populations. Screening for *N. gonorrhoeae* every 3–12 months is typically recommended in clinical guidelines for sexually active MSM (*33*). A notable exception to these guidelines is that from the US Preventive Service Task Force, which concluded that the absence of randomized controlled trials (RCTs) evaluating the merits of screening in men precluded recommendations on the matter (*34*). In MSM-PrEP programs, screening is recommended every 3–6 months (*35*). Longitudinal analyses of PrEP studies typically show high *N. gonorrhoeae* prevalences that do not decline despite frequent screening (*15*,*35*,*36*). A recent PrEP study found that the prevalence of *N. gonorrhoeae* remained static in the pharynx and rectum and increased in the urethra despite quarterly screening (*35*). Modeling studies have found that increasing screening intensity in MSM populations results in either a modest (*37*) or dramatic (*38*) reduction in *N. gonorrhoeae* prevalence.

Some authors have gone further and argued that screening is an important component for containing AMR emergence in *N. gonorrhoeae* (*2*,*20*). A recent paper on this topic for example outlined the argument as follows: "Gonococcal AMR will only be effectively mitigated when the global gonorrhea burden is reduced. Increased detection and effective treatment of asymptomatic gonorrhea in general and pharyngeal gonorrhea in particular are critical, because these infections are potential gonococcal reservoirs in which AMR (especially extended spectrum cephalosporin AMR) can emerge. Oropharyngeal infections are prevalent, mostly asymptomatic, and more difficult to treat; accordingly, screening and treatment in high-risk patients are important" (*20*). For similar reasons, the World Health Organization (WHO) has made the early detection and treatment of asymptomatic *N. gonorrhoeae* a key component of its plan to reduce the prevalence of *N. gonorrhoeae* infection by 90% by 2030 as well as *N. gonorrhoeae* AMR (*39*).

Future Evaluation of the Connectivity–AMR Hypothesis

Increasing screening of high-risk patients to combat AMR is diametrically opposed to our connectivity–AMR thesis. Given the stakes involved (including untreatable infections), establishing the validity of the connectivity–AMR hypothesis in general and the place of screening in high-prevalence populations specifically is imperative. Part of the answer lies in accurately describing the mechanisms underpinning AMR in *N. gonorrhoeae* compared with other organisms. In some pathogens, such as *Mycobacterium tuberculosis,* resistance emerges primarily through mutations during treatment in hosts (*18*). For these pathogens, screening and treating infected persons is crucial for containing the spread of AMR (*18*). For other bacteria, such as *Streptococcus pneumonia*, *Enterococcus* spp., *Staphylococcus aureus*, and *N. gonorrhoeae*, horizontal gene transfer is the predominant means of acquisition of AMR (*40*). For these bacteria, AMR is driven predominantly by indirect population-level mechanisms of selection (Table 2) (*18*). Although screening for these organisms may reduce prevalence, it may also increase AMR by these indirect mechanisms. Two types of study could assess the connectivity–AMR thesis and the net benefits and harms of *N. gonorrhoeae* screening programs in MSM: RCTs and modeling studies.

Community RCTs in high-connectivity populations, including MSM who are taking PrEP, could assess the effect of *N. gonorrhoeae* screening and treatment (vs. no screening and limiting therapy to patients with symptomatic *N. gonorrhoeae* infection) on several parameters: prevalence of *N. gonorrhoeae* infection; susceptibility to other STIs, including HIV; effect on adaptive immunity to *N. gonorrhoeae*; effect on individual and population resistome and microbiome; and emergence of AMR. Researchers could increase the probability of reducing *N. gonorrhoeae* prevalence in these studies by including aggressive contact tracing strategies, such as by using sexual networking apps. A practical challenge would be the large cohort size required to demonstrate a difference in the probability of AMR between the screening and no-screening arms of the study because AMR emergence is a rare event. Nonetheless, establishing whether *N. gonorrhoeae* screening reduces infection prevalence in dense networks and at what cost to the resistome (individual and population) would be informative. If screening is found to have little or no effect on *N. gonorrhoeae* prevalence but a large effect on the population resistome, it may cause a reevaluation of screening policies. Researchers could also assess the significance of altered resistomes to AMR in *N. gonorrhoeae* in vitro by assessing whether *N. gonorrhoeae* is able to acquire AMR via transformation with DNA extracts from posttreatment microbiomes. Such studies could also provide the probabilities of resistome alteration following specific therapies (including the decay curves of these alterations). These data could then be used to construct more realistic models of AMR induction in *N. gonorrhoeae* (*41*).

Recent modeling studies have found that screening high-connectivity MSM populations could reduce *N. gonorrhoeae* prevalence by ≈50% (*38*), but at the expense of an 11-fold increase in antimicrobial drug exposure (*37*). Few studies have evaluated the effect of screening on the emergence of AMR. One such study found evidence of a screening paradox: although screening the core group was crucial to reduce prevalence of *N. gonorrhoeae,* this strategy involved the highest risk of inducing AMR (*42*). However, that study used a compartmental model of the underlying sexual network and examined only 1 type of *N. gonorrhoeae* AMR, chromosomally mediated AMR (*42*). Future models that evaluate the probability of AMR emergence should use individual-based models that can model AMR via horizontal gene transfer. These models could assess if the combination of high connectivity and antimicrobial exposure is more likely to produce and disseminate AMR than is the combination of low connectivity and high antimicrobial exposure or of high connectivity and low antimicrobial exposure. Using models could also help establish the level of intensity required of screen-and-treat programs for highly connected sexual networks to reduce the prevalence of *N. gonorrhoeae* to a level with minimal risk for reinfection during the period when the resistomes of treated persons are altered. Our discussion has focused on MSM populations taking PrEP, but similar arguments would apply to other segments of the MSM sexual network, such as HIV-infected MSM who are excluded from PrEP programs and MSM without HIV infection who are not taking PrEP. Modeling studies could explore how differential network connectivity and antimicrobial drug exposure in different sections of MSM sexual networks may interact to produce AMR.

Allodemics of Resistance in MSM

The connectivity–AMR theory makes 2 other predictions regarding AMR in MSM. The first is that *N. gonorrhoeae* will become resistant to the full range of antimicrobial drugs to which the population is exposed. In accordance with the connectivity–AMR theory, any widely used antimicrobial drug that reduces *N. gonorrhoeae* prevalence in MSM populations is at risk for AMR. Although not all studies have reached the same conclusion, the data from the national *N. gonorrhoeae* surveillance projects in the United States and United Kingdom have generally found this to be true (*3*,*43*). In the United States, for example, MSM were statistically more likely to have AMR *N. gonorrhoeae* for all classes of antimicrobial drugs tested (*3*).

The second prediction is that AMR in other bacterial STIs will be likely to emerge or to become more prevalent

in MSM. Although the link is not as clearly established as with *N. gonorrhoeae*, there is some evidence that this is the case. For example, in the United States and Australia, macrolide resistance in *Treponema pallidum* first emerged in predominantly MSM populations at roughly the same time as azithromycin resistance in *N. gonorrhoeae* (*44*,*45*). In addition, the prevalence of macrolide resistance in *Mycoplasma genitalium* in MSM in 1 Australia study was found to be approximately double that of heterosexual men (*46*). Outbreaks have occurred in MSM of macrolide- or quinolone-resistant sexually transmissible enteric organisms *Shigella* spp. (*47*) and *Campylobacter* spp. (*48*). One phylogenetic analysis of *Shigella flexneri* infections from 29 countries concluded that the 3a serotype had emerged and acquired multiple AMR mutations while circulating sexually in international MSM sexual networks characterized by high rates of reinfection with this same serotype (*47*). Outbreaks of sexually transmitted methicillin-resistant *Staphylococcus aureus* have also been described in MSM (*49*).

The variations in population shifts of *N. gonorrhoeae* MICs to various antimicrobial drugs by sexual orientation are also compatible with the connectivity–AMR thesis. The earliest available data for *N. gonorrhoeae* sensitivity by sexual orientation from the United Kingdom reveal that MSM have a higher proportion of *N. gonorrhoeae* with high MICs than women do (Figure 3). Furthermore, the evolution of *N. gonorrhoeae* MIC distributions in MSM from 2010–2015 reveals a right shifting of the whole distribution curve, indicating a reduction in the proportion of MSM with low MICs (Figure 4).

Considered together, these findings support the hypothesis that the problem of AMR in MSM could constructively be viewed as an allodemic of AMR. Baquero et al. first introduced this term to describe how the spread of extended spectrum β lactamase (ESBL)–producing bacteria in a hospital in Spain was best described by an increase in ESBL production in a range of bacteria (an allodemic) rather than epidemics of single species or clones (*50*). They argued that appreciating this polyclonal spread of resistance as an allodemic enabled them to address the underlying environmental determinant of AMR: excess use of antimicrobial drugs that induce ESBL production in multiple bacteria species rather than traditional approaches targeting individual clones or species (*50*). The problem of polyclonal AMR in MSM may likewise benefit from efforts to address the underpinning environmental determinants.

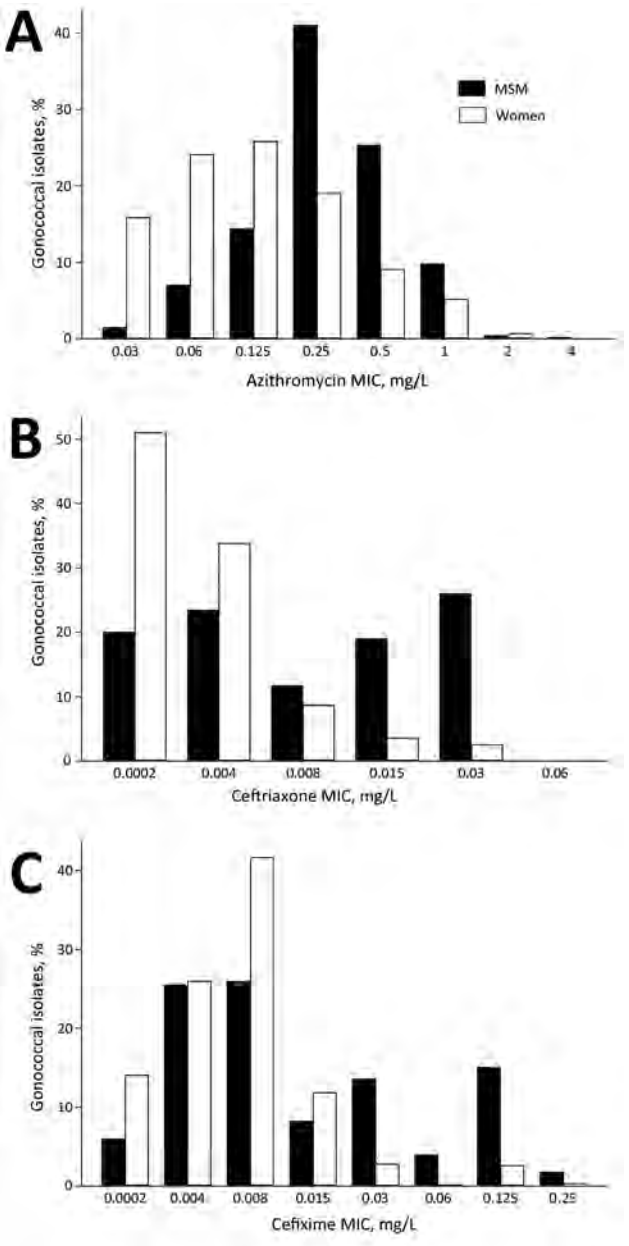

Figure 3. Comparison of distribution of drug MICs for *Neisseria gonorrhoeae* isolates from MSM and from women as determined by surveillance reports from the United Kingdom. A) Azithromycin, 2015; B) ceftriaxone, 2010; C) cefixime, 2011. Data from the Gonococcal Resistance to Antimicrobials Surveillance Programme. MSM, men who have sex with men.

Conclusions

Although we have focused our discussion on the connectivity–AMR thesis in MSM, similar considerations would also apply to other high connectivity populations. The emergence of *N. gonorrhoeae* AMR in sex workers, for example, has been linked to extensive antimicrobial drug use (*2*). Various studies have also concluded that high rates of STIs in various populations in sub-Saharan Africa are underpinned by dense sexual networks (*7*). In keeping with WHO directives, interventions are being planned to detect and treat asymptomatic STIs in South Africa and elsewhere. If the connectivity–AMR thesis applies to these populations, then due caution should be exercised if screening and antimicrobial drug use are used to reduce STI prevalence.

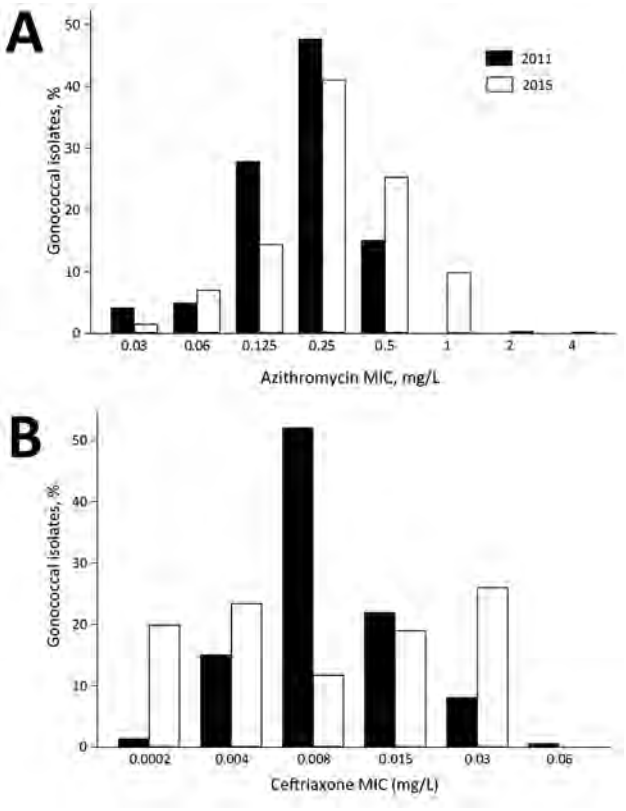

Figure 4. Comparison of distribution of drug MICs for *Neisseria gonorrhoeae* isolates by year as determined by surveillance reports from the United Kingdom. A) Azithromycin, 2011 and 2015; B) ceftriaxone, 2010 and 2015. Data from Gonococcal Resistance to Antimicrobials Surveillance Programme.

In high-connectivity populations, particular consideration should be given to the use of nonantimicrobial STI therapies such as local disinfectants (e.g., for pharyngeal STIs), bacteriophage therapy, and vaccines. If antimicrobial drugs are used, research is required to guide their selection on the basis of efficacy and resistogenicity of therapies. Genotypic resistance profiling before therapy could also be considered. If STI prevention and control programs are unable to attain the level of screen-and-treat coverage required to eradicate STIs (or make negligible the risk for reinfection during the period of posttreatment resistome alteration), then they should prioritize STI reduction strategies that minimize the risk for AMR selection. These strategies would include methods to fragment sexual network connectivity (e.g., through decreasing rates of partner change) and treat STIs with nonantimicrobial therapies (e.g., bacteriophages and antiseptics).

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Large Outbreaks of Fungal and Bacterial Bloodstream Infections in a Neonatal Unit, South Africa, 2012–2016

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Release date: June 14, 2018; Expiration date: June 14, 2019

Learning Objectives

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

- Describe the background of *Candida krusei* infection among neonates
- Assess the clinical picture and prognosis of *C. krusei* candidemia among neonates
- Distinguish risk factors for *C. krusei* candidemia among neonates
- Analyze results of an environmental study in the neonatal intensive care unit (NICU) to find the source of *C. krusei*

CME Editor

Jean Michaels Jones, BSN, Technical Writer/Editor, Emerging Infectious Diseases. *Disclosure: Jean Michaels Jones has disclosed no relevant financial relationships.*

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Candidemia is a major cause of healthcare-associated infections. We describe a large outbreak of *Candida krusei* bloodstream infections among infants in Gauteng Province, South Africa, during a 4-month period; a series of candidemia and bacteremia outbreaks in the neonatal unit followed. We detected cases by using enhanced laboratory surveillance and audited hospital wards by environmental sampling and epidemiologic studies. During July–October 2014, among 589 patients, 48 unique cases of *C. krusei* candidemia occurred (8.2% incidence). Risk factors for candidemia on multivariable analyses were necrotizing enterocolitis, birthweight <1,500 g, receipt of parenteral nutrition, and receipt of blood transfusion. Despite initial interventions, outbreaks of bloodstream infection caused by *C. krusei*, rarer fungal species, and bacterial pathogens continued in the neonatal unit through July 29, 2016. Multiple factors contributed to these outbreaks; the most functional response is to fortify infection prevention and control.

On August 4, 2014, the National Institute for Communicable Diseases (NICD) received a report of 11 neonates infected with candidemia from a university-affiliated hospital in Gauteng Province, South Africa. A large outbreak of candidemia caused by *Candida krusei* ensued over 4 months in the neonatal unit. We investigated to identify the possible source and mode of transmission of the outbreak, to identify risk factors for the development of candidemia, and to recommend control measures. After this outbreak, and despite the initial interventions, a series of ≥4 outbreaks of bacterial and fungal bloodstream infections (BSI) lasting until July 29, 2016, occurred. We investigated the first outbreak extensively; we report the results of this and subsequent investigations.

Candidemia may result in substantial long-term illness among hospitalized premature neonates, and reported crude mortality rates are 32%–46% (*1*–*3*). In a recent point prevalence survey among hospitalized adults and children in the United States, *Candida* was the leading pathogen causing BSI (*4*). *C. krusei*, a less common cause of BSI, is intrinsically resistant to fluconazole, a first-line antifungal agent (*5*).

Known risk factors for candidemia among neonates include very low birthweight (VLBW), prematurity, central venous catheter use, necrotizing enterocolitis (NEC), total parenteral nutrition (TPN), and prior or prolonged broad-spectrum antibacterial drug use, among others (*1*,*2*,*6*–*10*). Worldwide, outbreaks of candidemia in neonatal intensive care units (NICUs) are often caused by *C. parapsilosis* and associated with suboptimal adherence to infection prevention and control practices (*5*,*11*–*13*). In South Africa, *C. parapsilosis* is the most common *Candida* species among neonates; 2% of candidemia case-patients among all age groups test positive for *C. krusei* (*14*).

Methods

Outbreak Setting

Hospital A is a 1,500-bed public-sector hospital in a semi-urban area of South Africa that serves as a referral center for 9 hospitals in 3 provinces in the region. The metropolitan area had a population of ≈3.1 million in 2014 (*15*). The infant mortality rate was estimated at 19.3/1,000 live births in Gauteng in 2014 (*16*), and the antenatal HIV prevalence was 28% (*17*).

The neonatal unit at hospital A has 55 beds, comprising 14 intensive-care beds, 20 high-care beds, and a nursery area that has 15 cots and 6 beds for surgical patients. The ward is largely of open-plan design: it has areas not fully separated by floor-to-ceiling divisions. An average of 154 patients are admitted to the unit every month. The unit is often overcrowded, and infants share cots when capacity is exceeded. Fluconazole is not routinely used as prophylaxis but was used as first-line treatment for suspected or confirmed fungemia and other invasive fungal infections before this outbreak. Amphotericin B deoxycholate was the other systemic antifungal agent available for therapeutic use; penicillin G and amikacin were used as empiric therapy for suspected bacteremia. The unit protocol requires that blood culture samples be collected for every admitted neonate at birth and for all infants in whom sepsis is suspected. A confirmatory blood culture specimen is completed before appropriate treatment is initiated. All specimens are referred to an onsite hospital laboratory with a full microbiology service.

First Outbreak

Case Definition

For the outbreak investigation, we defined a case-patient as any neonate admitted to the neonatal unit during July 1–October 31, 2014, whose blood sample was positive for *C. krusei*. Any specimen positive for *C. krusei* from the same patient within 30 days of the first positive specimen was considered to be part of a single case. We defined a neonate as an infant ≤28 days of age; however, infants remaining in the unit or whose sample tested positive for candidemia beyond the 28th day of life were also included in this investigation.

Baseline Data Extraction, Confirmation of the Outbreak, and Identification of Cases

We extracted data for all cases of laboratory-confirmed candidemia during January 2012–December 2013 from the National Health Laboratory Service (NHLS) Corporate Data Warehouse (CDW), which archives demographic and laboratory data from patients whose diagnostic laboratory tests are performed by any NHLS laboratory. NICD began conducting active, laboratory-based surveillance for

candidemia at enhanced surveillance sites in South Africa in 2012. Hospital A became an enhanced site in January 2014, which meant that a nurse surveillance officer at the hospital collected clinical data on a standardized case report form (including age, gestational age, gender, birthweight, mode of delivery, feeding method, and HIV exposure status and outcome) and isolates were submitted to a reference laboratory at NICD. We extracted demographic, clinical, and laboratory data for cases of candidemia from January–June 2014 from the surveillance database. We used the C2-CUSUM method (*18*) to establish a baseline of expected cases, by *Candida* species, in the unit. We detected outbreak cases through ongoing surveillance.

Reference Laboratory Methods

We confirmed identification and susceptibility testing of bloodstream *Candida* isolates, as previously described, with modifications (*14*). This included the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics, Bremen, Germany) and sequencing of the internal transcribed spacer region of the ribosomal gene to confirm species-level identification. We did not genotype specimens.

Epidemiologic Studies

To determine risk factors for *C. krusei* candidemia, we conducted a retrospective cohort study. All neonates admitted for ≥72 hours to the neonatal unit during July 1–October 31, 2014, were included. We analyzed data from an existing ward database containing clinical data on all admitted patients and their mothers.

We collected and analyzed additional data (unavailable in the ward database) for a subset of patients by using a nested matched case–control design. We retrospectively reviewed patient and laboratory records for data pertaining to antibacterial and antifungal treatment, other medication administered (with emphasis on medication from multidose vials), intravenous fluids, TPN, blood transfusions, and laboratory parameters. Data for the presence, sites, and duration of insertion of peripheral and central venous catheters were not available. We selected 41 control-patients and 41 case-patients from the same neonatal unit who were admitted during a similar time period (±1 week) and matched by gender and birthweight (±500 g).

Statistical Analysis

By dividing the number of new cases by the total number of admissions to the neonatal unit during the 4-month outbreak period, we calculated the incidence of *C. krusei* and other fungal and bacterial BSI. Data on patient-days were not available.

We compared clinical and demographic characteristics of case-patients and non–case-patients in the cohort by using the Pearson χ^2 and Fisher exact tests or Student *t*-test and Wilcoxon rank-sum test, as appropriate. We evaluated exposure variables as risk factors for candidemia by univariate analysis. Variables with p values <0.2 were included in a multivariable logistic regression model. We used conditional logistic regression to determine additional risk factors for candidemia in matched case-control pairs. We conducted all statistical analyses in Stata version 13 (StataCorp LLC, College Park, TX, USA).

Infection Prevention and Control Interventions

Upon recognition of the outbreak, the hospital infection control department conducted a hand hygiene campaign, and infection prevention and control (IPC) was intensified. We recommended the use of amphotericin B as the empiric antifungal agent of choice, instead of fluconazole, for all neonates with suspected candidemia. We conducted 2 IPC audits (initial, December 2014, and follow-up, March 2015), to determine whether suboptimal practices had contributed to the outbreak and to encourage improvement in IPC. We describe details of the audits in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/7/17-1087-Techapp1.pdf).

We conducted 4 types of surveys during 2 IPC audit periods: during the first period, administration of IPC knowledge and perception questionnaires and targeted environmental sampling with submission of samples for fungal culture; and in both periods, a cross-sectional observational audit and observation of hand hygiene behavior. We sampled high-touch surfaces (such as procedure trolleys, intravenous fluid stands, computer monitor touchscreens and keyboards, and incubator door handles), fluids (such as TPN, a container of communal hand cream shared by staff, and a tube of water-based lubricant), contents of multidose vials (such as heparin), staff member hands, and stethoscopes.

Subsequent Outbreaks

Ongoing surveillance identified ≥4 subsequent outbreaks. We performed a 1-time retrospective audit of the NHLS CDW for 2014–2015 for 3 common bacterial pathogens: *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter cloacae*. We compared these data with candidemia surveillance data (beginning on January 1, 2014, and ending on December 31, 2016). Results are shown in the online Technical Appendix Figure.

Ethics

NICD acquired approval for retrospective data collection for surveillance purposes and outbreak investigation activities from the Human Research Ethics Committee (Medical) of the University of Witwatersrand (reference numbers M140159 and M160667). In addition, an epidemiologic study protocol was approved (M1411112). We obtained permission to conduct the investigation from hospital A.

The hospital Department of Paediatrics and Child Health granted permission for secondary data use.

Results

First Outbreak

In a 5-year period (January 2012–December 2016) before, during, and after the first outbreak, 262 cases of candidemia (caused by numerous *Candida* species) were detected in the neonatal unit at hospital A (Figure 1). We identified 10 different species of *Candida*; the most common was *C. krusei* (91/262; 35% of cases), followed by *C. albicans* (75/262; 29%) and *C. parapsilosis* (41/388; 16%). Cases of candidemia caused by *C. albicans* were diagnosed continually through the 5-year period; other species were identified intermittently. Before onset of the outbreak in July 2014, a single case of *C. krusei* candidemia was recorded in October 2012. During July–October 2014, of 589 neonatal admissions, 48 cases of *C. krusei* candidemia occurred, an incidence of 8.2/100 admissions. During July (n = 14), August (n = 18), and September 2014 (n = 11), *C. krusei* was the only *Candida* species detected from blood cultures in the neonatal unit. This represented a total species replacement and was above the expected baseline of 0 cases for the unit.

The *C. krusei* index case sample was collected on July 5, 2014. Overlapping collection dates suggested a propagated outbreak with horizontal transmission of *C. krusei* among case-patients (Figure 2). The last outbreak case was confirmed from a sample collected on October 20, 2014. In samples from 48 case-patients, C. krusei was isolated >1 time in 29 (60%) case-patients (mean 2.5 positive isolates/case-patient). All 118 C. krusei isolates had amphotericin B MICs<2 μg/mL.

Characteristics of Outbreak Case-Patients

Among the cohort of 589 infants admitted to the neonatal unit during the 4-month outbreak period, the mean gestational age of infants with *C. krusei* candidemia (33 wk) was lower than that of infants whose samples tested negative (35 wk; p<0.001) (Table 1). Mean birthweight was also lower among positive (1,356 g) than negative (2,300 g) infants (p<0.001). Among case-patients, 26 infants (54%) had a very low birthweight and 8 infants had an extremely low birthweight (<1,000 g). Median chronological age at onset of candidemia was 13 days (interquartile range [IQR] 7.5–17.5 days). Of 35 case-patients for whom HIV exposure status data were available, 16 (46%) had antenatal exposure to HIV; not all infants who were treated for candidemia had been tested for HIV at birth. Infants in whom candidemia was diagnosed had a longer duration of hospitalization (median 39 days, IQR 25–55 days) than did infants who tested negative (median 7 days, IQR 1–17 days; p<0.001). Of 48 infants who tested positive for candidemia, 7 died (crude case-fatality ratio 15%), compared

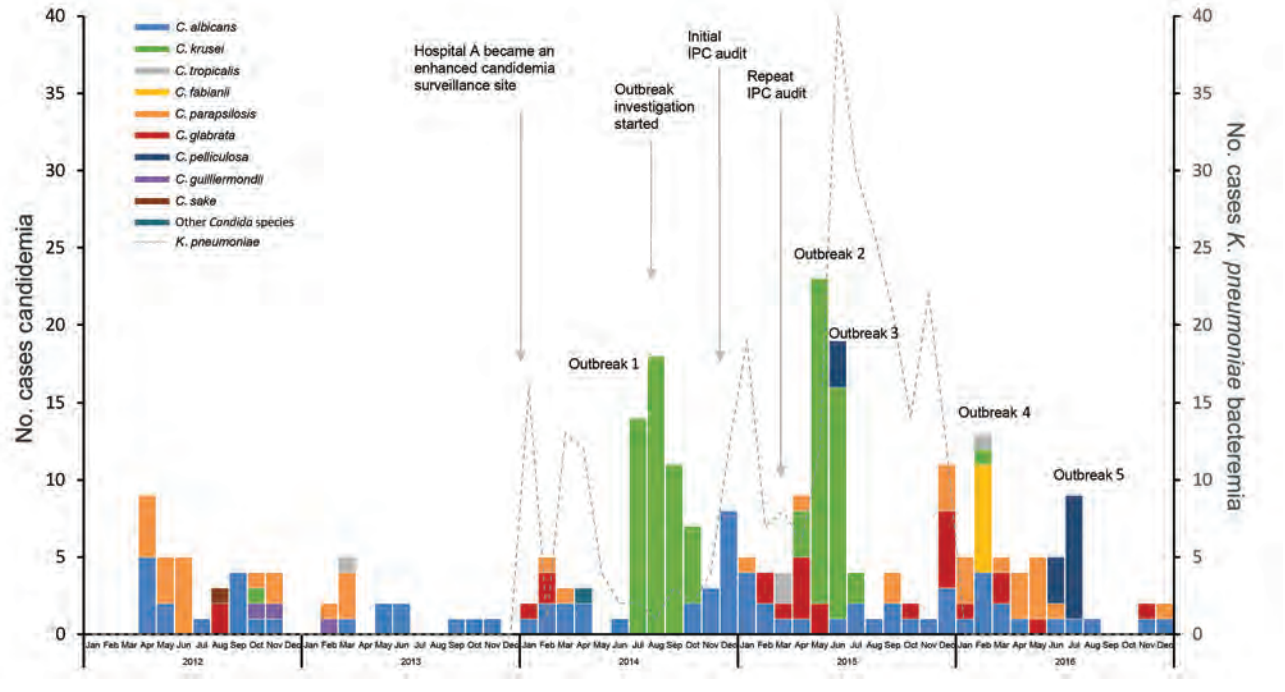

Figure 1. Cases of candidemia (n = 262), by *Candida* species, and bacteremia caused by *Klebsiella pneumoniae* (n = 298) in the neonatal unit at hospital A, Gauteng, South Africa, January 2012–December 2016. Individual outbreaks caused by the following *Candida* species: outbreak 1, *C. krusei*; outbreak 2, *C. krusei*; outbreak 3, *C. pelliculosa*, outbreak 4, *C. fabianii*; outbreak 5, *C. pelliculosa*. Specific points during the outbreak investigation are labeled. IPC, infection prevention and control.

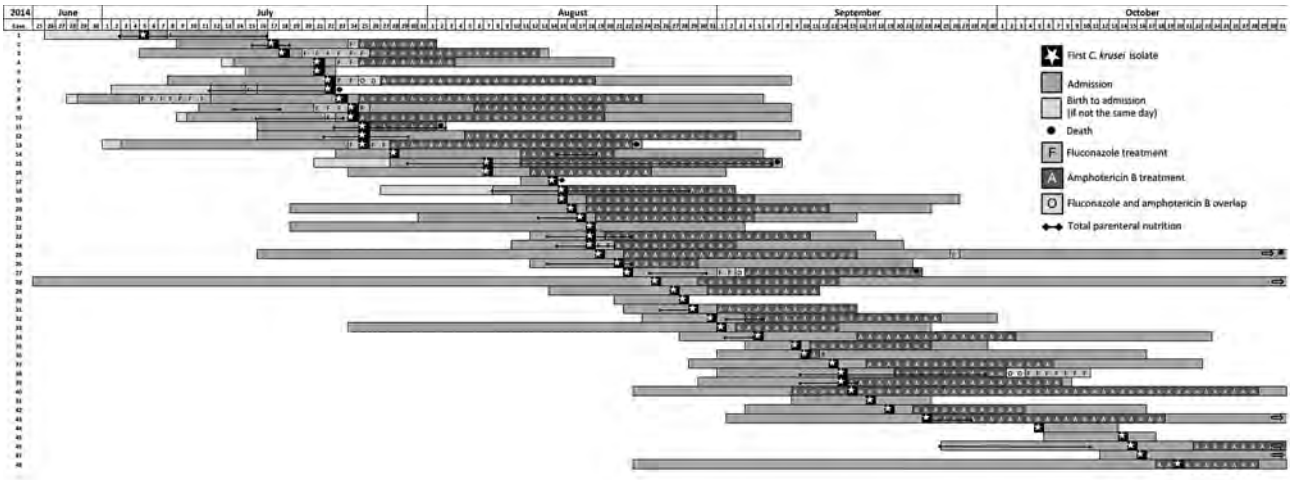

Figure 2. Gantt chart illustrating the timeline of an outbreak of 48 cases of *Candida krusei* bloodstream infection among neonates admitted to the neonatal unit at hospital A, Gauteng, South Africa, July 1–October 31, 2014.

to 62 of 538 infants who tested negative (crude case-fatality ratio 12%) (p = 0.5).

Risk Factors for C. krusei Candidemia

After adjustment for possible confounders in the multivariable regression model, infants diagnosed with NEC were 3 times more likely to develop candidemia than those who tested negative (adjusted odds ratio [aOR] 3.1, 95% CI 1.4–6.7) (Table 2). Neonates weighing 1,000–1,500 g at birth were 6 times more likely to have candidemia than those who had a birthweight >2,500 g (aOR 6.1, 95% CI 2.1–17.2). Infants who had extremely low birthweight also had a higher risk for candidemia (aOR 6.5, 95% CI 1.9–21.6). In addition, having been admitted to the unit during July and August was associated with positive test results for candidemia (Table 2).

Case-patients and controls received a median of 3 (IQR 2–5) antibacterial drugs during their entire hospital stay. During the first 13 days after admission (a censored time-point corresponding to the median age of onset of candidemia), case-patients received a median of 3 (IQR 2–3) antibacterial drugs, whereas controls received a median of 2 (IQR 0–3) antibacterial drugs (p = 0.001). Of 41 case-patients, 37 (90%) received courses of antifungal therapy; 6 of these occurred before the first positive culture of *C. krusei* (fluconazole, n = 4; amphotericin B, n = 2). Of the 4 case-patients who received fluconazole, 3 were subsequently given amphotericin B, and 1 case-patient

Table 1. Characteristics of a cohort of 589 neonates, with and without *Candida krusei* candidemia, admitted to the neonatal unit at hospital A, Gauteng, South Africa, July 2014–October 2014*

Patient characteristics	*C. krusei* candidemia, n = 48	No *C. krusei* candidemia, n = 541	Total	p value
Sex				
M	28/48 (58.3)	309/539 (57.3)	337/587 (57.4)	0.878
F	20/48 (41.7)	230/539 (42.7)	250/587 (42.6)	
Median chronological age at onset of candidemia, d (IQR)	13 (7.5–17)	NA	NA	NA
Mean gestational age at birth, wk (±SD)	33 (±3.8)	35 (±4.1)	35 (±4.1)	**<0.001**
Median birthweight, g (IQR)	1,365 (1,130–1,970)	2,300 (1,635–3,070)	2,225 (1,580–3,030)	**<0.001**
Median length of hospital stay, d (IQR)	39 (25–55)	7 (1–17)	8 (2–20)	**<0.001**
Twin infants or triplets	4/48 (8.3)	54/541 (10)	58/589 (9.8)	1.000
Born in hospital A	42/46 (91.3)	490/541 (90.6)	532/587 (90.6)	1.000
Died	7/48 (14.6)	62/538 (11.5)	69/586 (11.8)	0.468
Received antibacterial drugs during hospital stay	40/41 (97.6)	28/41 (68.3)	68/82 (82.9)	**0.001**
Median no. (IQR) antibacterial drugs received in first 13 d	3 (2–3)	2 (0–3)	2 (0–3)	**0.001**
Received TPN during hospital stay	24/40 (60)	5/41 (12.2)	29/81 (35.8)	**<0.001**
Received ≥1 blood transfusion during hospital stay	38/41 (92.7)	18/41 (43.9)	56/82 (68.3)	**<0.001**

*Values are no. in category/total no. (%) except as indicated. Bold indicates statistically significant values. In the No *C. krusei* candidemia group, data were unavailable for the following variables: sex (n = 2), gestational age (n = 22), birthweight (n = 1), length of hospital stay (n = 3) and death (n = 3). In the *C. krusei* candidemia group, data was unavailable for the following variables: gestational age (n = 3), birthweight (n = 2), length of hospital stay (n = 4), place of birth (n = 2). Data for the following variables were only available for a subset of patients from the nested case-control study (cases: n = 41, controls: n = 41): antibacterial drugs during hospital stay, number of antibacterial drugs in first 13 d, TPN during hospital stay and blood transfusions. IQR, interquartile range; NA, not applicable; TPN, total parenteral nutrition.

Table 2. Univariate and multivariable logistic regression analysis of factors associated with candidemia caused by *Candida krusei* among infants admitted to the neonatal unit at hospital A, Gauteng, South Africa, July 1–October 31, 2014*

Characteristics	Candidemia positive, no. in category/total no. (%)	Univariate analysis		Multivariable analysis	
		OR (95% CI)	p value	aOR (95% CI)	p value
Sex					
M	27/336 (58.7)	Reference		Reference	
F	19/249 (41.3)	0.9 (0.5–1.8)	0.857	0.9 (0.4–1.7)	0.671
Gestational age at birth, wks†					
<28	3/27 (6.7)	3.1 (0.7–12.1)	0.111	ND	ND
28–31	18/99 (40.0)	5.4 (2.3–12.6)	**<0.001**	ND	ND
32–36	15/209 (33.3)	1.9 (0.8–4.5)	0.141	ND	ND
≥37	9/229 (20.0)	Reference		ND	ND
Birthweight, g					
<1,000	8/44 (17.4)	8.7 (2.8–26.7)	**<0.001**	6.5 (1.9–21.6)	**0.002**
1,000–1,499	16/87 (34.8)	8.9 (3.3–23.5)	**<0.001**	6.1 (2.1–17.2)	**0.001**
1,500–1,999	11/120 (23.9)	4.0 (1.4–11.1)	**0.008**	3.4 (1.1–10.0)	**0.023**
2,000–2,499	5/93 (10.9)	2.2 (0.6–7.6)	0.193	2.5 (0.7–8.8)	0.139
≥2,500	6/242 (13.0)	Reference		Reference	
Necrotizing enterocolitis					
No	31/521 (67.4)	Reference		Reference	
Yes	15/65 (32.6)	4.8 (2.3–9.4)	**<0.001**	3.1 (1.4–6.7)	**0.005**
HIV exposed					
No	19/314 (54.3)	Reference		ND	ND
Yes	16/178 (45.7)	1.5 (0.7–3.1)	0.226	ND	ND
Month admitted					
July	21/152 (45.7)	8.9 (2.6–30.6)	**<0.001**	9.3 (2.5–33.1)	**0.001**
August	15/128 (32.6)	7.4 (2.0–26.2)	**0.002**	8.6 (2.3–31.5)	**0.001**
September	7/137 (15.2)	3.0 (0.7–11.9)	0.117	3.5 (0.8–14.4)	0.080
October	3/170 (6.5)	Reference		ND	ND
Underlying conditions					
Respiratory					
No	9/195 (19.6)	Reference		ND	ND
Yes	37/390 (80.4)	2.2 (1.0–4.6)	0.043	ND	ND
Cardiovascular					
No	25/387 (58.1)	Reference		ND	ND
Yes	18/149 (41.9)	2.0 (1.0–3.8)	0.035	ND	ND
Jaundice				ND	ND
No	17/313 (37.0)	Reference		ND	ND
Yes	29/274 (63.0)	2.1 (1.0–3.9)	0.023	ND	ND
Mother's antenatal care					
None	13/76 (28.2)	Reference		ND	ND
1–5 visits	26/392 (56.5)	0.3 (0.1–0.8)	**0.004**	ND	ND
6–10 visits	7/116 (15.2)	0.3 (0.1–0.9)	**0.018**	ND	ND
>10 visits	0/3 (0)	1		ND	ND
Mother's educational level					
<Grade 10	7/134 (16.7)	Reference		ND	ND
≥Grade 10	35/426 (83.3)	1.6 (0.7–3.8)	0.255	ND	ND

*Bold typeface indicates statistically significant values. Variables with a p value of <0.2 in the univariate analysis were added to a multivariable model and only variables that remained in the final model are displayed in the last 2 columns. Sex was included in the multivariable model as an *a priori* confounder. OR, odds ratio; aOR, adjusted OR; NA, not applicable; ND, no data (variables were not included in the final multivariable model).
†World Health Organization classification.

received 1 dose of fluconazole as prophylaxis on the day of surgery, 7 days before she had positive *C. krusei* culture results.

Among 40 case-patients for whom nutritional source data were available, 24 received TPN during their hospital stay; 19 had started TPN before the first positive culture for *C. krusei* (median 4 days, IQR 3–7 days). Having received TPN at any point during hospitalization (aOR 14.1, 95% CI 1.3–143.6) and having received furosemide after blood transfusion (aOR 12.0, 95% CI 1.1–139.5) were associated with having candidemia. The number of antibacterial drugs received was not associated with candidemia in the regression model. We found no difference in median duration of TPN between cases (6 days, IQR 4–9.5 days) and controls (3 days, IQR 2–11 days) (p = 0.6).

Evaluation of IPC Interventions

At the time of the audit, conducted almost 2 months after the first outbreak ended, the patient census was 12% above the unit's capacity. General cleanliness and handwashing facilities were adequate, but ventilation was poor. Isolation facilities were inadequate. A period of interrupted municipal water supply reportedly occurred in June 2014. Staff hand hygiene compliance was 76% (72 actions performed/95 opportunities). Although we isolated other bacterial and fungal species from surfaces, solutions, and staff

hand samples, we were unable to find a source of *C. krusei* in the environment.

Subsequent Outbreaks

During April–July 2015, another large outbreak consisting of 41 identified cases of *C. krusei* candidemia occurred (Figure 1, outbreak 2). Three cases of candidemia caused by *Candida pelliculosa* were retrospectively identified; these cases occurred during the second *C. krusei* outbreak in June 2015 (Figure 1, outbreak 3). Because this species had not been identified in the neonatal unit before, this cluster constituted an outbreak. Similarly, in February 2016, 7 cases of candidemia caused by *Candida* (*Cyberlindnera*) *fabianii* were detected (Figure 1, outbreak 4). In June 2016, another 8 cases of candidemia caused by *C. pelliculosa* occurred (Figure 1, outbreak 5). During January 2014–December 2015, a total of 298 cases of *K. pneumoniae* bacteremia occurred in the neonatal unit, with an overall incidence of 8/100 admissions. We retrospectively identified ≥3 outbreaks of *K. pneumoniae* bacteremia that appeared to closely precede or follow outbreaks of candidemia.

Discussion

We have documented a large outbreak of *C. krusei* candidemia in a neonatal unit, reporting 48 cases occurring during 4 months. Candidemia-positive infants had a lower gestational age and birthweight than did infants negative for candidemia. NEC, birthweight <1500 g, administration of TPN, and blood transfusion were identified as risk factors. An environmental source of the outbreak was not identified, but infection was likely transmitted among infants by contact with healthcare workers and fomites.

Nosocomial outbreaks caused by other *Candida* species (mostly *C. parapsilosis*) have been reported in NICU settings in the United States, Mexico, Taiwan, and Brazil (*5*,*11*–*13*,*19*,*20*). However, none of these outbreaks was as large as the outbreak we describe. The root causes of an outbreak spanning a 4-month period are likely multifactorial; delayed recognition of the outbreak and a slow response in implementing control measures were probable contributing factors, as were broader issues such as interrupted water supply, structural problems of the building that precluded appropriate isolation of infected infants, and overcrowding in the unit. Suboptimal IPC practices, however, are usually a major contributing factor in outbreaks of this nature. In an outbreak of *C. parapsilosis* among 3 patients in a Mexico NICU, molecular testing confirmed that the hands of a healthcare worker were a source of infection (*12*). A point source from a bottle of intravenous multielectrolyte solution was identified in a 7-case outbreak of *C. krusei* fungemia in a NICU in India (*21*). Often, the sources of such outbreaks are not found.

Of neonates infected with *C. krusei* candidemia in this outbreak, >50% had very low birthweights and were born earlier than neonates who tested negative for candidemia. This finding is in agreement with other reports highlighting prematurity and low birthweight as well-recognized risk factors for candidemia (*2*,*3*,*7*). Host factors such as an immature immune system and a fragile skin barrier predispose neonates to invasive infection. Disruption of the intestinal lining, as seen in conditions like NEC, may also facilitate invasion of *Candida* into the bloodstream (*8*,*10*). We found a clear association between NEC and candidemia in this outbreak; however, we could not establish the order of onset. We used the modified Bell's staging criteria (*22*) to diagnose and stage NEC in this unit, but the date of onset of symptoms or diagnosis was not routinely recorded. Therefore, we were unable to determine whether NEC preceded candidemia.

Administration of TPN likely represents a critical event during which *Candida* entered the bloodstream, in addition to suboptimal adherence to IPC protocols. The possibility of contaminated TPN could not be ruled out, but is unlikely in view of the propagated nature of the outbreak. Previous studies have found a longer duration of TPN to be associated with an increased risk for candidemia in older patient populations, hypothesized to be associated with prolonged exposure to glucose and lipid-rich solution, and subsequent *Candida* biofilm formation (*23*,*24*). We did not, however, find such an association, possibly because of the low number of patients who received TPN.

It is standard practice in this neonatal unit to administer a dose of furosemide after blood is administered. As with TPN, blood transfusion is not a risk factor in itself but more likely indicates exposure to an invasive procedure or a breach in IPC.

Source of the Outbreak

Although the source of this outbreak could not be definitively established, overcrowding and suboptimal IPC practices likely contributed to transmission of *C. krusei* (online Technical Appendix). This assumption is supported by the overlap of collection dates for the first positive specimen, suggesting a propagated outbreak, as well as subsequent outbreaks of bacterial and fungal pathogens in the unit, for which similar findings were documented. *C. krusei* has been isolated from healthcare workers' hands, hospital surfaces, and medical devices in previous studies (*25*,*26*). Although *C. krusei* was not isolated from the environment in our investigation, propagation on hands or fomites was the probable mode of transmission in this outbreak.

Limitations

This outbreak investigation had several limitations. First, delayed recognition and initiation of a response limited the

team's ability to intervene in a timely manner. The outbreak response team had limited jurisdiction to become involved without appropriate permission from the hospital authorities; such permission to conduct an investigation was not obtained until October 2014. Second, our secondary analysis of routine ward clinical data was limited by the variables originally collected. Although we obtained additional data from patient and laboratory records, the retrospective nature of data collection meant that data were inevitably incomplete. Nonetheless, we were able to assess associations between well-established risk factors and candidemia in both epidemiologic studies. Third, because the investigation involved a closed population with a limited number of appropriately matched controls admitted to the unit during the outbreak period, the case–control study was statistically underpowered to detect true differences between the 2 groups. In addition, because identification of laboratory-confirmed cases is dependent on specimen collection practices and blood cultures have low sensitivity as a diagnostic test method, we may have misclassified potential case-patients as controls and therefore underestimated associations between risk factors and the development of candidemia. Fourth, we compiled the IPC audit after the outbreak was over, thereby reducing the probability of isolating the causative pathogen in the environment or identifying the source of the outbreak. Information on the exact location and relocation of infants within the ward was not available. We were also not able to assess staff allocations and determine which staff members were allocated to care of infants. Although we assessed the action of performing hand hygiene, we did not measure the effectiveness of those actions. We did not evaluate invasive procedures, such as administration of TPN or blood transfusions and practices around central or peripheral intravenous line maintenance.

Recommendations Made after the Outbreak Investigation

As a result of the outbreak investigation, we re-emphasized adherence to IPC protocols at all opportunities and made further detailed recommendations (online Technical Appendix). Active surveillance for candidemia has continued at this hospital. Although there were recurrent outbreaks, response has improved.

Conclusions

Multiple factors contributed to this outbreak of *C. krusei* candidemia and the series of subsequent outbreaks, the most critical being suboptimal adherence to IPC practices at the point of patient care. This investigation highlights the need for early detection and timely interventions in outbreaks of this nature. We did not attempt to report the resolution of a single outbreak, because contributing factors have been and are still present in this neonatal unit. Like many healthcare facilities in low- and middle-income countries, hospital infrastructure and maintenance, access to reliable water and sanitation services, and broader healthcare system and socioeconomic issues contribute to a scenario ripe for outbreaks of this magnitude to occur. A proactive approach to prevention of neonatal sepsis, with a focus on IPC and antimicrobial stewardship, is needed in this unit.

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Typhus Group Rickettsiosis, Germany, 2010–2017[1]

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Typhus group rickettsiosis is caused by the vectorborne bacteria *Rickettsia typhi* and *R. prowazekii*. *R. typhi*, which causes murine typhus, the less severe endemic form of typhus, is transmitted by fleas; *R. prowazekii*, which causes the severe epidemic form of typhus, is transmitted by body lice. To examine the immunology of human infection with typhus group rickettsiae, we retrospectively reviewed clinical signs and symptoms, laboratory changes, and travel destinations of 28 patients who had typhus group rickettsiosis diagnosed by the German Reference Center for Tropical Pathogens, Hamburg, Germany, during 2010–2017. Immunofluorescence assays of follow-up serum samples indicated simultaneous seroconversion of IgM, IgA, and IgG or concurrence in the first serum sample. Cytokine levels peaked during the second week of infection, coinciding with organ dysfunction and seroconversion. For 3 patients, *R. typhi* was detected by species-specific nested quantitative PCR. For all 28 patients, *R. typhi* was the most likely causative pathogen.

Typhus group rickettsiosis (TGR) is caused by *Rickettsia typhi* and *R. prowazekii*, 2 Biosafety Level 3 organisms of the family *Rickettsiaceae*, which comprises obligate intracellular gram-negative zoonotic bacteria. *R. typhi* is responsible for murine typhus, the endemic fleaborne form of typhus, which is emerging in predominantly tropical coastal regions. *R. prowazekii* is responsible for epidemic louseborne typhus in temperate and tropical regions. *R. prowazekii* is classified as a Centers for Disease Control and Prevention category B bioweapon pathogen.

The clinical picture of TGR caused by either pathogen is similar: fever, headache, and exanthema (*1*,*2*). Inoculation eschars, which are classically seen in patients with spotted fever group rickettsioses (SFGR), are usually absent in patients with TGR. Cardiac, pulmonary, and central nervous system (CNS) complications can occur during the course of infection (*1*,*2*). *R. typhi* infection is usually milder than *R. prowazekii* infection. Fatality rates among patients with untreated typhus are ≈4% (*3*,*4*) among patients with *R. typhi* infection and 13%–30% (*2*) among those with *R. prowazekii* infection.

To learn more about the immunology of human infection with typhus group rickettsiae, we retrospectively analyzed TGR cases diagnosed at the National Reference Center for Tropical Pathogens in Hamburg, Germany, during 2010–2017. We collected clinical data and analyzed patient serum. Antibody kinetics were determined from follow-up serum samples. Serum cytokine responses were measured by flow cytometry from all available serum samples. In addition, we used novel nested quantitative PCRs targeting the *prsA* genes of *R. typhi* and *R. prowazekii* on archived clinical material.

Patients, Materials, and Methods

Cases and Inclusion Criteria

We screened the database of the German Reference Center for Tropical Pathogens at the Bernhard Nocht Institute for Tropical Medicine in Hamburg for autochthonous and imported (travel- or migration-associated) TGR cases diagnosed from January 1, 2010, through December 31, 2017. Written general consent had been obtained from patients before the study. TGR cases were defined as a clinically compatible disease with ≥1 of the following laboratory test results: a positive PCR and sequencing result, seroconversion to TGR antigens in an indirect immunofluorescence assay (IFA), parallel TGR IgM and IgG detection in a single sample by IFA, or a single IFA IgG or total Ig titer of ≥320. In addition, antibodies against SFGR antigens, when available, had to be lower than TGR antigens in the IFA. Serologic testing results for leptospirosis (in-house ELISA), scrub typhus (in-house IFA), and dengue fever (in-house IFA) had to be negative.

Serologic Assays

We performed in-house TGR IFA by using *R. typhi* strain Wilmington and *R. prowazekii* strain Madrid E grown in

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L929 mouse fibroblast cell culture. IFA reference values for *R. typhi* and *R. prowazekii* were <40 (IgM), <20 (IgA), and <80 (IgG and total Ig). In parallel, we performed in-house SFGR IFA with *R. conorii* strain 7 (ATCC VR-613) by using the same culture conditions and with reference values of <20 (IgM and IgA) and <40 (IgG and total Ig). All reference values were determined with serum from 200 healthy Caucasian blood donors.

Molecular Assays

We performed panrickettsial real-time quantitative PCR (qPCR) targeting the *ompB* gene (*5*). A nested qPCR detecting specifically the *prsA* gene of *R. typhi* (*6*) was used, and a nested qPCR for amplifying the *prsA* gene of *R. prowazekii* was developed by using outer primers GCTTGCAGAAGAATTCTCTCTTG (forward) and GGCACAGGTTTTTTTTCAAGCAC (reverse) and nested primers CAGCGTCAAATGGTGGGATT (forward) and TGCCAACCGAAACTTGTTTTG (reverse) with established cycling conditions (*6*). Probes were 6FAM-ATCAATCAGGGCAATTAGTACCAGAA-BHQ1 for *R. typhi* and 6FAM-ATCAACCAGGGCAGTTAGTACCAGAA-BHQ1 for *R. prowazekii*. We used conventional gel PCRs for later sequencing, followed by BLAST analysis (http://www.blast.ncbi.nlm.nih.gov). We performed PCRs from DNA extracts from blood in EDTA, from the first archived serum sample if available, and in 1 case from a formalin-fixed paraffin-embedded liver biopsy sample.

Cytokine Measurements

For all available serum samples, we analyzed serum cytokine responses by using LEGENDplex (BioLegend, Fell, Germany). For controls, we used 16 serum samples from healthy blood donors. For cytokine analysis, we assigned blood sampling dates from the patients as acute phase of infection (days 1–7 and days 8–14 of illness), prolonged phase (days 15–28 of illness), and convalescent phase (days 29–56 of illness).

Results

We identified 28 TGR patients (Table 1); age range was 4–80 years (mean age 38.3 years), and male:female ratio was 1.5:1. For 1 patient, no information about travel destination or medical history was available; only age and sex information was available. TGR infections had been acquired during travel (Figure 1), primarily to Southeast Asia (Indonesia, Thailand, Cambodia, [13 (46%) cases]); most infections were acquired in Indonesia (8 [29%] cases). Other cases were acquired in Europe (Germany, Greece, Canary Islands; 6 [21%] cases), Africa (The Gambia, Burkina Faso, Cameroon, Namibia; 4 [14%] cases), North America (Florida and Texas, USA; 2 [7%] cases), Costa Rica (1 case), and Nepal (1 case). Three infections were locally acquired in Germany by patients without a travel history (patients 9, 15, and 21). No patients had been asked about exposure to rats.

Of note, patients were examined on different days of illness at different hospitals. At the time of initial examination, the most frequently reported sign or symptom was fever (79%), followed by exanthema (50%; Figure 2), headache (46%), myalgia/arthralgia (25%), cough/pneumonia (15%), and splenomegaly (11%). Only 6 (21%) patients had the classical TGR triad of fever, headache, and exanthema. The following were recorded in the medical records of 1 (4%) patient each: neurologic signs, lymphadenopathy, herpes simplex reactivation, acute kidney injury, dysuria, diarrhea, and ear redness. Hospitalization was necessary for 18 (64%) patients. Patients had received doxycycline (200 mg/d for 5–14 d) in the country of travel or after return; all recovered from infection without sequelae.

At the time of initial examination, laboratory changes were reported in the medical records of 23 patients and included increased levels of C-reactive protein (70%), liver enzymes (65%), lactate dehydrogenase (LDH, 13%), and creatine kinase (13%); thrombocytopenia (26%); anemia (17%); and leukocytosis (17%). A positive PCR for *R. typhi* was obtained for 3 (10.7%) patients: on days 7 and 10 of infection from whole blood by panrickettsial *ompB*-qPCR and *R. typhi*–specific nested *prsA*-qPCR (patients 28 and 17), and on day 10 from a liver biopsy sample by *R. typhi*–specific nested *prsA*-qPCR only (patient 13). We confirmed identity of *R. typhi* by sequencing of the 856 bp *ompB* gene fragment, which showed 100% identity to GenBank entries of *R. typhi* clone 4, strain B9991CWPP, strain TH1527, and strain Wilmington (GenBank accession nos. KF241858, CP003398, CP003397, and AE017197). Sequencing of the 140 bp *prsA* gene fragment showed 98% identity to the same strains. No positive PCR for *R. prowazekii* was obtained (Table 1).

The earliest that antibodies against TGR antigens were detected was day 7 of illness. The median day for seroconversion was day 12. The percentages of seroconversion and parallel and single antibody class detection against TGR antigens are shown in Table 2. For 5 patients, only limited serologic information was available because a limited quantity of serum had been stored for retesting. Antibody titers of any class varied among patients despite illness onset occurring on the same day. TGR-specific IgA titers were identical or lower and even undetectable for some patients but never higher than TGR-specific IgM titers except for 1 patient for whom specific IgA and IgG but not IgM were detected. No serologic differentiation between *R. typhi* and *R. prowazekii* was achieved by IFA; titer differences between the 2 species for all antibody classes were ≤2 for all patients.

Serum cytokines (Figure 3) could be measured for 21 (75%) patients; for 11 patients, they were measured as kinetics at different time points (2–5 time points). Illness was

Table 1. Characteristics of 28 patients with typhus group rickettsiosis, Germany, 2010–2017*

Patient no.	Age, y/sex	Year of diagnosis	Travel history	PCR result†	Signs and symptoms	Hospitalized
1	26/M	2016	Indonesia (Java), Singapore	Negative	Fever, exanthema, femoral lymphadenitis	Yes
2	4/M	2016	The Gambia	Negative	Fever, myalgia, pneumonia	Yes
3	27/M	2015	Indonesia (Java, Bali, Gili, Lombok)	NA	Fever, headache	Yes
4	27/M	2015	Indonesia (Lombok, Komodo, Flores, Gili, Bali, Java)	NA	Fever, headache, exanthema	Yes
5	76/F	2015	Canary Islands	NA	Fever, exanthema, dysuria	Yes
6	21/F	2015	Thailand	NA	Fever, exanthema, genital herpes	No
7	27/F	2015	Cameroon	Negative	Fever, headache, acute kidney injury	Yes
8	26/F	2015	NA	NA	NA	NA
9	80/F	2015	No (Germany)	NA	Fever, headache, arthralgia, myalgia	No
10	42/M	2014	Thailand	Negative	Fever, exanthema	Yes
11	25/F	2014	Florida	NA	Cough, reddened ear	No
12	31/F	2014	Indonesia (Bali)	Negative	Fever, headache, exanthema	No
13	28/M	2014	Greece	*R. typhi*‡	Fever, fatigue	Yes
14	31/M	2014	Indonesia (Bali)	NA	Fever, exanthema	Yes
15	72/M	2014	No (Germany)	NA	Pneumonia, pleural effusion	Yes
16	34/F	2014	Costa Rica	NA	Headache	No
17	57/M	2013	Cambodia	*R. typhi*§	Fever, headache, exanthema, splenomegaly	Yes
18	75/M	2013	Texas	Negative	Exanthema, confusion, hallucination, fatigue	Yes
19	58/M	2013	Greece	NA	Fever, exanthema, arthralgia, fatigue	Yes
20	28/F	2013	Indonesia (Bali, Gili)	Negative	Fever, headache, myalgia, splenomegaly, fatigue	Yes
21	50/F	2012	No (Germany)	NA	Arthralgia	No
22	23/M	2012	Burkina Faso	Negative	Fever, diarrhea	Yes
23	21/M	2012	Namibia	NA	Fever, exanthema	No
24	53/M	2012	Cambodia	Negative	Fever, headache, exanthema, arthralgia	No
25	26/M	2011	Cambodia	NA	Fever, headache, exanthema	Yes
26	47/M	2011	Indonesia (Java, Bali)	Negative	Fever, headache, arthralgia, splenomegaly	No
27	22/M	2011	Indonesia (Bali, Lombok)	Negative	Fever, headache, arthralgia	Yes
28	34/F	2010	Nepal	*R. typhi*§	Fever, headache, exanthema, pneumonia	Yes

*NA, not available.
†*Rickettsia typhi*–specific and *R. prowazekii*–specific nested *prsA* quantitative PCR.
‡Nested *R. typhi*–specific *prsA* quantitative PCR from liver biopsy sample, positive; from serum sample, negative.
§Also positive *ompB* PCR from whole blood.

determined to be in the acute phase according to 17 serum samples (7 on days 0–7 and 10 on days 8–14), in the prolonged phase for 4 samples, and in the convalescent phase for 5. Concentrations of interferon γ–induced protein (IP) 10 and vascular endothelial growth factor (VEGF) were elevated in the first week of the acute phase, peaked during the second week, and then declined. This trend was also observed for interleukin (IL) 8, except for patient 26, for whom IL-8 increased continuously (from 101 pg/mL on day 5 of illness and 245 pg/mL on day 12 up to 12,380 pg/mL on day 29). Serum levels of interferon-γ, IL-1β, IL-6, IL-8, IP-10, macrophage inflammatory proteins 1α and 1β, and VEGF were substantially increased over those in healthy controls in the second week of the acute phase of illness. The serum concentrations of IL-21 and IL-22 started to elevate during the second week of the acute phase, peaked during the prolonged phase, and were not detectable in the convalescent phase. Serum levels of interferon-α were substantially increased during the second and third weeks of illness; IL-10 was also elevated during this time. The serum concentrations of the following were comparable between controls and patients at all analyzed time points (data not shown): basic fibroblast growth factor, eotaxin, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, IL-2, IL-4, IL-5, IL-9, IL-12p70, IL-13, IL-17A, IL-17F, monocyte chemotactic protein (MCP) 1, platelet-derived growth factor, RANTES (regulated on activation, normal T cell expressed and secreted), and tumor necrosis factor α.

Discussion

The rather benign course of illness and outcomes, the travel destinations, and the molecular identification of *R. typhi* for a few patients indicate that murine (endemic) typhus is the most likely diagnosis for all TGR patients in our study. We found no cases of definitive *R. prowazekii* infection (i.e., epidemic typhus). IFA testing did not allow for TGR species discrimination because no significant

Figure 1. Countries and US states in which 27 of 28 patients acquired typhus group rickettsiosis diagnosed in Germany, 2010–2017. For 1 of the 28 patients, no information was available. Most infections were acquired in Southeast Asia, although 3 autochthonous cases were found in Germany. Each dot symbolizes 1 patient.

titer differences were noted between serum tested for *R. typhi* and *R. prowazekii*.

As expected, most TGR cases diagnosed in our study were travel associated. Nearly half of the infections were acquired in Southeast Asia. A surveillance study also found Southeast Asia to be the most common region of exposure for patients with murine typhus (*7*). Another study found Southeast Asia to rank second after Africa (*8*). We identified 3 autochthonous cases in patients from Germany, but the circumstances of infection and the exposure (rats or flea bites) could not be identified. For these 3 patients (50, 72, and 80 years of age), we could not exclude the possibility of a relapse of *R. prowazekii* infection (Brill-Zinsser disease) acquired during World War II or in early post-war Germany. For Europe, exposure to *R. typhi* had been previously recorded in Spain (*9*); Canary Islands and Greece (*8*); and Cyprus, Italy, France, Croatia and Slovenia (*10*).

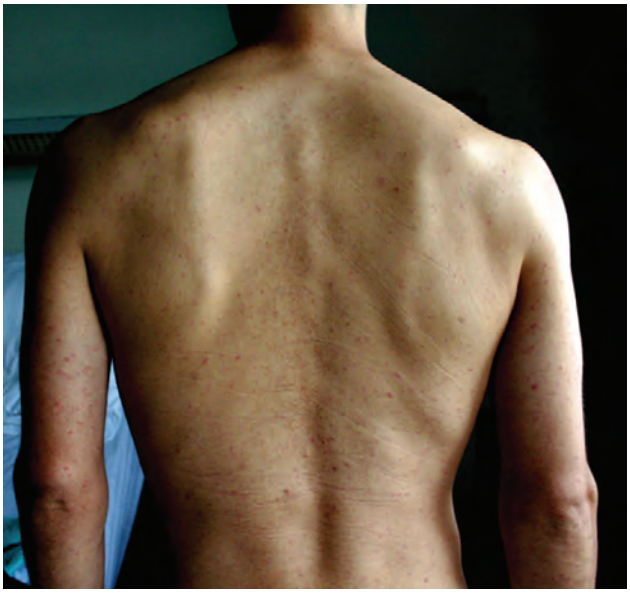

Figure 2. Typical exanthema in a typhus patient after travel to Thailand. The rash is maculopapular and nonpruritic.

The clinical signs of illness found in our study were undifferentiated, except for a maculopapular rash that had developed for half of the patients. The typical triad for murine typhus (fever, headache, and exanthema) occurred in less than one fourth of the patients reported here. According to similar findings by a study of comparable size in France, the triad was considered nonspecific (*8*); the triad occurred in one third of patients according to a study from Texas (*11*) and a recent review by others (*1*). In our study, nearly two thirds of the patients were hospitalized during travel or after return home. A similar high rate of 60% for TGR-associated hospitalization in Texas has been described (*11*). Complications, mostly pulmonary or CNS, have reached 26% among patients with murine typhus (*1*). In our study, the complication rate was 18%; complications included pneumonia, acute kidney injury, and CNS involvement.

In our study, laboratory data, which could not be retrieved in detail for all patients, often showed elevated levels of C-reactive protein, LDH, and liver enzymes,

Table 2. Serologic testing results for 28 patients with typhus group rickettsiosis, Germany, 2010–2017

Category	Result, % patients	Days of illness during which serologic results were obtained, range (median)
Observed seroconversion	18 (always with parallel IgM, IgA, and IgG detection)*	10–41 (12)†
Parallel IgM and IgG/total Ig detection in first sample	64	7–180 (10)‡
Parallel IgM, IgA, and IgG detection in first sample	46	7–39 (10)
Only 1 IgG or total Ig titer of ≥320 in any sample	14	10–99 (14)§

*1 patient showed 1 typhus group rickettsiae–specific IgM in the first sample, followed by IgA and IgG seroconversion in the follow-up sample.
†1 patient returned 41 d after the initial visit and showed seroconversion.
‡For 1 patient, serum was collected 180 d after a disease compatible with typhus group rickettsiosis had occurred.
§For 1 patient, serum was collected 99 d after a disease compatible with typhus group rickettsiosis had occurred.

paralleled by thrombocytopenia. More than 70% of patients with murine typhus had increased liver enzyme and LDH levels, and nearly half had thrombocytopenia, as described in a recent review (*1*). Because many patients in our study had fever, exanthema, elevated liver enzymes, and thrombocytopenia, the differential diagnoses for travelers, especially to Asia, include scrub typhus, dengue fever, and leptospirosis. All patients in our study were negative for these infections. Thus, infection with *R. typhi* should be considered for patients with fever, headache, exanthema, and concurrent thrombocytopenia and elevated levels of liver enzymes, particularly patients who have recently traveled to Southeast Asia.

In our study, molecular detection of TGR species was positive for a liver biopsy and 2 whole blood samples but negative for archived serum. This finding is in line with the higher sensitivity of rickettsial PCR from whole blood or buffy coat than from serum (*12*).

Typically, diagnostic IgM and IgG are simultaneously detected 7–15 days after onset of symptoms (*13*) and titers are detected by IFA for 50% of patients at the end of the first week of symptoms and for nearly all after

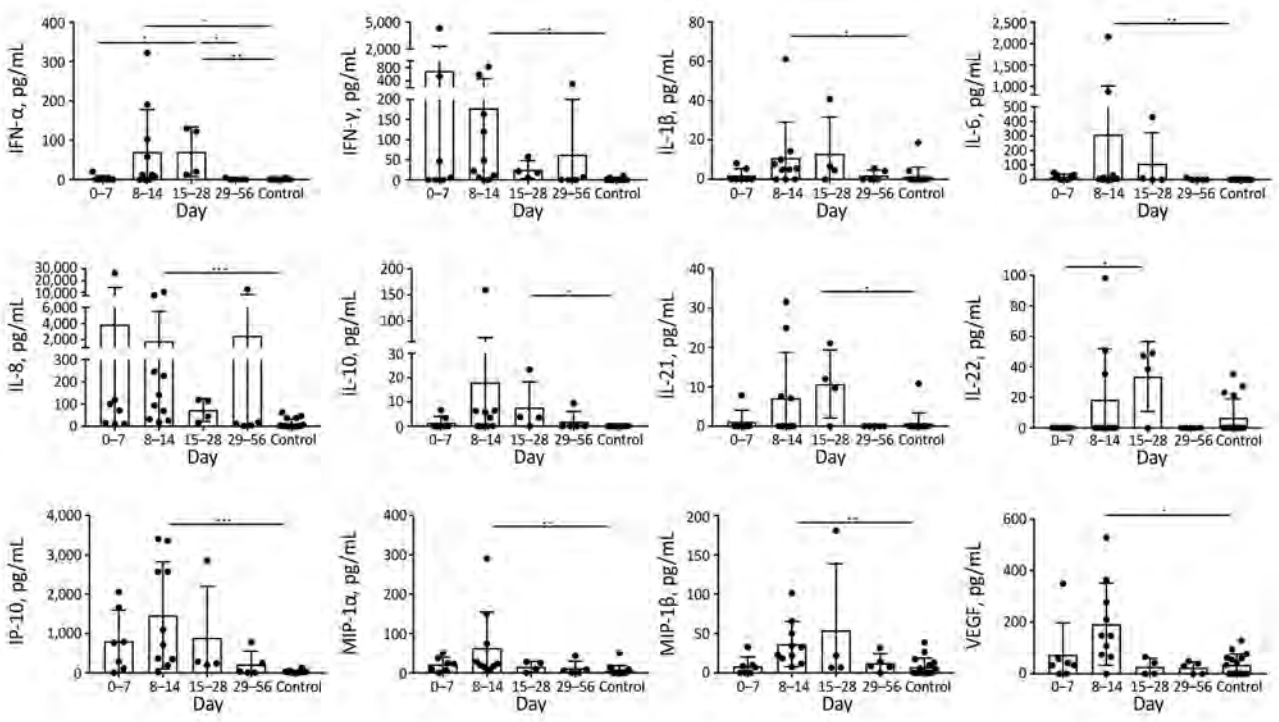

Figure 3. Cytokine and chemokine levels in serum from patients with imported and autochthonous typhus group rickettsiosis and controls, Germany, 2010–2017. Using a bead-based LEGENDplex assay (BioLegend, Fell, Germany), we analyzed 16 serum samples from healthy persons without rickettsial disease and 26 samples from 21 patients with typhus group rickettsiosis in parallel. We assigned 17 serum samples to the acute phase of illness (7 on days 0–7 and 10 on days 8–14), 4 to the prolonged phase, and 5 to the convalescent phase. Most serum cytokine levels started to increase in the first week of illness, peaked in the second week, and then started to decline again, except for IL-21 and IL-22, which reached their highest levels in the third week after symptom onset. Data are expressed as mean ± SD. Statistical analyses were performed by using the Kruskal-Wallis test and subsequently the Dunn multiple comparisons test. IFN, interferon; IL, interleukin; IP, interferon γ–induced protein; MIP, macrophage inflammatory protein; VEGF, vascular endothelial growth factor. Asterisks indicate statistically significant differences: *p<0.05; **p<0.01; ***p<0.001.

2 weeks (*4*). In our study, the earliest detection of antibodies against TGR was day 7 of illness, and the median day of seroconversion was day 12. Seroconversion, which is delayed in patients with TGR rickettsiosis, involved IgM, IgA, and IgG simultaneously. In nearly half of the patients in our study, antibodies of these 3 classes were detected in parallel against TGR in the first serum sample collected. It remains tempting to speculate about an initial depression of B-cell responses by TGR rickettsiae. IgA testing had been included in the study to test for possible earlier seroconversion with antibodies in this class, but IgA detection proved less sensitive than IgM detection. Of note, because serum had not been collected from all patients on the same day and was not collected every day, the time points can only serve as estimates.

We report data on the systemic inflammatory response in patients with TGR during 8 weeks after illness onset. Data on circulating levels of inflammatory mediators are available for only a few SFGR diseases, probably because of the limited availability of patient samples. Most cytokine and chemokine elevations in TGR patients in our study occurred in the second week of infection, coinciding with organ dysfunction and seroconversion. The mechanisms expected to contribute to the vascular permeability observed in patients with clinical disease include the effects of inflammatory cells and their mediators (*14*). Of note, vascular dysfunction and damage in the infected host most likely contribute to the pathogenesis of human rickettsial diseases. Because endothelial cells are the major target cells for rickettsial infections (*15*) and have emerged as key immune-reactive cells involved in host defense and inflammation (*16*,*17*), prior studies have focused on the behavior and inflammatory phenotype of these cells after infection in vitro. Endothelial cells react to infection with SFGR species by increased expression of cytokines such as IL-1 and IL-6 and chemokines such as IL-8 and MCP-1, which favor the migration of leukocytes (*18–20*). Experiments with cultured endothelial cells showed that they also become activated by *R. prowazekii*, which induces the expression of proinflammatory cytokines and chemokines (*21*). Endothelial cells upregulate the expression of the cytokines tumor necrosis factor-α, IL-1α, and IL-6 and the chemokines IP-10, MCP-1, and RANTES, which leads to transmigration of peripheral blood mononuclear cells that acquire an inflammatory transcriptional profile. Infection of endothelial cells with *R. typhi* resulted in enhanced expression of IP-10, MCP-1, and RANTES in infected endothelial cells; the expression of IL-8 was also upregulated (*22*). Although different intracytoplasmic behavior of SFGR and TGR pathogens has been shown (*22–25*), these findings indicate that endothelial cells react in a comparable way after infection with pathogens of either group. In fact, the elevations of the chemokines IL-8, IP-10, macrophage inflammatory protein 1α, IL-6, and IL-10 that we found in the serum of the patients in our study during the acute phase of illness were also detectable in patients with SFGR, such as African tick bite fever caused by *R. africae* and Mediterranean spotted fever caused by *R. conorii* (*26–28*). Moreover, inflammatory cytokines such as IL-1β, IL-6, and IL-8 upregulate VEGF expression (*29*). Of note, the serum concentrations of all these mediators were substantially elevated during the same period in the patients in our study (precisely in the second week of illness). VEGF and IL-8 are important mediators of angiogenesis and might contribute to initiation of repair mechanisms after endothelial damage through rickettsial growth and spread (*30*). In addition, IL-8 induces neutrophil mobilization and activation (*31*). Neutrophils are the first cells of the innate immune system that migrate to the site of infection and participate in bacterial defense.

In our study, interferon-γ levels were substantially elevated in the serum of patients in the acute phase of disease and seem to play a crucial role in antirickettsial immune responses. Interferon-γ has protective features in host defense during infection of susceptible mouse strains with SFGR and TGR species (*32–37*), and it adversely affects the growth of TGR species in various host cells (*36–39*). Therefore, the early interferon-γ response could activate intracellular bactericidal mechanisms to further control the spread of infection.

The concentrations of IL-21 and IL-22 in the serum of TGR patients started to increase in the second week of illness, peaked in the third week, and were no longer detectable 1 month after symptom onset. IL-22 seems to have protective functions because it increases the production of neutrophilic granulocyte-attracting chemokines such as IL-8, protects tissues from damage, and enhances tissue regeneration (*40*,*41*). IL-22–responsive cells are distributed throughout the body in several organs including those from the digestive (pancreas, liver, colon) and respiratory (lung, trachea) systems and the skin (*42*). Whether endothelial cells express the IL-22 receptor complex remains to be determined. IL-22 is produced by several types of cells of the lymphoid lineage and include activated T-cells as well as innate lymphoid cells such as natural killer cells, lymphoid tissue inducer cells, and lymphoid tissue inducer–like cells (*43–50*). Which cell types are responsible for IL-22 secretion and the protective potential of this cytokine during rickettsial infections remains to be elucidated.

In conclusion, our data broaden the knowledge of TGR immunology and diagnosis and shed light on immunologic changes that occur during successive weeks of illness. However, more investigations of immunologic changes, including analyses of human B and T cells, are needed.

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Molecular Epidemiology of Human Adenovirus–Associated Febrile Respiratory Illness in Soldiers, South Korea[1]

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During January 2013–April 2014, we subjected nasopharyngeal specimens collected from patients with acute febrile respiratory illness in a military hospital to PCR testing to detect 12 respiratory viruses and sequence a partial hexon gene for human adenovirus (HAdV) molecular typing. We analyzed the epidemiologic characteristics of HAdV infections and compared clinical characteristics of HAdV types. Among the 305 patients with acute febrile respiratory illness, we detected respiratory viruses in 139 (45.6%) patients; HAdV was the most prevalent virus (69 cases). Of the 40 adenoviruses identified based on type, HAdV-55 (29 cases) was the most prevalent, followed by HAdV-4 (9 cases). HAdV-55 was common in patients with pneumonia (odds ratio 2.17; 95% CI 0.48–9.86) and hospitalized patients (odds ratio 5.21; 95% CI 1.06–25.50). In soldiers with HAdV infection in Korea, HAdV-55 was the most prevalent type and might be associated with severe clinical outcomes.

Human adenoviruses (HAdVs) are considered the most important causative agent of acute respiratory infection in soldiers, particularly in new recruits (*1*,*2*). A total of 79 HAdV types have been documented (*3*). The distribution of HAdV types differs substantially by geographic region and environmental factors (*4*,*5*). HAdVs are prevalent in training facilities or military barracks for soldiers, but the prevalent types of HAdV have changed over time (*6*). Historically, HAdV-4 and HAdV-7 have been the most prevalent causes of acute febrile respiratory illness (AFRI) among US military personnel since the 1950s (*7*). Vaccination against HAdV-4 and HAdV-7 has been effective in reducing AFRI among US military trainees to date (*8*,*9*). However, since 2007, the emergence of new adenovirus types such as HAdV-14, which is distinct from the prototype in the United States, has been associated with outbreaks of AFRI and severe pneumonia (including several deaths) in military populations (*10*,*11*).

HAdV is most prevalent in patients with acute lower respiratory tract infection and is the most common cause of pneumonia among military personnel in South Korea (*12*). However, studies evaluating the types of HAdV in this population are limited because of the lack of knowledge about HAdVs among military physicians. In 2012, HAdV-55 was identified in patients with severe pneumonia, and an outbreak of AFRI among military personnel in South Korea was recorded (*13*,*14*). HAdV-55, which is a novel HAdV type characterized by genome recombination between HAdV-B11 and HAdV-B14, caused outbreaks of acute respiratory diseases in military camps in Turkey, China, and Singapore (*15*–*18*). Although cases of pneumonia caused by HAdV-55 among military personnel in South Korea have been recorded, information on the epidemiology and characteristics of type-specific HAdV respiratory infections among military personnel in South Korea is limited. Thus, our study aimed to investigate the epidemiology of HAdV infections and to compare the clinical characteristics by type of HAdVs in soldiers in South Korea via hospital-based surveillance on viral respiratory infections.

Methods

Characteristics of the Study Population and Case Definition

The study was approved by the Institutional Review Board of the South Korea Armed Forces Medical Command. During January 2013–April 2014, we enrolled in the study all new recruits and active duty soldiers with AFRI who were required to visit the emergency department or to undergo

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hospitalization at the Armed Forces Capital Hospital in Seongnam, South Korea. We defined AFRI as a history of fever or measured fever ≥37.6°C and the presence of ≥1 respiratory symptom such as cough, sore throat, or rhinorrhea with onset within the last 7 days. The Armed Forces Capital Hospital is the only tertiary care hospital in the South Korea military healthcare system. Furthermore, the hospital offers primary and secondary medical services to soldiers in the city of Seoul and Gyeonggi Province. Almost all soldiers in South Korea, except officers, use military hospitals for free health services.

Respiratory Virus Multiplex Reverse Transcription PCR

We collected nasopharyngeal or throat swab specimens from the patients with AFRI within 24 hours after their hospital visit by using a flocked swab. We stored the specimen at 4°C in viral transport media until further testing. Within 3 days of collection, we sent specimens to a commercial laboratory center (GC Labs, Yongin, South Korea), where they were subjected to respiratory virus multiplex reverse transcription PCR.

We extracted total viral nucleic acid from the specimens by using the Chemagic Viral DNA/RNA Extraction Kit (Chemagen Inc., Baesweiler, Germany) and performed cDNA synthesis by using the CapFishing Full-Length cDNA Premix Kit (Seegene Inc., Seoul, South Korea). We performed PCR by using the Seeplex RV12 ACE Detection Kit (Seegene Inc., South Korea), which is used for identifying influenza viruses A and B, respiratory syncytial viruses A and B, adenovirus, parainfluenza virus types 1–3, rhinovirus group A, human coronavirus 229E/NL63, human coronavirus OC43, and human metapneumovirus.

Molecular Analysis of HAdVs

We extracted DNA from the adenovirus-positive respiratory specimens by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). We amplified the partial nucleotides of the hexon gene by using PCR as described elsewhere with some modifications (*19*). We amplified viral sequences by using oligonucleotide primers producing a 475-bp fragment: ADHEX1F (5′-CAACACCTAYGASTACATGAA-3′) and ADHEX1R (5′-KATGGGGTARAGCATGTT-3′). PCR conditions were as follows: initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, elongation at 68°C for 1 min, and final extension at 68°C for 5 min. For samples that tested negative in the first PCR reaction, we performed heminested PCR by using primers (273 bp) ADHEX1F (5′-CAACACCTAYGASTACATGAA-3′) and ADHEX2R (5′-ACATCCTTBCKGAAGTTCCA-3′) with the same temperature and time profiles. We determined DNA sequences in both directions by using the Applied Biosystems Automatic Sequencer ABI 3730xl and ABI Prism BigDye Terminator v3.1 sequencing system (Applied Biosystems, Foster City, CA, USA). We identified the type of HAdV by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

We generated phylogeny on the basis of the 232-bp nucleotide sequences of the hexon gene of HAdVs. For the phylogenetic analysis, we selected the sequences of each type of HAdV from GenBank. We used MEGA 6 software to generate the phylogenetic tree and evaluated topologies by performing a bootstrap analysis of 1,000 iterations (*20*).

Collection of Clinical Data

We obtained clinical information of the patients with HAdV respiratory infection during the study period from standardized case report forms. The case report forms, which included clinical diagnosis, intensive care unit stay, requirement for mechanical ventilation or vasopressor, and symptoms at presentation, were completed within 7 days of the hospital visit by an attending physician.

Statistical Analysis

We conducted Pearson's χ^2 and Fisher exact test for the demographic and clinical variables by using the SPSS for Windows version 20 (IBM Corp., Armonk, NY, USA). For all analyses, we defined statistical significance as $p<0.05$.

Results

Epidemiology of HAdVs in Soldiers with AFRI

During January 2013–April 2014, we enrolled 305 patients with AFRI in the study. We detected a total of 157 respiratory viruses in 139 (45.6%) soldiers with AFRI. HAdV was the most prevalent virus (49.6% [69/139]), followed

Table 1. Respiratory viruses in soldiers with acute febrile respiratory illness, South Korea, January 2013–April 2014

Respiratory virus	No. (%) with virus identified*	No. (%) with ≥2 viruses identified
Adenovirus	69 (49.6)	12 (17.4)
Influenza A or B	40 (28.8)	3 (7.5)
Rhinovirus group A	17 (12.2)	6 (35.3)
Coronavirus 229E/NL63 or OC43	11 (7.7)	5 (45.4)
Respiratory syncytial virus A or B	10 (7.9)	4 (40.0)
Parainfluenza virus 1, 2, or 3	9 (6.5)	5 (55.6)
Metapneumovirus	1 (0.7)	1 (100.0)

*Among the 139 soldiers in whom a respiratory virus was detected.

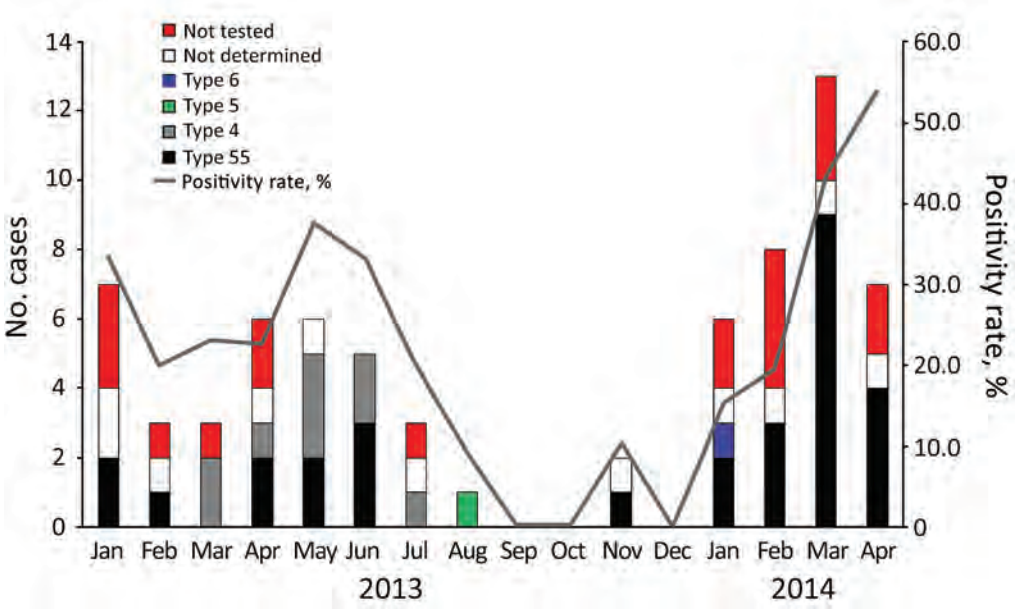

Figure 1. Temporal distribution of acute febrile respiratory illness from human adenovirus (HAdV) infection among soldiers (no. cases) and overall HAdV positivity rate among collected specimens, by HAdV type, South Korea, January 2013–April 2014. We observed HAdV respiratory infection primarily during winter and spring. In 2014, acute febrile respiratory illness in soldiers in South Korea was almost always associated with HAdV-55. Co-circulation of HAdV-55 and HAdV-4 occurred during spring and early summer of 2013.

by influenza A or B virus (28.8% [40/139]) and rhinovirus group A (12.2% [17/139]). Among 139 cases in which respiratory viruses were identified, ≥2 viruses were detected in 18 cases (12.9%) (Table 1).

We commonly observed cases of HAdV infection during winter and spring (Figure 1). Among the tested cases of AFRI, the monthly positive rate for HAdV ranged from 0% to 53.8%; average positivity rate was 22.6%. A peak positive rate for HAdV infection occurred in soldiers with AFRI during March–April 2014. The mean age of the patients with HAdV infection was 21.7 years. Among the 69 patients with HAdV infection, 40.6% were new recruits and 75.4% were hospitalized (Table 2).

From the 69 patients with HAdV infection, 51 respiratory specimens were available for further molecular analysis. For the 51 samples tested, we identified the HAdV type in 40 samples (Figure 2). HAdV-55 (72.5% [29/40]) was the most prevalent type in soldiers with HAdV infection, followed by HAdV-4 (22.5% [9/40]). We detected HAdV-55 and HAdV-4 in 2013, but we did not detect HAdV-4 in 2014. We detected HAdV-5 and HAdV-6 in 1 case each.

Clinical Characteristics of HAdV-55 Infection versus Other Types of HAdV Infections

We observed no statistically significant difference in the demographic characteristics or signs and symptoms of the patients with HAdV-55 infection compared with those with other types of HAdV infection (Table 3). Furthermore, we observed no statistically significant difference between the patients in terms of laboratory and radiographic findings. Co-infections with other bacteria or viruses were similar in both groups. The patients with HAdV-55 infection were more likely to have onset pneumonia (44.8% vs. 27.3% [odds ratio (OR) 2.17; 95% CI 0.48–9.86]) and be hospitalized (86.2% vs. 54.5% [OR 5.21; 95% CI 1.06–25.50]) than those infected with other types of HAdV. In particular, we identified HAdV-55 infection in all patients who required hospitalization in the intensive care unit or mechanical ventilation caused by acute respiratory distress syndrome (ARDS).

Table 2. Demographic characteristics of soldiers with HAdV respiratory infection, by HAdV type, South Korea, January 2013–April 2014*

Characteristic	HAdV type						
	Type 55	Type 4	Type 5	Type 6	Not determined	Unable to type	Total
No. (%) cases	29 (42.0)	9 (13.0)	1 (1.4)	1 (1.4)	11 (15.9)	18 (26.1)	69 (100.0)
Age, y, mean ±SD	21.41 ±1.92	21.33 ±1.32	21	20	21.42 ±1.75	21.63 ±1.38	21.69 ±1.49
Military rank, no. (%)							
New recruits	12 (41.4)	3 (33.3)	1 (100.0)	0	5 (45.5)	7 (38.9)	28 (40.6)
Active-duty soldiers	17 (58.6)	6 (66.7)	0	1 (100.0)	7 (63.6)	10 (55.6)	41 (59.4)
Smokers, no. (%)	15 (51.7)	5 (55.6)	0	0	7 (63.6)	11 (61.1)	38 (55.1)
Hospitalized, no. (%)	25 (86.2)	6 (66.7)	0	0	8 (72.7)	13 (72.2)	52 (75.4)

*All soldiers were male. Some HAdV types were not verified despite our performing molecular analyses. Molecular analyses for typing of HAdV type were not performed. HAdV, human adenovirus.

Discussion

In our study, HAdV was the most prevalent virus detected among soldiers with AFRI in South Korea, representing 49.6% of the cases. HAdV-55 was the most prevalent type among the cases that could be identified using PCR. Although HAdV-55 has recently received public attention as an emerging pathogen that causes outbreaks of respiratory illness and severe pneumonia in the general population and soldiers, acute respiratory illness associated with HAdV-55 has rarely been reported in the civilian population in South Korea (*6*,*16*,*21*–*23*). In children in South Korea, HAdV-3 and HAdV-7 are prevalent serotypes and have also been associated with the outbreaks of adenoviral respiratory illness since the 1990s (*24*,*25*). Severe pneumonia cases associated with HAdV-55 infection were recently reported in soldiers in South Korea (*13*,*14*). Our finding that HAdV-55 was the most prevalent type in soldiers in South Korea differs from the epidemiology of adenovirus in children in South Korea.

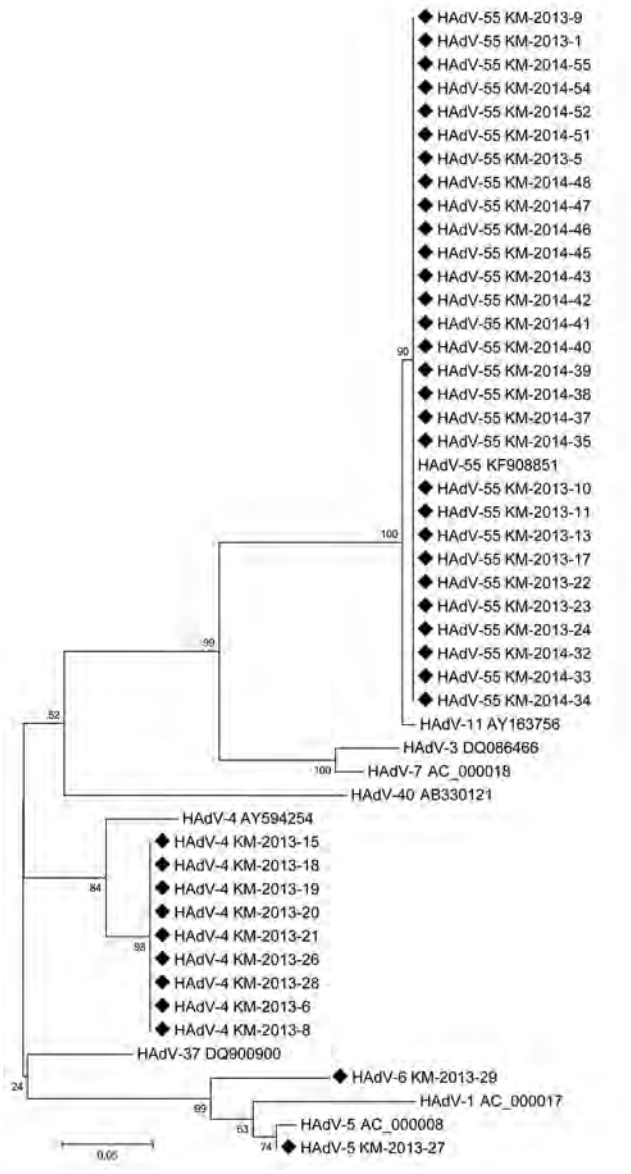

Figure 2. Phylogenetic tree human adenoviruses detected among soldiers with acute febrile respiratory illness from human adenovirus (HAdV) infection, South Korea, January 2013–April 2014. Tree was constructed by the neighbor-joining method on the basis of a 232-bp nucleotide sequence of the hexon gene. We used MEGA 6 software (*20*) to generate the phylogenetic tree and evaluated topologies by using bootstrap analysis of 1,000 iterations. GenBank accession numbers of sequences of HAdV from the soldiers with acute febrile respiratory illness (indicated by black diamonds) were KX227462–KX227469 and KX513954–KX513985. GenBank accession numbers of reference adenovirus sequences are shown in the tree. Scale bar indicates nucleotide substitutions per site. HAdV, human adenovirus.

Another notable finding of this study was the changing epidemiologic trend from the co-circulation of HAdV-4 and HAdV-55 in 2013 to the predominant circulation of HAdV-55 in 2014. HAdV-4 was the second most common type after HAdV-55 among soldiers with AFRI in South Korea in 2013. However, HAdV-4 has not been identified in soldiers in South Korea since November 2013. Although the shift in the HAdV types in soldiers in South Korea could not be fully understood because of the relatively short study period, HAdV-55 infection has been prevalent among soldiers in South Korea since 2014 (*14*,*26*).

Pneumonia and hospitalization associated with HAdV-55 infection were more frequent than those associated with the other types of HAdV infection in soldiers in South Korea. In particular, HAdV-55 infection was associated with severe pneumonia or ARDS. Nevertheless, we observed no significant differences between HAdV-55 cases and non–HAdV-55 cases in terms of the frequency of clinical diagnosis of pneumonia, hospitalization, and ARDS. These findings could be explained by 2 assumptions. First, specific HAdV types such as HAdV-3, HAdV-7, or HAdV-14 might be more virulent than other types (*25*,*27*,*28*). Considering that the HAdV-55 genome is more similar to the HAdV-14 genome than the HAdV-11 genome, HAdV-55 infection could be associated with severe respiratory infection in certain patients (*17*). Second, the lower levels of herd immunity against HAdV-55 could have an influence on the epidemic of HAdV-55–associated respiratory infection in soldiers in South Korea (*29*,*30*). Severe respiratory illness and outbreaks associated with HAdV-55 in soldiers in South Korea might be similar to those observed in military personnel in China (*13*,*14*,*21*,*22*).

In this study, the proportion of new recruits with HAdV infection among soldiers in South Korea was not as high as expected. More than half of the South Korean soldiers with HAdV infection were on active duty. These findings contrast with previous data in which HAdV-associated respiratory infection has been common among new recruits

Table 3. Comparison of demographic and clinical features of soldiers with acute febrile respiratory illness, by HAdV type 55 infection status, South Korea, January 2013–April 2014*

Characteristic	HAdV-55 infection status		OR (95% CI)	p value
	HAdV-55, n = 29	Non–HAdV-55, n = 11		
Age, y, mean ±SD	21.41 ±1.92	21.18 ±1.40	NA	0.717†
New recruits	12 (41.4)	5 (45.5)	0.84 (0.21–3.43)	0.816‡
Signs and symptoms at presentation				
Fever ≥5 d	17 (58.6)	5 (45.5)	1.70 (0.42–6.88)	0.455‡
Nausea/vomiting	5 (17.2)	2 (18.2)	0.93 (0.15–5.73)	1.000§
Diarrhea	6 (20.7)	3 (27.3)	0.70 (0.14–3.45)	0.686§
Dyspnea/tachypnea	8 (27.6)	1 (9.1)	3.81 (0.42–34.8)	0.399§
Conjunctival injection	4 (13.8)	2 (18.2)	0.72 (0.11–4.63)	1.000§
Laboratory findings, mean ±SD				
Leukocyte count, cells/μL	5,200 ±1,818	6,322 ±2,656	NA	0.134†
Platelet count, × 10^3 cells/μL	177 ±57	172 ±77	NA	0.848†
C-reactive protein, mg/dL	6.7 ±3.9	5.0 ±1.8	NA	0.193†
Radiographic findings				
Bilateral involvement	6 (20.7)	1 (9.1)	2.61 (0.28–24.59)	0.650§
Consolidation	11 (37.9)	2 (18.2)	2.75 (0.50–15.14)	0.286§
Patchy infiltration	3 (10.3)	1 (9.1)	1.15 (0.11–12.44)	1.000§
Effusion	3 (10.3)	1 (9.1)	1.15 (0.11–12.44)	1.000§
Clinical diagnosis				
Pharyngitis	12 (41.4)	5 (45.5)	0.85 (0.21–3.43)	0.815‡
Tracheobronchitis	4 (13.8)	3 (27.3)	0.43 (0.08–2.33)	0.369§
Pneumonia	13 (44.8)	3 (27.3)	2.17 (0.48–9.86)	0.473§
Co-identified bacteria	2 (6.9)	1 (9.1)	0.74 (0.06–9.09)	1.000§
Streptococcus pneumoniae	1 (3.4)	1 (9.1)	–	–
Mycoplasma pneumoniae	1 (3.4)	0	–	–
Co-identified viruses	5 (17.2)	1 (9.1)	2.08 (0.22–20.17)	1.000§
Rhinovirus	3 (10.3)	0	–	–
Coronavirus	1 (3.4)	1 (9.1)	–	–
Parainfluenza virus	1 (3.4)	0	–	–
Metapneumovirus	1 (3.4)	0	–	–
Hospitalized patients	25 (86.2)	6 (54.5)	5.21 (1.06–25.50)	0.083§
Admission to the intensive care unit	7 (24.1)	0	NA	0.159§
Acute respiratory distress syndrome	5 (17.2)	0	NA	0.298§
Length of hospital stay, d, mean ±SD	16.2 ±9.9	14.6 ±5.6	NA	0.619†
Death	0	0	NA	NA

*Values are no. (%) soldiers except as indicated. HAdV, human adenovirus; NA, not available; OR, odds ratio; –, not applicable.
†By Student *t*-test.
‡By χ^2 test.
§By Fisher exact test.

(*7*). The patients included in this study might have had clinically severe illness rather than mild illness because our institute is the only central referral hospital in the military system in South Korea. Moreover, HAdV easily spreads to advanced training sites or military barracks and it can spread in geographically dispersed military barracks by the movement of soldiers because of prolonged viral shedding (*31*). In this study, some of the patients with HAdV-55 infection who were active duty soldiers were identified in the clusters of patients in 4 military barracks (data not shown). Although the outbreak of HAdV-55 infection was not directly confirmed in this study, outbreaks of HAdV-55–associated respiratory infection might be occurring among active duty soldiers.

Our study has some limitations. First, the study was performed for a relatively short period (16 months). Although the data might be insufficient to reflect the epidemiology of respiratory HAdV infection among military personnel in South Korea, the variation of HAdV type over time and the impact of the emergence of HAdV-55 on clinical severity can be observed. Considering the long-term experience of the US military with respiratory HAdV infection, the distribution of HAdV types might show a substantial difference in the military and community populations in South Korea. Thus, a surveillance system must be established to detect the circulation of the HAdV type among military personnel. Second, the study population included patients who needed hospitalization or an emergency department visit. Among soldiers in South Korea with HAdV infection, patients who had severe clinical signs and symptoms might be those who were primarily enrolled in this study. Nevertheless, our results suggest that of the several HAdV types, HAdV-55 and HAdV-4 might be implicated in HAdV respiratory infections among soldiers in South Korea. Furthermore, active duty soldiers and new recruits could have a substantial disease burden caused by respiratory HAdV infection. Third, we could not determine HAdV types in 11 cases. The viral titer in the clinical specimen might have been low and the

viral DNA degraded because some respiratory specimens were not frozen until the molecular experiment. Using the 2-step hemi-nested PCR, we did not observe a DNA band in 11 samples.

This study is important because it is a prospective study on patients with AFRI. Studies on the HAdV type in soldiers in South Korea since 2012 are limited. However, the previous studies provided data on the epidemiology of the HAdV type in patients with severe clinical manifestations or in those with HAdV respiratory infection. These conditions might result in bias in the epidemiology of HAdV infections among military personnel in South Korea.

In conclusion, our study found that HAdV was the most prevalent virus among soldiers with AFRI in South Korea. In particular, HAdV-55 and HAdV-4 were the prevalent types in soldiers with HAdV-associated respiratory infection. HAdV-55 was associated with severe clinical outcomes. Further studies are needed to verify which HAdV types are associated with AFRI in military recruits. In addition, studies on the introduction or development of an effective vaccine against HAdV-55 and HAdV-4 should be considered.

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etymologia

Cytokines [si′to-kīnes]

Thomas J. Gryczan

From the Greek *cyto* (cavity or cell) and *kine* (movement), cytokines are proteins involved in cell signaling and function as immunomodulating agents. Cytokines are produced by immune cells (e.g., macrophages, B and T lymphocytes, mast cells, neutrophils, natural killer cells), endothelial cells, fibroblasts, and stromal cells.

Although the term cytokine had not yet even been defined, interferon-α, the first cytokine known, was identified in 1957 as a protein that interfered with virus replication. Activities of interferon-γ and interleukin-2 were identified in 1965. Macrophage migratory inhibitory factor was identified in 1966. In 1969, Dumonde and colleagues proposed the term lymphokine to describe proteins secreted from lymphocytes. Proteins derived from macrophages and monocytes were later called monokines. In 1974, Cohen and colleagues reported production of macrophage migration inhibitory factors in virus-infected fibroblasts, which led (finally) to proposal of the term cytokine.

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Diversity of Influenza A(H5N1) Viruses in Infected Humans, Northern Vietnam, 2004–2010

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Influenza viruses exist in each host as a collection of genetically diverse variants, which might enhance their adaptive potential. To assess the genetic and functional diversity of highly pathogenic avian influenza A(H5N1) viruses within infected humans, we used deep-sequencing methods to characterize samples obtained from infected patients in northern Vietnam during 2004–2010 on different days after infection, from different anatomic sites, or both. We detected changes in virus genes that affected receptor binding, polymerase activity, or interferon antagonism, suggesting that these factors could play roles in influenza virus adaptation to humans. However, the frequency of most of these mutations remained low in the samples tested, implying that they were not efficiently selected within these hosts. Our data suggest that adaptation of influenza A(H5N1) viruses is probably stepwise and depends on accumulating combinations of mutations that alter function while maintaining fitness.

Highly pathogenic avian influenza (HPAI) A(H5N1) viruses are not readily transmitted among humans, although a few cases of human-to-human transmission have been reported (*1*–*3*). However, recent laboratory experiments have demonstrated that a small number of amino acid changes can render avian H5 influenza viruses transmissible via respiratory droplets among ferrets (*4*–*6*) or guinea pigs (*7*). The evolutionary processes by which influenza A(H5N1) viruses might adapt to mammals are poorly understood. Because of error-prone genome replication, influenza viruses exist in an infected host as a collection of genetic variants; this within-host genetic diversity is believed to facilitate rapid adaptation to changing selective pressures (*8*). Recent mathematical models have highlighted the role of virus mutation rates, the number of replication cycles in a given host, and natural selection in assessing the likelihood with which an influenza A(H5N1) virus transmissible among mammals might emerge, but little information on avian influenza virus diversity within infected hosts was available to inform these studies (*9*,*10*).

We recently found that selection pressure on hemagglutinin (HA) can impose a strong population bottleneck during virus transmission: a variant representing only 5.9% of the total virus population in animals infected with a reassortant influenza A(H5N1) virus infected an animal via aerosol transmission (*11*). These findings prompted us to seek a deeper understanding of virus populations in infected humans. We used deep-sequencing and functional analyses to evaluate the genetic and functional diversity among influenza A(H5N1) viruses in infected humans.

Materials and Methods

Biosafety and Biosecurity

The studies were conducted after the University of Wisconsin–Madison Office of Biologic Safety completed risk assessments for the proposed experiments and the Institutional Biosafety Committee approved the experiments. All experiments used the biosafety and biosecurity practices and procedures that were developed in conjunction with the University's Select Agent Program and approved by the Centers for Disease Control and Prevention Division of Select Agents and Toxins and by the US Department of Agriculture, Animal and Plant Health Inspection Service, Agriculture Select Agent Services. The studies and subsequent data were reviewed for potential dual use research of concern in accordance with the US Government Policy for Oversight of Life

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Sciences Dual Use Research of Concern. All experiments conducted in laboratories at the University of Tokyo (Tokyo, Japan) were approved by the appropriate committees at the University of Tokyo. Isolation and amplification of HPAI A(H5N1) viruses from clinical specimens were performed in enhanced Biosafety Level 3 laboratories at the University of Tokyo, which are approved for such use by the Ministry of Agriculture, Forestry, and Fisheries, Japan.

RNA Extraction, cDNA Synthesis, and PCR Amplification

We obtained clinical specimens (throat swabs and tracheal aspirates) from 7 patients infected with influenza A(H5N1) virus in northern Vietnam during 2004–2010. To avoid the emergence of mutations during virus amplification in cultured cells, we extracted total RNA directly from clinical specimens by using the QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany). Because of the limited amounts of viral RNA (vRNA), we reverse transcribed vRNA segments encoding known determinants of mammalian adaptation or transmissibility (i.e., polymerase basic [PB] 2, PB1, polymerase acidic [PA], nucleoprotein [NP], HA, and nonstructural [NS] segments) by using SuperScript III (Invitrogen, Carlsbad, CA, USA). We PCR amplified the segments by using iProof High-Fidelity DNA polymerase (Bio-Rad, Hercules, California, USA) with primers specific for the terminal sequences of each vRNA segment. If we failed to PCR amplify the full-length vRNAs, we used primers specific for the internal sequences (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/7/17-1441-Techapp1.pdf).

Illumina MiSeq Sequencing

We quantified PCR-amplified and agarose gel–purified PCR products by using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen). Amplified genes were pooled, and sample barcodes were added by using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Barcoded samples were pooled in equimolar amounts and loaded onto a 500-cycle kit for sequencing on the Illumina MiSeq. Illumina MiSeq fastq files are publicly available in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra; SRA: SRP103022).

Computational Methods

We imported Illumina MiSeq sequences into CLC Genomics Workbench version 7.3 (CLC bio, Aarhus, Denmark) in fastq format. We trimmed reads by using a quality-limit threshold of 0.001 and retained only reads ≥100 bp. Trimmed reads were de novo assembled to generate a consensus sequence for each gene segment of each patient sample. We then used these within-host consensus sequences, defined by the nucleotides found in more than half of the sequence reads at each position, as reference sequences for further mapping. Reads were remapped to each sample's own consensus sequence to identify within-host single-nucleotide polymorphisms (SNPs), requiring that nucleotide positions be covered by ≥100 sequence reads, that they have a central base quality score ≥Q30, and that SNPs be detected in at least 1 forward and 1 reverse read. We only considered SNPs present in ≥1% of sequence reads within a population; we previously showed that this stringent cutoff excludes false-positive SNPs generated by using our approach (*11*). Amino acid variations in HA were mapped onto the A/Vietnam/1203/2004 (H5N1) HA structure (Protein Data Bank accession no. 2FK0) by using Pymol (http://www.pymol.org).

Cells

MDCK cells (ATCC, Manassas, VA, USA) were maintained in minimal essential medium with 5% newborn calf serum. Human embryonic kidney 293T cells (ATCC) were maintained in Dulbecco modified Eagle medium with 10% fetal calf serum. Both cell lines were cultured at 37°C in 5% CO_2.

Viruses

Reassortants possessing an HA polymorphism in the genetic background of the respective H5 HA gene and the remaining genes derived from A/California/04/2009 (H1N1) (CA04) were generated in 293T cells by using reverse genetics (*12*) and amplified in MDCK cells; the resulting viruses are referred to as in the following example: CA04/UT3040I-HA-138V. Sanger sequence analysis of all virus stocks revealed that the CA04/UT36282I/II-HA-138V virus possessed a mixture of valine and alanine at HA position 138 and that the HA-203P residue of CA04/UT31312III-HA-203P was replaced by leucine. We were unable to rescue the CA04/UT31413II-HA-486H reassortant virus and therefore omitted it from further studies. Because of the research pause imposed on certain gain-of-function studies (http://www.phe.gov/s3/dualuse/Documents/gain-of-function.pdf), we were not able to generate 1 variant (CA04/UT31413II-HA-511I) and therefore will not discuss it further. All experiments with infectious viruses possessing the H5 HA with a polybasic cleavage site were performed in enhanced Biosafety Level 3 containment laboratories.

Solid-Phase Binding Assay

We coated 96-well plates with sodium salts of sialylglycopolymers (poly-l-glutamic acid backbones containing N-acetylneuraminic acid linked to galactose through either an α2,3 [Neu5Acα2,3Galβ1,4GlcNAcβ1-Pap] or an α2,6 [Neu5Acα2,6Galβ1,4GlcNAcβ1-Pap] bond) (*13*), synthesized at Chubu University. We assessed virus binding to the sialylglycopolymers as previously described (*4*,*14*).

Glycan Arrays

We performed glycan array analysis as previously described (*15*,*16*). Viruses were amplified in MDCK cells and inactivated by mixing the supernatants with 0.1% β-propiolactone (final concentration). A complete list of glycans on the array is provided in online Technical Appendix Figure 1.

Virus Tissue Binding Assay

We diluted inactivated viruses with phosphate-buffered saline to equal amounts of viral M1 protein (as assessed by Western blot). Diluted viruses were labeled with FITC isomer I (Life Technologies, Carlsbad, CA, USA). Virus binding to human adult normal trachea sections was assessed as previously described (*17*,*18*).

Thermostability Assay

We diluted amounts of virus equivalent to 64 hemagglutinating units in minimal essential medium supplemented with 0.3% bovine serum albumin and heat treated the diluted virus samples at 55°C for the indicated times. We then determined the hemagglutination activity of the heat-treated viruses by using HA assays with 0.5% turkey red blood cells (Rockland Immunochemicals Inc., Limerick, PA, USA). Virus infectivity was determined by performing plaque assays in MDCK cells.

Mini-Replicon Assay

We transfected human 293T cells with pCAGGS plasmids (*19*) encoding wild-type or mutant PB2, PB1, PA, and NP protein with a plasmid expressing the firefly luciferase gene from a virus-like RNA (pPolWSNNA F-Luc) (*20*) and with the control plasmid pGL4.74[hRluc/TK] (expressing *Renilla* luciferase; Promega, Madison, WI, USA) by using TransIT293 (Mirus, Madison, WI, USA). Cells were incubated at 33°C or 37°C for 24 h and then lysed with 1× Passive Lysis Buffer (Promega). We determined luciferase activity by using the Dual-Luciferase Reporter Assay System (Promega).

Interferon Reporter Assays

We assessed the effect of NS1 amino acid changes on interferon production as previously described (*21*,*22*) with some modifications. We transfected human 293T cells with pCAGGS plasmids expressing wild-type or mutant NS1 and with a plasmid expressing luciferase under the control of the interferon-β promoter (pGL-interferonβ luc) (*23*). Luciferase activity was determined 24 h after treating cells with 10^6 focus-forming units of Sendai virus to induce interferon production (*21*,*22*).

To assess the effect of NS1 on interferon signaling, we transfected 293T cells with a plasmid encoding wild-type or mutant NS1 and a plasmid encoding luciferase under the control of an interferon-stimulated response element (pISRE-Luc; Clontech, Kusatsu, Japan). Luciferase activity was determined after treating cells with human interferon-β1a (PBL Assay Science, Piscataway, NJ, USA).

Statistical Analyses

For the mini-replicon assay, we used the Dunnett test to compare the viral polymerase activity of each mutant with that of the respective majority variant in each experiment. In the interferon reporter assays, we analyzed the data by using a one-way analysis of variance, followed by the Tukey post hoc test to compare the interferon antagonistic properties among the empty vector, UT36250I-NS1, and UT36250I-NS1-124M.

Results

Genetic Diversity of Influenza A(H5N1) Viruses in Humans

For each of the 7 patients with confirmed influenza A(H5N1) virus infection, 2–3 samples were available that had been collected on different days or from different anatomic sites (i.e., throat swabs or tracheal aspirates) (Table). To estimate the genetic diversity of these viruses in humans, we first defined a within-host consensus sequence for each viral gene segment, the sequences of which we obtained by deep sequencing with RNA extracted from patient clinical samples. We then counted the SNPs relative to this consensus that were detected at ≥1% frequency within each host, detecting a total of 251 nonsynonymous and 146 synonymous mutations (online Technical Appendix Table 2). Within-host genetic diversity varied considerably among patients, anatomic site of sample (nasal swab or tracheal aspirate), and day of isolation (online Technical Appendix Figure 2, Table 3). Nonetheless, 91 variants were detected ≥2 times in samples from the same patient.

Previously, we demonstrated that a viral variant detected at a frequency of only 5.9% in 1 ferret founded infection after respiratory droplet transmission to a contact ferret (*11*). Therefore, we focused our functional analyses on mutants present in ≥5% of the virus population, yielding 29 nonsynonymous polymorphisms in the viral HA, PB2, PB1, PA, NP, and NS1 proteins of viruses obtained from 6 patients (Figure 1; online Technical Appendix Table 3). One more variant (PA-85T/M) was detected at a frequency of <5% at the first time point, but its frequency had increased greatly by the second time point; therefore, we included this variant in our subsequent analyses, for a total of 30 variants examined (Figure 1; online Technical Appendix Table 3).

Receptor-binding Specificity of HA Variants

Several of the HA sequence polymorphisms detected were located in the vicinity of the receptor-binding pocket (Figure 2, panel A). We therefore tested the receptor-binding

Table. Characteristics of patients infected with influenza A(H5N1) virus and samples collected, northern Vietnam, 2004–2010*

Patient no., virus isolated	HA clade	Sample	Date			Outcome
			Symptom onset	Hospitalization	Sample collection	
UT3040						
A/Vietnam/UT3040I/2004 (UT3040I)	1	TS	NA	NA	2004 Jan 6	Died
A/Vietnam/UT3040II/2004 (UT3040II)	1	TA	NA	NA	2004 Jan 7	Died
UT31312						
A/Vietnam/UT31312I/2007 (UT31312I)	2.3.4	TA	NA	NA	2007 Jul 25	Died
A/Vietnam/UT31312II/2007 (UT31312II)	2.3.4	TA	NA	NA	2007 Jul 26	Died
A/Vietnam/UT31312III/2007 (UT31312III)	2.3.4	TS	NA	NA	2007 Jul 25	Died
UT31394						
A/Vietnam/UT31394I/2008 (UT31394I)	2.3.4	TS	NA	NA	2008 Jan 17	Died
A/Vietnam/UT31394II/2008 (UT31394II)	2.3.4	TA	NA	NA	2008 Jan 17	Died
UT31413						
A/Vietnam/UT31413I/2008 (UT31413I)	2.3.4	TS	2008 Feb 3	NA	2008 Feb 13	Died
A/Vietnam/UT31413II/2008 (UT31413II)	2.3.4	TA	2008 Feb 3	NA	2008 Feb 13	Died
UT36250						
A/Vietnam/UT36250I/2010 (UT36250I)	2.3.4.2	TS	2010 Mar 5	2010 Mar 10	2010 Mar 10	Survived
A/Vietnam/UT36250II/2010 (UT36250II)	2.3.4.2	TA	2010 Mar 5	2010 Mar 10	2010 Mar 11	Survived
UT36282						
A/Vietnam/UT36282I/2010 (UT36282I)	2.3.4.1	TS	2010 Mar 27	2010 Apr 2	2010 Apr 1	Survived
A/Vietnam/UT36282II/2010 (UT36282II)	2.3.4.1	TS	2010 Mar 27	2010 Apr 2	2010 Apr 3	Survived
UT36285						
A/Vietnam/UT36285I/2010 (UT36285I)	2.3.4.1	TS	2010 Apr 2	2010 Apr 4	2010 Apr 4	Survived
A/Vietnam/UT36285II/2010 (UT36285II)	2.3.4.1	TS	2010 Apr 2	2010 Apr 4	2010 Apr 8	Survived

*HA, hemagglutinin; NA; not available; TA, throat swab; TS, tracheal aspirate.

properties of these variants by using a solid-phase binding assay with synthetic α2,3- or α2,6-linked sialylglycopolymers (Figure 2, panels B–O). A virus possessing the HA gene from the human A/Kawasaki/173/2001 (H1N1) virus and the remaining genes from CA04 (CA04/K173) served as a control virus for human virus receptor-binding specificity. As expected, this virus preferentially bound to α2,6-linked sialylglycopolymers (Figure 2, panel B). In contrast, CA04-reassortant H5 viruses encoding the majority and minority variants from infected persons preferentially bound to avian-type α2,3-linked sialylglycopolymers (Figure 2, panels C–O). In the context of CA04/UT3040II-HA, the HA-186K variant bound more efficiently to human-type receptors than did consensus CA04/UT3040II (encoding HA-186N) (Figure 2, panels C and E); however, this effect was not detected with the same viruses in the glycan arrays (Figure 3, panels A, C), or when we tested the same mutation in UT31312III HA (Figure 2, panels F, G). Therefore, more rigorous evaluation is needed to assess the role of 186K in human-type receptor specificity.

We also performed a glycan array analysis on an array containing 135 synthetic glycans representing O-linked and N-linked glycans and linear glycan fragments with sialic acid in α2,3- and α2,6-linkages (online Technical Appendix Figure 1) (*15*). Glycan arrays can be used to evaluate the specificity of viruses for numerous glycans simultaneously and to assess avian- and human-type specificity with high stringency; however, they are less effective for assessing changes in avidity (*24*). Tested wild-type and mutant influenza A(H5N1) viruses interacted primarily with α2,3-linked glycans (Figure 3, panels A–G); however, some mutations affected the binding intensity to receptor glycans (e.g., compare Figure 3, panels F and G). Two human control viruses (CA04/K173 and A/Brisbane/10/2007) that we used in previous assays bound primarily to α2,6-linked glycans (Figure 3, panels H, I). Collectively, these data demonstrate that the H5 HA variants detected in infected humans retained their preferential binding to avian-type receptors.

Binding of HA Variants to Human Respiratory Tissues

We also tested the HA variants for their ability to bind to human respiratory airway samples (online Technical Appendix Figure 3). The human CA04/K173 virus bound extensively to epithelial cells of tracheal tissue cross-sections. The CA04/UT3040I/II-HA virus bound weakly to mucus secreted by goblet cells but not to epithelial cells. Unlike the results of our solid-phase binding assays, CA04/UT36250I/II-HA-138V, CA04/UT36282I/II-HA, and CA04/UT36282I/II-HA-138V/A displayed appreciable binding to epithelial cells in human tracheal tissues (online Technical Appendix Figure 3). To confirm virus binding specificity to sialic acids, we pretreated the tracheal tissues with *Arthrobacter ureafaciens* neuraminidase, which removes terminal sialic acids; as expected, virus binding to these samples was greatly reduced (online Technical Appendix Figure 4). The remaining viruses displayed little to no binding to tracheal epithelial cells. These findings show that although we detected polymorphisms at HA amino acid positions known to affect receptor binding, the variants tested did not acquire preferential binding to human-type receptors, although the HA-138V mutation in UT36250I

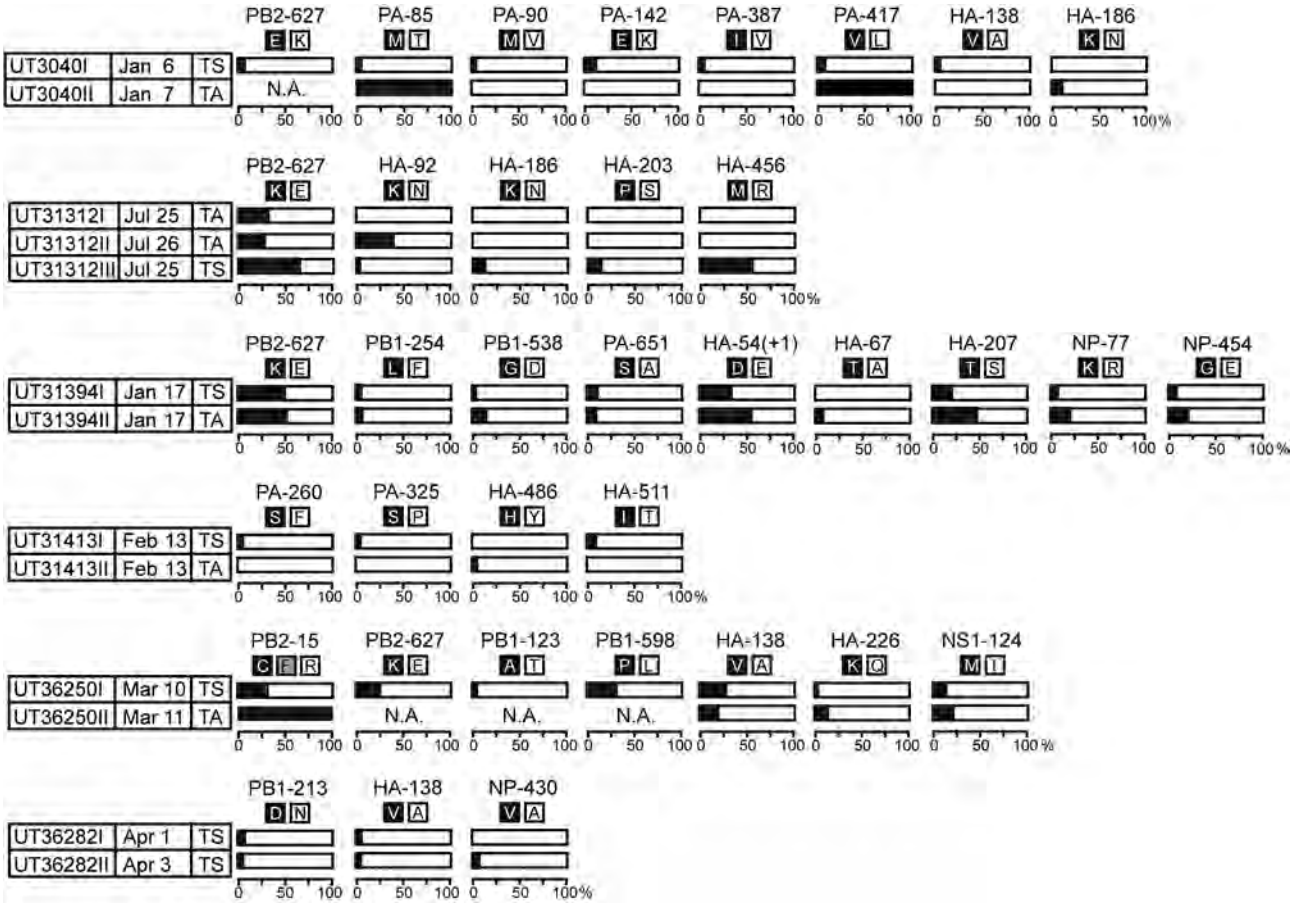

Figure 1. Frequencies of nonsynonymous single-nucleotide polymorphisms detected in ≥5% of sequence reads obtained from humans infected with influenza A(H5N1) virus, northern Vietnam, 2004–2010. Bar graphs depict the percentages of the indicated single-nucleotide polymorphisms from TS or TA samples. The amino acids at the respective positions are shown by the single-letter code. HA-54(+1) refers to an H5 HA amino acid insertion after position 54 that was not found in H3 HAs (which was used as a reference for the numbering of HA amino acid positions). HA, hemagglutinin; NP, nucleocapsid; NS, nonstructural; PA, polymerase acidic; PB, polymerase basic; TA, tracheal aspirate; TS, throat swab.

and the HA-138V/A mutation in UT36282I/II enhanced HA binding to epithelial cells in human respiratory tissue.

Stability of HA Variants

We (*4*) and others (*25*) have shown that increased HA stability is needed for the respiratory droplet transmissibility of H5 viruses among ferrets. We therefore tested the HA polymorphisms that were located away from the receptor-binding domain (Figure 4, panel A) for their effect on HA thermostability (Figure 4, panels B–G). The selected variants were incubated at 55°C for the indicated time intervals, after which loss of infectivity was determined by means of a plaque assay and HA hemagglutination activity by hemagglutination assay.

The human CA04/K173 virus displayed appreciably higher thermostability than the avian control virus CA04/VN1203 (possessing the A/Vietnam/1203/2004 [H5N1] HA gene in the genetic background of CA04). No appreciable differences in thermostability were detected between the minority and majority variants in CA04/UT31312II/III (Figure 4, panels B, E) and in CA04/UT31394I/II (Figure 4, panels C, F) except that CA04/UT31394II-HA-67T lost hemagglutination activity more rapidly than the other variants for unknown reasons. We did not, therefore, identify HA-stabilizing mutations that arose during replication in humans.

Polymerase Activity of Polymerase and NP Variants

Amino acid variations were located in multiple domains of each polymerase subunit. Our mini-replicon assay demonstrated that PB2-627K conferred significantly higher polymerase activity in mammalian cells than did PB2-627E (Figure 5), as demonstrated previously by us (*26*) and others (*25*). In addition, the UT36250I PB1-598P minority variant showed an ≈80-fold increase in polymerase activity at 37°C, but not at 33°C, relative to the PB1-598L majority variant (Figure 5, panels A and B). These potentially

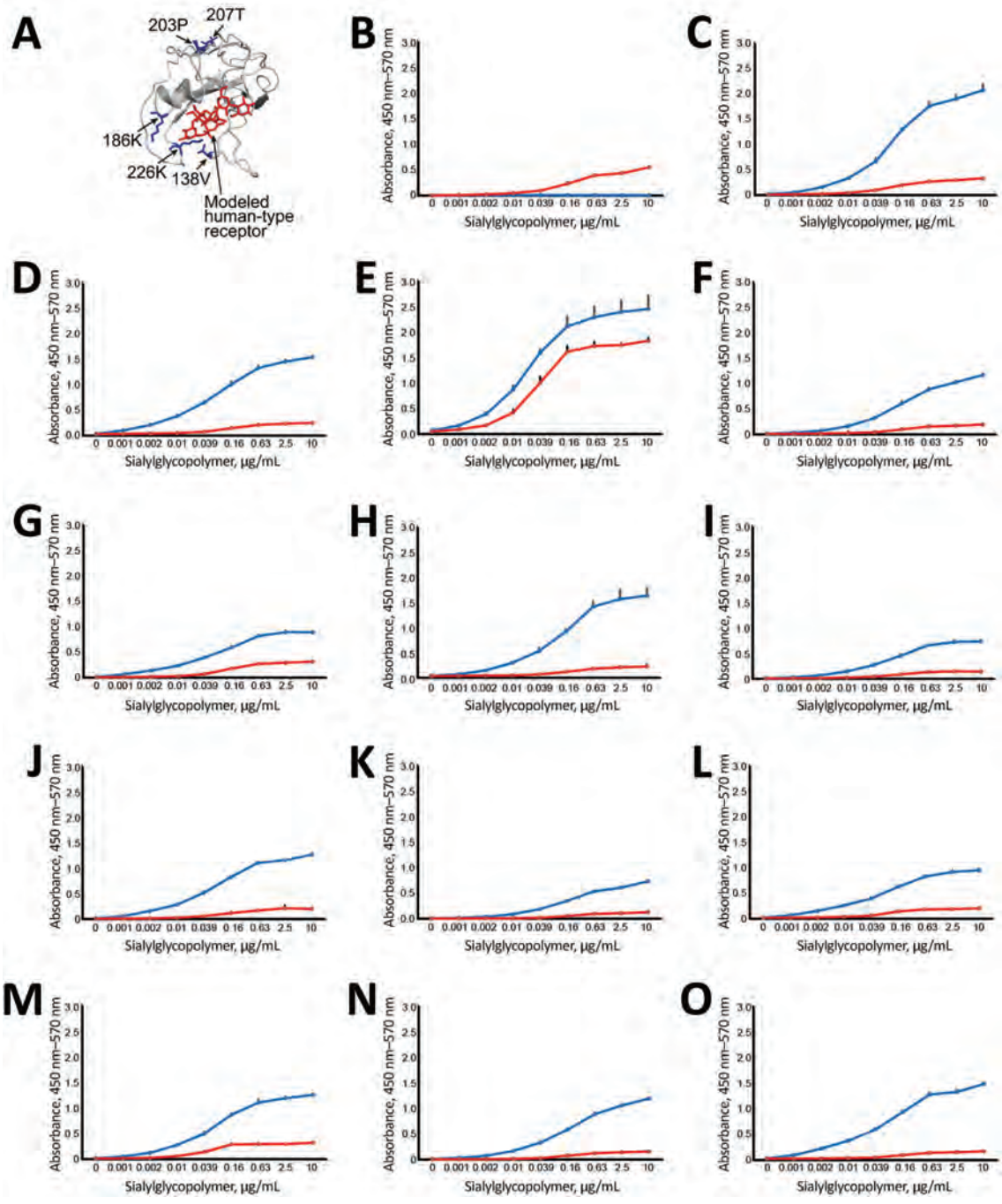

Figure 2. Effect of amino acid variations in hemagglutinin (HA) on influenza virus receptor binding specificities in influenza A(H5N1) virus isolates from humans, northern Vietnam, 2004–2010. A) Localization of selected amino acid variations detected in clinical samples. The detected HA changes were mapped onto the receptor-binding domain (aa positions 117–265) of a monomer of A/VN1203/2004 (H5N1) HA (Protein Data Bank accession no. 2FK0). Red indicates modeled human-type receptor; blue indicates positions of amino acid variations on the receptor-binding domain. B–O) Receptor-binding specificities of HA variants. Direct binding of virus to α2,3-linked (blue) or α2,6-linked (red) sialylglycopolymers was assessed. Shown is the mean receptor-binding specificity + SDs of triplicates of a single experiment. If 2 isolate numbers are listed (e.g., CA04/UT3040I/II), we tested the major sequence variant (which is identical between the 2 samples: B) CA04/K173; C) CA04/UT3040I/II-HA; D) CA04/UT3040I-HA-138V; E) CA04/UT3040II-HA-186K; F) CA04/UT31312III-HA; G) CA04/UT31312III-HA-186K; H) CA04/UT31312III-HA-203P-to-L (the proline residue introduced at position 203 of UT31312III HA mutated to leucine); I) CA04/UT31394II-HA; J) CA04/UT31394II-HA-207T; K) CA04/UT36250I/II-HA; L) CA04/UT36250I/II-HA-138V; M) CA04/UT36250II-HA-226K; N) CA04/UT36282I/II-HA; O) CA04/UT36282I/II-HA-138V/A (after introduction of valine at position 138 of UT36282I/II HA, we detected both valine and alanine at this position).

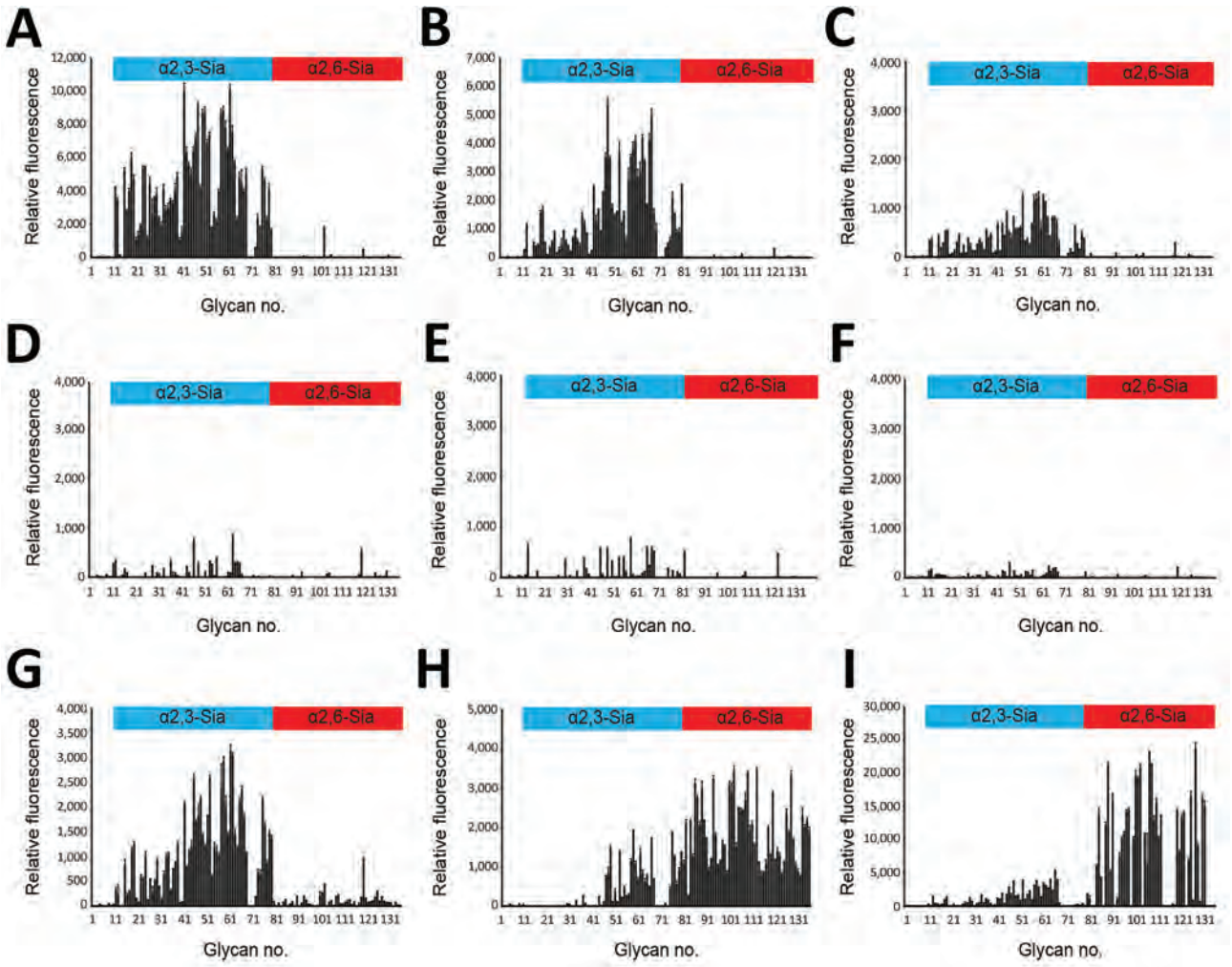

Figure 3. Glycan microarray analysis of selected influenza A(H5N1) virus isolates from humans, northern Vietnam, 2004–2010. The labeled viruses were applied to a microarray that included α2,3-linked (blue) and α2,6-linked (red) glycans, which are indicated by numbers on the *x*-axis (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/7/17-1441-Techapp1.pdf). Shown are the binding signals with error bars reflecting SEMs calculated from 4 of 6 replicates on the array after discarding the highest and lowest signals; note that the scales on the y-axis (relative fluorescence) vary because of differences in binding strength: A) CA04/UT3040I/II-HA; B) CA04/UT3040I-HA-138V; C) CA04/UT3040II-HA-186K; D) CA04/UT36250I/II-HA; E) CA04/UT36250I/II-HA-138V; F) CA04/UT36282I/II-HA; G) CA04/UT36282I/II-HA-138V/A; H) CA04/K173; I) Brisbane/10/2007.

advantageous mutations did not become dominant in some of the samples available for this study; however, we cannot know whether these mutations became dominant at later times for which samples are not available. Two mutations (PA-85M and PA-417V) occurred at a low frequency in UT3040I yet represented the only population in UT3040II. Of note, both mutations reduced polymerase activity at 1 or both temperatures tested (Figure 5).

In summary, we detected a complex pattern of phenotypic effects of avian virus polymerase complex mutations in infected humans. With the exception of PB2-627K, some mutations improved polymerase activity in mini-replicon assays but did not become dominant in the virus samples tested, whereas other mutations replaced the wild-type amino acid although they appeared to reduce polymerase activity in vitro. Thus, enhancement of polymerase activity in the mini-replicon assay did not necessarily correlate with mutations that became dominant in vivo.

Interferon-antagonistic Activity of NS1 Variants

The influenza A virus NS gene encodes 2 proteins, 1 of which (NS1) counteracts host innate interferon responses (*27*). HPAI A(H5N1) viruses are relatively resistant to the antiviral effects of host interferon responses (*28*), which might, in part, result from specific point mutations in the NS1 proteins of some of these viruses (*29*). We detected only 1 nonsynonymous polymorphism in NS1, a methionine (minority variant) or isoleucine (majority variant) at

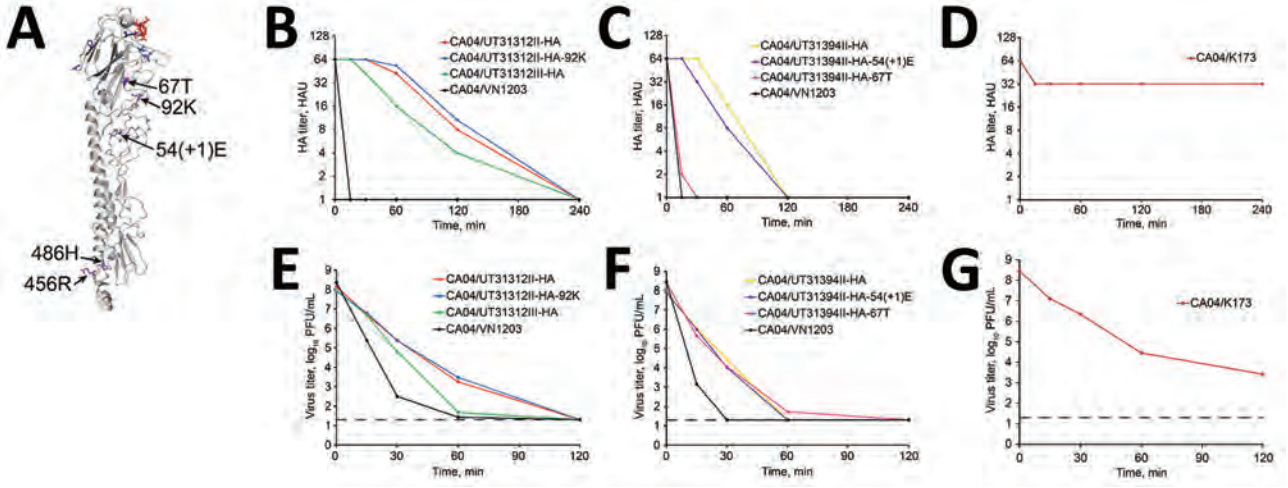

Figure 4. Effect of amino acid variations in HA on virus thermostability in influenza A(H5N1) virus isolates from humans, northern Vietnam, 2004–2010. A) Amino acid substitutions in non–receptor-binding domains mapped on the 3-dimensional structure of the monomer of VN1203 HA (Protein Data Bank accession no. 2FK0). Red indicates modeled human-type receptor; purple indicates positions of amino acid variations on the non–receptor-binding domains; blue indicates positions of amino acid variations on the receptor-binding domain corresponding to Figure 2, panel A. B–G) Thermostability of HA variants depicted in panel A. Amounts of viruses equivalent to 64 HA units were incubated at 55°C for 15, 30, 60, 120, and 240 min. B–D) HA titers in heat-treated samples were determined by performing HA assays with 0.5% turkey red blood cells: B) CA04/UT31312II-HA, CA04/UT31312II-HA-92K, CA04/UT31312III-HA, CA04/VN1203; C) CA04/UT31394II-HA, CA04/UT31394II-HA-54(+1)E, CA04/UT31394II-HA-67T, CA04/VN1203; D) CA04/K173. E–G) Virus titers of heat-treated samples determined by means of plaque assays in MDCK cells. Shown are the mean HA or virus titers of triplicates from a single experiment. Dashed lines indicate the detection limit for virus titration (20 PFU/mL). E) CA04/UT31312II-HA, CA04/UT31312II-HA-92K, CA04/UT31312III-HA, CA04/VN1203; F) CA04/UT31394II-HA, CA04/UT31394II-HA-54(+1)E, CA04/UT31394II-HA-67T, CA04/VN1203; G) CA04/K173. CA04/K173 virus was used as a control. HA, hemagglutinin.

position 124 of NS1 in samples UT36250I and UT36250II (Figure 1); the frequency of the NS1-124M minority variant increased slightly in samples collected on consecutive days. We compared the ability of both NS1 variants to interfere with interferon production and signaling. The UT36250I-NS1 (encoding the NS1-124I majority variant) and UT36250I-NS1-124M proteins showed similar ability to antagonize interferon production (online Technical Appendix Figure 5, panel A). However, UT36250I-NS1-124M was slightly more efficient than UT36250I-NS1 at suppressing interferon signaling (online Technical Appendix Figure 5, panel B). This finding might explain the slightly increased frequency of NS1-124M on the second day of sampling, although such slight differences in variant frequency and interferon signaling suppression might not be biologically significant.

Discussion

Worldwide, HPAI A(H5N1) viruses have infected ≈850 persons but have yet to adapt to humans. Because viral genetic and phenotypic diversity might facilitate adaptation in infected persons, we performed deep-sequencing and functional assays for influenza A(H5N1) viruses isolated from humans. We had access to only throat swab and tracheal aspirate samples; virus populations in other anatomic sites, such as alveoli, might differ. Nonetheless, the virus populations we observed were diverse, but most variants were detected only transiently, at low frequencies, or both, including variants with potentially beneficial traits (e.g., the PB1-598P mutation).

We found only a few sequence variants in >1 patient; they included PB2-627E/K, HA-138A/V, and HA-186N/K. PB2-627K is a known determinant of mammalian adaptation that increases the replicative ability of avian influenza virus polymerase complexes in mammals (*26*, *30*,*31*). Despite its strong effect on adaptation in mammals, in some samples, it coexisted with PB2–627E (Figure 1). Similarly, HA-138V and HA-186K, which can increase binding to human-type receptors (*14*,*32*–*34*), were detected in 3 and 2 patient samples, respectively. Although the HA-186K mutation exhibited increased binding to human-type receptors for 1 strain in the sialylglycopolymer binding assay, it did not alter the overall avian-type receptor specificity of the variants assessed by the glycan arrays, and both HA-138V and HA-186K coexisted with other viral variants in the samples tested. All variants bound to a wide variety of avian-type receptors, with no clear differences in specificity. Besides PB2-627K, the PB1-598P minority variant conferred a sizable increase in polymerase activity when compared with the majority population (encoding PB1-598L) (Figures 1, 5). Position 598 of PB1 lies in the so-called thumb domain of PB1 (*35*),

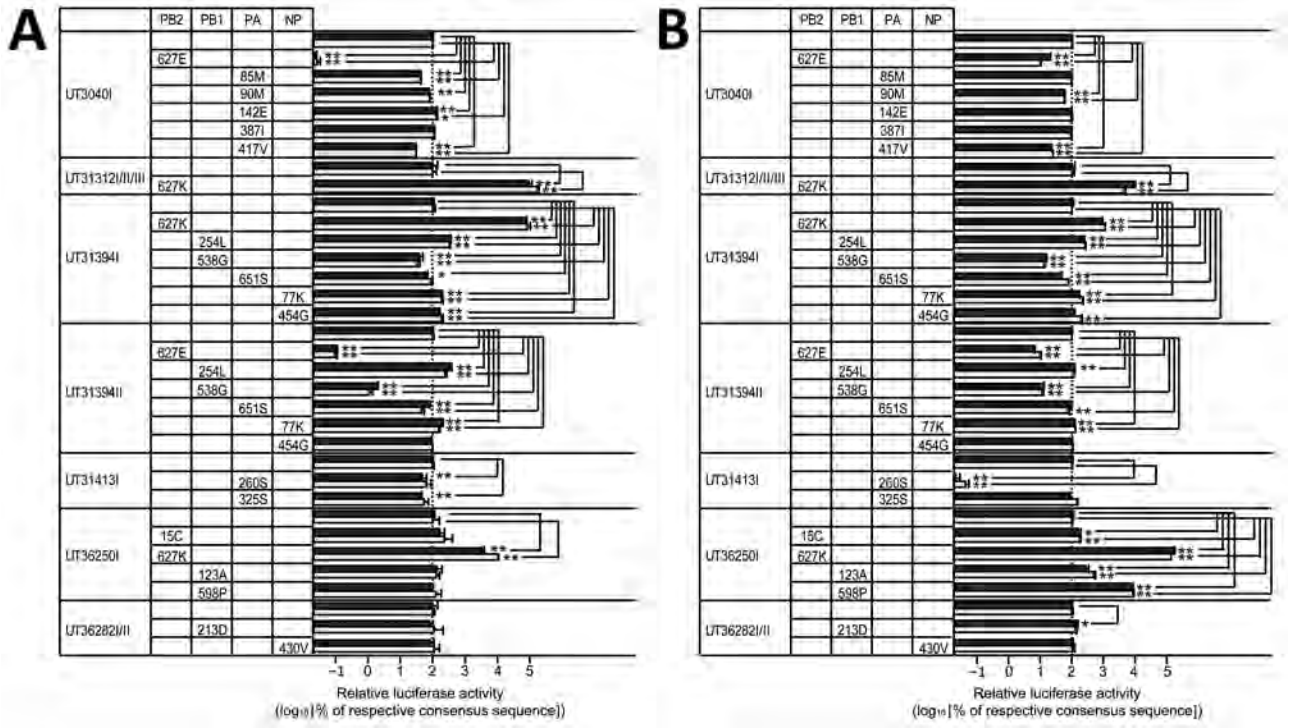

Figure 5. Effect of amino acid variations in polymerases and NP on viral polymerase activity in influenza A(H5N1) virus isolates from humans, northern Vietnam, 2004–2010. 293T cells were transfected with plasmids encoding the viral replication complex (PB2, PB1, PA, and NP), with a plasmid for the expression of an influenza virus mini-genome that encodes the firefly luciferase gene, and with a plasmid encoding *Renilla* luciferase (transfection control). If 2 or 3 isolate numbers are listed, we tested the major sequence variant, which is identical among the samples. The cells were incubated at 33°C (A) or at 37°C (B) for 24 h, and firefly and *Renilla* luciferase activities were measured by use of the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The firefly luciferase values were divided by the *Renilla* luciferase values to normalize for variances in transfection efficiency. The experiments (each in triplicate) were independently repeated twice. The mean relative viral polymerase activities plus SDs of each independent experiment are shown as black and white bars. The viral polymerase activity of the respective majority variant was set to 100%. NP, nucleocapsid; PA, polymerase acidic; PB, polymerase basic; *p<0.05; **p<0.01 (both by Dunnett test).

and the amino acid at this position might interact with the PA polymerase subunit. We do not know how the L-to-P change would affect influenza virus replication; mechanistic follow-up studies will be necessary to answer this question. There was no clear association between particular virus mutations and clinical outcomes of patients in this study. Indeed, even the PB2-627K mutation was not always detected in patients who died (e.g., patient UT31413).

The evolutionary forces that govern mammalian adaptation and the emergence of viruses capable of transmission among humans are largely unknown. RNA virus replication generates within-host genetic diversity that can rapidly change because of selective pressures (*36*,*37*). One might assume that in infected humans, positive selection favors the rapid outgrowth of variants that possess mammalian-type traits and that variants with human-adapting mutations might commonly be found as subpopulations within infected persons even if they were not present in most viruses. However, we detected few variants with such traits; only the known mammalian-adapting PB2-627K substitution became dominant in several virus populations. These findings might imply that for a variant to become dominant, additional potentiating mutation(s) are necessary within the same genetic background as the adapting mutation. In other words, adaptive mutations might alter virus phenotypes but might require the presence of potentiating mutations to maintain viral fitness. For instance, we (*4*) and others (*25*) have shown the crucial role of another phenotypic trait, increased HA stability, for the respiratory droplet transmissibility of H5 viruses among mammals. Thus, avian influenza virus adaptation to humans probably does not occur in a steady linear fashion; rather, it probably depends on the stepwise accumulation of potentiating mutations that favor the emergence of a particular adaptive mutation, followed by the accumulation of additional potentiating mutations that favor further adaptive mutations, and so on. Our study provides a framework for testing this hypothesis by using deep sequencing to analyze avian influenza virus populations within humans, followed by phenotypic characterization in the laboratory.

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Geographically Diverse Clusters of Nontoxigenic *Corynebacterium diphtheriae* Infection, Germany, 2016–2017

Alexandra Dangel,[1] Anja Berger,[1] Regina Konrad, Heribert Bischoff, Andreas Sing

From 2016 through the middle of 2017, the German Consiliary Laboratory on Diphtheria noted an increase in nontoxigenic *Corynebacterium diphtheriae* isolates submitted from cities in northern Germany. Many patients for whom epidemiologic data were available were homeless, alcohol or drug abusers, or both. After performing routine diagnostics and multilocus sequence typing (MLST), we analyzed isolates of sequence type (ST) 8 and previously submitted isolates by whole-genome sequencing. Results were analyzed for phylogenetic relationship by core genome MLST (cg-MLST) and whole-genome single-nucleotide polymorphism profiles. Next-generation sequencing–based cg-MLST revealed several outbreak clusters caused by ST8; the geographic focus was in the metropolitan areas of Hamburg and Berlin. To achieve enhanced analytical depth, we used additional cg-MLST target genes and genome-wide single-nucleotide polymorphisms. We identified patient characteristics and detected transmission events, providing evidence that nontoxigenic *C. diphtheriae* infection is a potential public health threat in industrialized countries.

Diphtheria and its causative pathogen, *Corynebacterium diphtheriae*, have been drifting out of focus in Western countries because of effective vaccination programs. In 1994, the World Health Organization aimed to eliminate these infections by the year 2000, but epidemics such as that in the former Soviet Union and outbreaks in other countries show that the goal of elimination by 2000 was not reached (*1*) and nationwide vaccination programs are still essential (*2*). Despite high vaccination coverage in most Western countries, cases of wound and bloodstream infections caused by *C. diphtheriae* are rising because vaccination with toxoid prevents only diphtheria toxin–associated symptoms. Therefore, *C. diphtheriae* is again causing severe public health problems in many Western industrialized countries (*1*,*3*). Health conditions associated with certain socioeconomic factors that enhance the risk for infection have been identified, including heart disease (*4*), cirrhosis, dental caries (*5*), diabetes mellitus (*6*), and skin colonization (*6*). The most serious risk factors are intravenous drug or alcohol abuse along with their various resultant health complications, as well as homelessness (*7*,*8*).

From the middle of 2016 through the middle of 2017, the German Consiliary Laboratory on Diphtheria noted an increase in nontoxigenic *C. diphtheriae* isolates submitted from diagnostic microbiological laboratories or local public health authorities in several cities of northern Germany, especially Berlin and Hamburg. A smaller number of isolates came from surrounding areas and other bigger cities in northwestern Germany. Although epidemiologic data were not available for each patient, many patients colonized or infected with nontoxigenic *C. diphtheriae* were reported to be homeless, abusers of alcohol or intravenous drugs, or any combination of these factors. To determine if an outbreak with a common strain was ongoing among members of this risk group in northern Germany, we characterized the isolates in more detail by conducting molecular typing analyses to study phylogenetic relationships in an approach with stepwise increased resolution.

As a first step, we performed multilocus sequence typing (MLST), which has developed over the past 20 years (*9*) as a standard molecular typing assay with clear and reproducible nomenclature for >90 bacterial pathogenic species, including the 2 potentially toxigenic species *C. diphtheriae* (*10*) and *C. ulcerans* (*11*). However, with current next-generation sequencing (NGS) techniques that enable highly parallel sequencing of whole bacterial genomes in a few days and within a reasonable budget, investigations of outbreaks or clinical events can benefit from this enhanced discriminatory power. Therefore, we

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used NGS together with core genome MLST (cg-MLST) as a second step, applying high discriminatory depth with clear nomenclature. This technique has been successfully applied in outbreak investigations of a broad range of bacteria (*12*–*14*) and has recently been used for *Corynebacterium* (*15*). To confirm and refine the results in highest resolution, we used phylogeny based on genome-wide single-nucleotide polymorphisms (SNPs).

Material and Methods

Bacteriology

We cultured samples on 5% sheep blood and serum tellurite agar plates (both Becton Dickinson, Heidelberg, Germany). Colonies suggestive of coryneform bacteria were subjected to MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry (Microflex; Bruker Daltonics, Bremen, Germany) and API Coryne (bioMérieux, Marcy-L'Ètoile, France) according to published methods (*16*) for species and biovar determination. We analyzed presence of the toxin gene by using a quantitative PCR approach (*17*).

Sequencing and Data Analyses

For DNA extraction, we used isolates from blood agar plates to inoculate liquid overnight cultures in brain–heart infusion broth (Thermo Scientific, Schwerte, Germany). Cells were harvested, resuspended in Tris buffer with 50 mg/mL lysozyme, and incubated 30 min at 37°C. We extracted genomic DNA by using a modified protocol of the Maxwell 16 LEV Blood DNA Kit on the Maxwell 16 instrument (Promega, Mannheim, Germany), starting with the addition of 150 μL incorporation buffer, 200 μL lysis buffer, 30 μL proteinase K, and 10 μL10 mg/mL RNase A, followed by incubation for 2 h at 65°C and then addition of 300 μL lysis buffer and transfer to the instrument. Genomic DNA was eluted in Tris buffer.

Routine MLST was performed by PCR of the 7 target regions *atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *odhA*, and *rpoB* by using a previously described protocol (*10*) and Sanger sequencing at GATC Biotech (Konstanz, Germany). Sequences were analyzed with the SmartGene IDNS (SmartGene, Lausanne, Switzerland). Unknown alleles were submitted to the *C. diphtheriae* PubMLST website (https://pubmlst.org/cdiphtheriae/; *18*) for assignment of new alleles or sequence types (STs).

Whole-genome libraries for NGS were prepared with the Nextera XT kit (Illumina, San Diego, CA, USA), and sequencing was performed with 2 × 250-bp paired-end reads on the Illumina MiSeq. Quality control of NGS sequencing runs was accomplished by using Illumina SAV software (http://emea.support.illumina.com/sequencing/sequencing_software/sequencing_analysis_viewer_sav.html).

We generated a *C. diphtheriae* cg-MLST scheme, defining specific target loci for whole-genome sequencing data typing, by using the SeqSphere+ target definer tool (Ridom, Munster, Germany) with default options (*19*). As reference, we used the genome of strain NCTC 13129 from the National Center for Biotechnology Information (NCBI) (accession no. BX248353.1/NC_002935.2). We used all 14 complete *C. diphtheriae* genomes available from NCBI as query sequences (accession nos. NC_016782.1, NC_016799.1, NC_016800.1, NC_016801.1, NC_016785.1, NC_016786.1, NC_016802.1, NC_016787.1, NC_016788.1, NC_016783.1, NC_016789.1, NC_016790.1, NZ_LN831026.1, NZ_CP018331). The resulting cg-MLST scheme consisted of 1,553 target loci. An accessory target scheme with 601 more loci was defined during the same process. The targets of the latter were genes not found in each query genome, or they were genes found multiple times in query genomes, overlapping in the reference, or showing an incorrect number of stop codons in >80% of the query genomes.

We performed next-generation–based MLST and cg-MLST with de novo assembled contigs after read-trimming and assembly by using Velvet in SeqSphere+ (Ridom, Munster, Germany) (*20*) with default settings. We performed in silico MLST by using the previously described 7 target loci and cg-MLST by using the generated cg-MLST or extended cg-MLST scheme of 1,553 or 2,154 target loci. After typing and assigning allele numbers, we calculated distances for tree building; during pairwise comparisons of allele profiles, we ignored missing values. Subsequently, minimum spanning trees were generated. We defined a cluster as a group of closely related cg-MLST–analyzed isolates differing by ≤5 alleles and subclusters with the same similarity threshold but after extended cg-MLST.

For SNP-based phylogeny, NGS reads were adapter-clipped and trimmed for quality and trimmed from short reads <50 bp with trimmomatic (*21*). By mapping against the *C. diphtheriae* reference genome, we performed SNP calling and filtering with the run_snp_pipeline script of the PHEnix pipeline by Public Health England (https://github.com/phe-bioinformatics/PHEnix). This process included bwa-mem mapping (*22*) with default settings and variant calling and filtering (frequency >0.7, mapping quality score >30, read depth >8) by GATK2 Unified Genotyper (*23*). We generated variant call files containing SNP positions passing filters and all positions not passing filters. SNPs were concatenated to FASTA-format alignments with the vcf2fasta-script from the PHEnix pipeline, converting bases at filter-failed positions to letter N, indicating an ambiguous base call, and allowing ≤90% missing data per sample and ≤20% missing data at each specific site within the sample set. We generated maximum-likelihood trees from SNP alignments by using RaxML (*24*), including 100

bootstrap replicates, and uploaded sequencing data to the NCBI sequence read archive (https://www.ncbi.nlm.nih.gov/sra; BioProject ID PRJNA416260).

Results

Samples and Epidemiologic Data

Our study started with the observation of an increasing number of *C. diphtheriae* isolates submitted to the German Consiliary Laboratory on Diphtheria by diagnostic microbial laboratories or local health authorities from different areas of northern Germany from mid-2016 through early 2017. The geographic regions from which increased *C. diphtheriae* isolates were submitted included Berlin; Hamburg and its surrounding cities Bremen, Bremerhaven, Kiel, and Schwerin; and other cities in northern Germany (Hanover, Dortmund, Bochum, Essen, Leverkusen, and Trier). Among many patients for whom epidemiologic data were available, the most common socioeconomic factors were homelessness, drug or alcohol abuse, or both. Initial routine diagnostic testing, including species identification of *C. diphtheriae* by MALDI-TOF and Coryne API, toxin gene presence testing by quantitative PCR, and random typing by MLST, identified 19 nontoxigenic cases caused by ST8, which led to suspicion of a potential outbreak. We selected those 19 ST8 isolates for NGS analysis, together with samples submitted from the same geographic regions, either from the same period and not yet analyzed by MLST or from an earlier period (2012–2015) and typed as ST8 or not yet typed. A total of 76 nontoxigenic *C. diphtheriae* isolates, submitted from April 2012 through July 2017 from the delineated geographic regions were subjected to the NGS-based in-depth outbreak investigation (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/7/17-2026-Techapp1.pdf).

The 76 analyzed isolates belonged mainly to biotype gravis (n = 45), followed by biotypes mitis (n = 30) and belfanti (n = 1). Most (63 [83%]) patients were male. For 25 patients, the common socioeconomic characteristics previously identified as risk factors (drug abuse, alcohol abuse, homelessness, or any combination) were confirmed (*6*,*8*). However, because of the retrospective design of the study, for many patients, epidemiologic information was not available or was incomplete. For 19 patients, travel to other countries or origin from other countries was reported. For 7 patients, mixed infections involving several pathogens were reported. Median patient age was 45 years; 38% were 40–60; 37%, 20–40; 13%, >60; and 5%, <20 years of age. Most (61 [80%]) strains were isolated from wound infections (e.g., 7 ulcers, 3 abscesses, 2 phlegmons, 1 deep wound after an amputation, 1 insect bite, 1 scratch, and 1 burn). Two wound infections, located on a patient's ear and hand, were associated with previous human bites. Most wound infections were reportedly on the lower (n = 26) or upper (n = 15) limbs. For the other patients, no wound specifics were reported. For 9 patients, severe invasive complications had developed, including 1 case of endocarditis, 2 cases of bacteremia, and 6 cases of sepsis. For the remaining patients, the type of infection was either not specified (n = 2) or the report listed diagnoses of single cases of peritonsillar abscess, olecranon bursitis, pharyngitis, tonsillitis, or investigation of carrier status.

Whole-Genome Sequencing Results

NGS runs with 2 × 250-bp paired-end reads resulted in 74.5%–79.5% of Q30 bases. An average coverage of 29.3–179.2-fold per sample was obtained; 75 of 76 samples reached coverage of >30, and 73 samples reached coverage of >50.

NGS reads were assembled de novo, and contigs of yet untyped isolates were analyzed by MLST with the 7-gene scheme (*10*). As expected from the sample selection, among the 20 STs identified, the largest group was formed by ST8 (n = 41 [54%] isolates), consistent with recent observations of ST8 being 1 of the 2 most abundant *C. diphtheriae* STs in PubMLST and thus probably in central Europe (*3*). The next most commonly identified STs were ST130 (10 isolates) and ST439 (5 isolates). All other STs were associated with only 1–5 isolates each (online Technical Appendix). cg-MLST with an in-house generated scheme, consisting of 1,553 *C. diphtheriae*–specific target loci, and visualization in a minimum spanning tree revealed that the different STs clustered in ST-specific branches (online Technical Appendix Figure). All 41 ST8 isolates were bundled in a distinct branch, separated from all other STs, different from all other isolates by ≥983 alleles. The ST8 branch was inspected in more detail and showed 4 clearly distinguishable clusters, which came from 2–17 isolates (Figure 1). The maximum difference between the isolates within each cluster ranged from 3 to 5 alleles. Of note, all 9 ST8 isolates from Hamburg belonged to 1 cluster of 14 isolates, together with isolates from Kiel, Essen, and Leverkusen, but none from Berlin. The other 2 bigger clusters with 17 (cluster 2) and 6 (cluster 3) isolates included isolates from Berlin and various other cities (Leverkusen, Bremen, and Hanover in cluster 2 and Leverkusen and Trier in cluster 3) but none from Hamburg. Cluster 4 consisted only of 2 isolates from Hanover, showing a difference of 3 alleles. Clusters 1, 2, and 4 consisted solely of biotype gravis, whereas cluster 3 consisted mainly of isolates of biotype mitis except for 1 isolate that was biotype gravis.

Enhanced Resolution Analysis by Accessory Targets and SNPs

To enhance resolution, we subsequently analyzed the branch of ST8 isolates showing the 4 described clusters

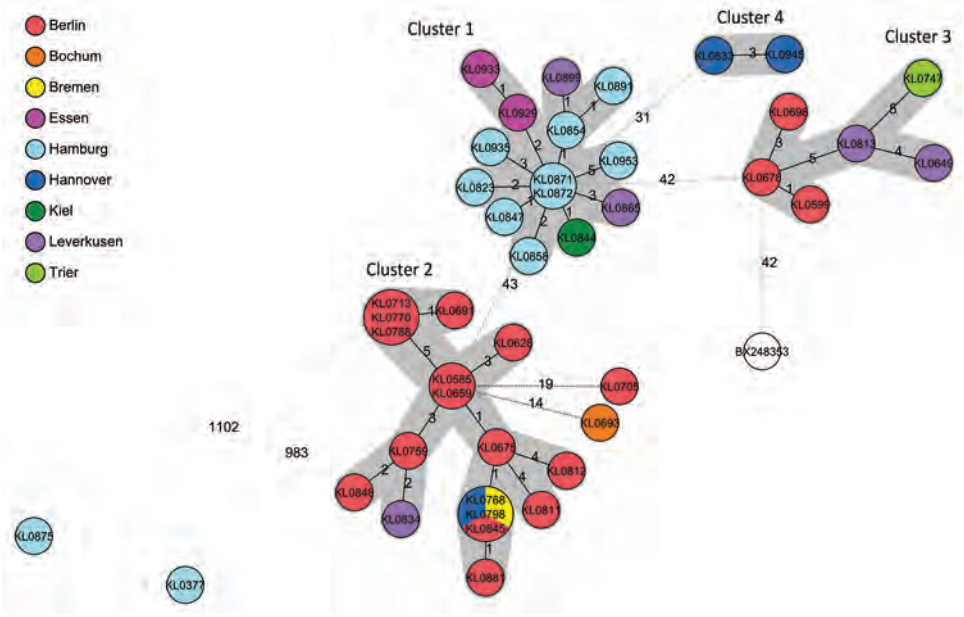

Figure 1. Minimum-spanning tree of core genome multilocus sequence typing with 1,553 targets of the nontoxigenic *Corynebacterium diphtheriae* sequence type 8 (ST8) isolate branch for isolates submitted from northern Germany, January 2015–June 2017. The branch of 41 ST8 typed isolates is shown, together with the 2 nearest isolates from other STs KL0875 (ST441) and KL0377 (ST123) and the reference genome used (GenBank accession no. BX248353). Allelic distances between isolates are indicated, clusters with allele difference ≤5 are shaded in gray, and the reference genome is shown in white.

by conducting a second, extended, cg-MLST analysis and whole-genome SNP phylogeny. Therefore, the cg-MLST scheme was enlarged by adding 601 accessory targets to the 1,553 cg-MLST targets. To confirm and further refine results, we performed SNP-based maximum-likelihood phylogeny and generated the phylogenic tree from concatenated SNP sequences after reference-based mapping and variant calling.

The general phylogenetic structure of the ST8 branch, with its 4 main clusters, comprising isolates from patients involved in the outbreak, could be confirmed by including more genomic positions in extended cg-MLST (Figure 2, panel A). In addition, the SNP-based phylogenetic tree organizes the isolates in cluster-confirming branches (Figure 2, panel B). The distances within the clusters were generally enhanced by including more target regions. Among the isolates of all 4 clusters, the maximum distance was 124 SNPs. In cluster 1, locally restricted to mainly Hamburg, and in cluster 4 with the 2 isolates from Hanover, the observed similarity of the isolates was still very high. In cluster 1, only the 2 most recent isolates from May and June 2017 drifted from the others with an increased maximum allelic difference of 8, corresponding to 13 SNPs. Considering the cluster threshold of 5 alleles previously defined in cg-MLST, the other 12 isolates still fell within a common cluster named subcluster 1. The maximum difference in cluster 4 was enhanced from 3 to 5 alleles. Distances between isolates of clusters 2 and 3 with a geographic focus in Berlin also increased to 8 alleles, corresponding to 32 SNPs within cluster 2 and 21 SNPs in cluster 3. This higher heterogeneity led to a partial decomposition of clusters 2 and 3. Cluster 2, especially, broke into 3 smaller subclusters (Figure 2, panel A) with 7, 4, and 3 isolates in subclusters 2, 3, and 4, differing in 19, 3, and 10 SNPs, respectively. For isolates KL0811 and KL0698 only, assignment to subcluster 2 could not be confirmed by SNP analysis, indicating, as expected, that the depth of SNP phylogeny was higher than that of extended cg-MLST.

Discussion

The phylogenetic analysis of the ST8 branch (n = 41 isolates) revealed 4 main outbreak clusters. Generally, the clusters were concentrated around geographic areas, especially Hamburg (cluster 1) and Berlin (clusters 2 and 3). Moreover, there were no clusters in which isolates from Hamburg and Berlin were found together. Three isolates from cluster 2 were genomically identical, although they were submitted from Bremen (KL0768), Hanover (KL0798), and Berlin (KL0845) during March–September 2016. Of note, transmission from 1 city to another occurred and is not surprising because of the considerable travel within the population group.

Generally, clusters 2 and 3, including the isolates from Berlin, seemed to be more heterogeneous and fragmented in smaller subclusters when resolution of analysis was enhanced by including more genes in the cg-MLST scheme or by SNP analysis. In contrast, within the cluster comprising the Hamburg isolates, very high genetic similarity was still noted. It is highly likely that within the 4 main clusters, and especially their resulting subclusters, several direct transmission events have taken place. For example, epidemiologic data proved a direct transmission event for isolates KL0929 and KL0933 in cluster 1, which differed by only 1 allele/1 SNP. They were submitted within 1 week in April 2017 and were isolated from 2 wound infections (from a homeless man and woman from Essen with a proven

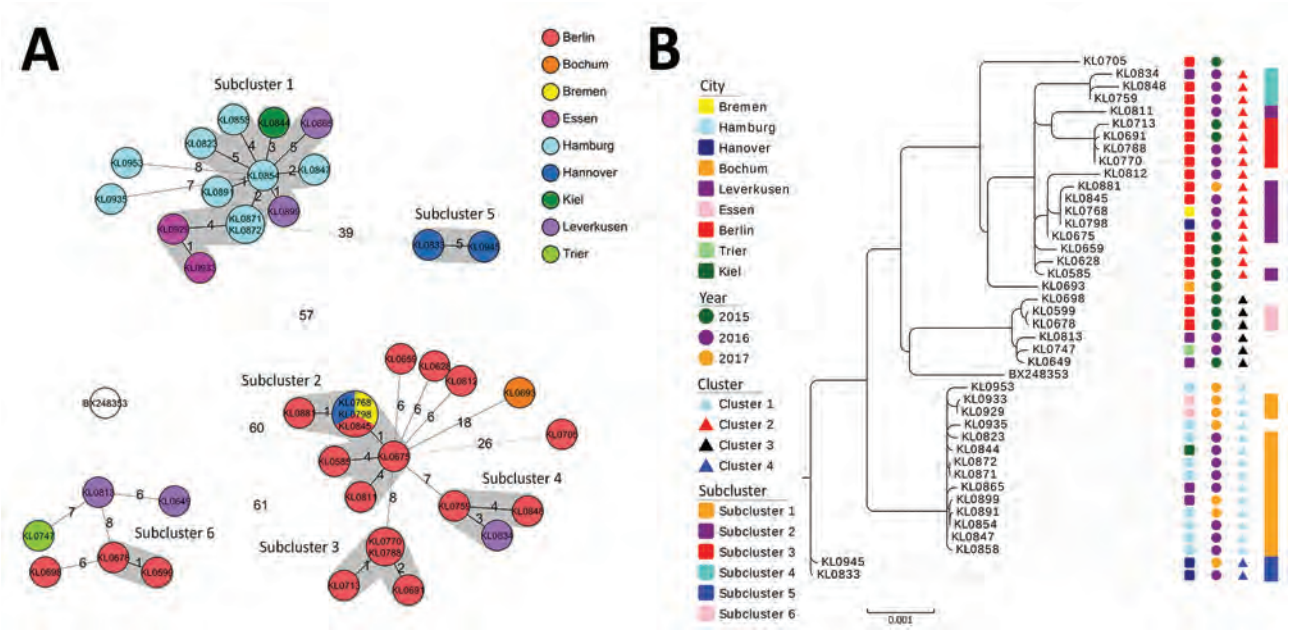

Figure 2. Enhancement of phylogenetic analysis depth of the 41 nontoxigenic sequence type 8 *Corynebacterium diphtheriae* isolates submitted from northern Germany, January 2015–June 2017, and reference genome. A) Phylogenetic minimum-spanning tree of isolates analyzed by extended core-genome multilocus sequence typing (cg-MLST), based on 2,154 target loci. Allele distances between isolates are indicated. Gray indicates subclusters with allele difference ≤5; white indicates the reference genome (BX248353). B) Phylogenetic maximum-likelihood tree based on genome-wide single-nucleotide polymorphism profiles. From left to right, the color-coded columns indicate isolate affiliations to submitting city, isolate submission year, cg-MLST cluster based on allelic difference of ≤5 alleles out of 1,553 cg-MLST target loci, and extended cg-MLST-subclusters based on allelic difference of ≤5 alleles out of 1,553 plus 601 extended cg-MLST target loci. Relative distances are based on 30 000 genomic positions. Scale bar indicates the portion of relative distances based on 14,920 distinct aligned positions.

epidemiologic link). Their high similarity to the remaining isolates from cluster 1, originating mainly from Hamburg, also suggests a tight transmission chain between these isolates. However, for the transmission between Essen and Hamburg, we found no epidemiologic link. Unfortunately, for many patients not enough information was available for us to draw a clear epidemiologic link or to identify further contact persons as part of the transmission chain, even for those with genetically identical isolates. For example, in 2 pairs of isolates, 1 from Hamburg (KL0871, KL0872; 0 SNP distance) and 1 from Berlin (KL0770, KL0788; 1 SNP distance), each pair came from a human bite–associated wound infection of a homeless man and a genetically virtually identical isolate from a limb-located wound infection of another patient. However, no epidemiologic link between the patients was available other than their isolates having been submitted from the same city. It is conceivable that these patients may not have infected their respective counterpart directly but rather that more undetected cases, possibly also of carrier status, may have contributed.

Even if epidemiologic data enabling links to cases are missing, the specific environment of the group of homeless and drug-using patients not only enhances the individual risk of acquiring infection but may also contribute to infection chains (e.g., by the reuse or shared use of needles and lack of hygiene measures) (*25*). For example, several patients had abscesses at a needlestick site. Several isolates of cluster 2 (KL0759, KL0788, KL0812, and KL0845) originated from patients who reportedly lived together in an abandoned house in Berlin, suggesting a common source or mode of transmission.

Pathogenic features such as invasiveness could generally not be connected to a specific outbreak clone. Unfortunately, information about the portal of entry was not submitted for all invasive strains. Wound infections for 4 of 9 patients and pneumonia for 1 patient were reported in addition to the underlying systemic disease. Both isolates from cluster 4 that were genetically similar (isolates KL0833 and KL0945) were responsible for endocarditis and sepsis. The other invasive isolates (KL0507, KL0675, KL0693, KL0768, KL0881, KL0954, KL0964) originated from persons in various cities and at various dates or belonged to different STs; thus, their invasiveness probably depended on host factors.

The more heterogeneous Berlin-focused clusters 2 and 3 were submitted from 2015 through January 2017. In contrast, isolates of homogeneous clusters 1 and 4 were submitted from June 2016 through June 2017. Expanded

cg-MLST and SNP-based phylogeny showed a less close relationship for clusters 2 and 3, with isolates submitted from an earlier and longer time frame, than within clusters 1 and 4, with isolates submitted from a later and shorter time frame. For several isolate connections within the clusters, a chronological order could be assumed from the SNP-based phylogenetic tree (e.g., between isolates KL0675, KL0768/KL0798/KL0845, and KL0881 in cluster 2) (Figure 2, panel B). To visualize these ordered connections, inclusion of earlier isolates in the dataset proved useful. Even in the very homogeneous cluster 1, the most recent isolates KL0935 and KL0953 veered away in the extended cg-MLST analysis (Figure 2, panel A) and showed an enhanced branch length in the maximum-likelihood tree (Figure 2, panel B). These observations show hints for evolution of the outbreak strains. However, it seems that evolution proceeded rather slowly because allele and SNP profiles showed high similarities in related isolates over longer periods of several months to almost a year (e.g., clusters 1 and 4). However, not much is known about distances characterizing clonal complexes or parentage within the genus *Corynebacterium*. As an example of a non–outbreak-related ST8 isolate, we included the used reference genome in cg-MLST and SNP phylogeny, which is a UK-derived representative of the former Soviet Union outbreak in the 1990s (*26*). This isolate showed a similar allelic difference to the 4 clusters as did the clusters between each other and was arranged at a superordinate branch of cluster 3 in the SNP dendogram. We concluded that the 4 geographically concentrated clusters have probably been evolving separately for years. To classify isolates more clearly into common or separated ancestorship in the future, allelic or SNP difference threshold definitions for clonal complexes would be of great advantage. To gain the underlying knowledge, more sequencing studies of carefully selected isolate sets will be needed.

In conclusion, by whole-genome sequencing analysis, we identified several outbreak clusters of nontoxigenic *C. diphtheriae* overlapping with specific geographic areas in metropolitan areas of northern Germany. Direct transmission between patients probably occurred within these local clusters. In contrast, the different clusters seem to have been separated from each other for years. Nonetheless, outbreak strains are persisting and show an ongoing, although slow, evolution. Our stepwise, high-resolution approach for NGS-based outbreak analysis of nontoxigenic *C. diphtheriae* showed that MLST analysis can still serve as a good first-level molecular analysis and standard classification (e.g., to confine possible outbreak candidates). At the next level, whole-genome sequencing brings deeper and more comprehensive insights during the investigation of *C. diphtheriae* outbreaks causing different clinical symptoms and distributed over wider geographic areas and longer periods. We also show that nontoxigenic *C. diphtheriae* can become a public health threat in industrialized countries because strains can persist, evolve within risk groups, and lead to outbreaks, which are difficult to register early enough to stop transmission. We suspect that this threat is not specific for Germany only but that it is a potential problem for specific risk groups in metropolitan areas in general. Therefore, clinicians and public health authorities should bear in mind the potential for nontoxigenic *C. diphtheriae* to cause disease (*7,8,27,28*) and should recognize infection and transmission events early.

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About the Author

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Avian Influenza A Virus Infection among Workers at Live Poultry Markets, China, 2013–2016

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We conducted a 3-year longitudinal serologic survey on an open cohort of poultry workers, swine workers, and general population controls to assess avian influenza A virus (AIV) seroprevalence and seroincidence and virologic diversity at live poultry markets (LPMs) in Wuxi City, Jiangsu Province, China. Of 964 poultry workers, 9 (0.93%) were seropositive for subtype H7N9 virus, 18 (1.87%) for H9N2, and 18 (1.87%) for H5N1. Of 468 poultry workers followed longitudinally, 2 (0.43%), 13 (2.78%), and 7 (1.5%) seroconverted, respectively; incidence was 1.27, 8.28, and 4.46/1,000 person-years for H7N9, H9N2, and H5N1 viruses, respectively. Longitudinal surveillance of AIVs at 9 LPMs revealed high co-circulation of H9, H7, and H5 subtypes. We detected AIVs in 726 (23.3%) of 3,121 samples and identified a high diversity (10 subtypes) of new genetic constellations and reassortant viruses. These data suggest that stronger surveillance for AIVs within LPMs and high-risk populations is imperative.

Avian influenza A viruses (AIVs) remain an important threat to human health. With new strains widely circulating in China, an increasing number of human infections with AIVs have been reported since 2013, including subtypes H7N9, H5N6, and H10N8 (*1*–*3*). In addition, more human infections with H9N2 have been reported since 2014 (*4*). Although no sustained human-to-human transmission has been observed for these viral subtypes, serious concern exists that the virus could become more efficient in causing human epidemics (*5*).

Most human infections with AIVs (e.g., subtypes H7N9, H5N1, and H5N6) have been associated with exposure to poultry and resulted in severe illness (*6*). However, these severely ill patients could represent the tip of the iceberg because mild and asymptomatic infections with H7N9, H9N2, and H5N1 subtypes have been observed by surveillance (*7*–*11*) and serologic studies (*12*–*18*). Surveillance might miss persons with mild or asymptomatic infection who do not seek medical care. Cross-sectional serologic studies have limited value for measuring incidence rates of AIV infections, resulting in poor understanding of the prevalence of infection and the proportion of cases that are mild or subclinical in humans.

The southern provinces of China have a high density of poultry and humans and are considered likely hot spots for the emergence of new reassortant influenza viruses (*19*). China's Jiangsu Province, one of the hot spots, has reported human infections with H7N9 and H5N1 subtypes. We conducted a 3-year longitudinal serologic study to estimate the seroprevalence and seroincidence of H7N9, H9N2, H5N1, and H5N6 subtypes among animal (poultry and swine) workers and general population controls and to identify the risk factors for seropositivity or seroconversion. We also conducted longitudinal surveillance to measure the diversity and genetic variation of AIVs at live poultry markets (LPMs) in the city of Wuxi, Jiangsu Province, China.

Materials and Methods

Study Population, Sampling, and Data Collection

During July 2013–September 2016, we conducted a longitudinal serologic survey among an open cohort of poultry and swine workers and general population controls in Wuxi. We recruited workers who were ≥18 years of age and were exposed to poultry and pigs or to poultry and pig manure as part of their daily activities (e.g., husbandry, slaughtering, sales). In addition, we recruited control participants from residents at community service centers who reported having no exposure to poultry or pigs or to animal manure as part of their daily activities. After enrolling

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participants in July 2013, we conducted follow-up visits at 1, 2, and 3 years. Because poultry and swine workers in China are often temporarily employed and different workers might be present each year, prospective follow-up of the same persons over the study period was not always feasible. Therefore, we enrolled new participants at each follow-up visit to maintain the number of active cohort participants at ≈2,000.

At participant enrollment, we used a comprehensive questionnaire to collect demographic data, exposure variables, information about any history of chronic medical conditions, influenza vaccination history, self-reported influenza-like illness during the past 12 months, and the extent and nature of exposure to animals or animal manure. At each follow-up visit, we used a shorter questionnaire to collect additional demographic data, recent history of exposure to poultry or pigs, and self-reported recent influenza-like illness. At enrollment and follow-up visits, we asked each participant to provide a 5-mL blood sample.

We obtained written informed consent from all participants before conducting interviews and collecting samples. The institutional review boards of the Beijing Institute of Microbiology and Epidemiology (no number given) approved the study protocol.

Poultry and Environmental Surveillance of AIVs

During the serologic study period, we also conducted prospective surveillance of AIVs at 9 LPMs in 9 districts of Wuxi (Figure 1). Once each month, we collected ≈54 cloacal swab samples (6 samples from each LPM) from chickens, ducks, or geese and preserved each sample in a tube containing 3 mL of viral transport medium (MT0301; Yocon, Beijing, China). In addition, 18 of each type of environmental swab and fecal/slurry samples were collected (2 samples of each type from each LPM). We collected environmental samples by swabbing surfaces of chicken epilators, chopping boards, cages, and sewage 4–8 times with separate cotton-tipped swabs. We then inserted the swabs into a tube containing 3 mL of viral transport medium (Yocon). Fecal (1 g) or slurry (1 mL) samples were collected at available sites and were diluted in viral transport medium (Yocon).

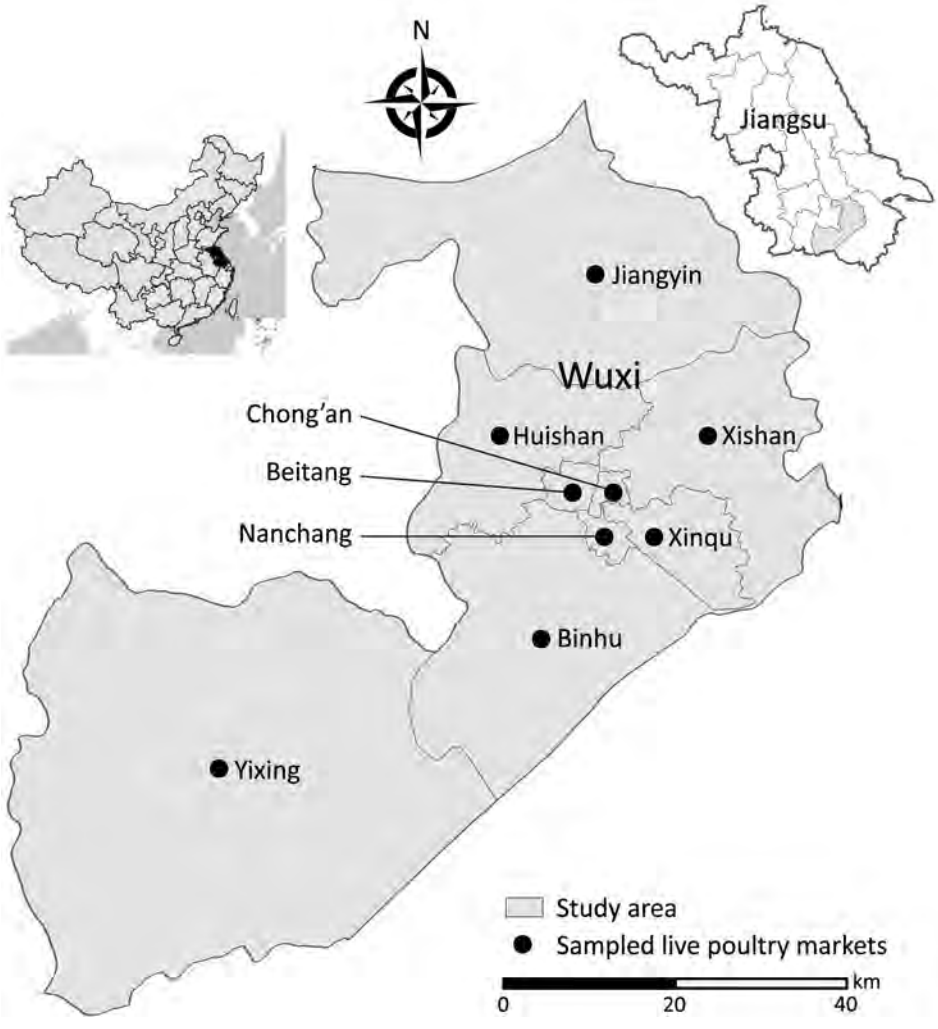

Figure 1. Location of study area where participants were enrolled and of live poultry markets where environmental and cloacal swab sampling was conducted in study of influenza A infection among workers at live poultry markets in 9 districts of Wuxi, Jiangsu Province, China, 2013–2016. Insets show location of Wuxi in Jiangsu Province and location of the province in China.

Sample Processing and Laboratory Analysis

All blood, cloacal, and environmental specimens were kept on frozen cold packs at 2°C–8°C after collection and transported to the local Center for Disease Control and Prevention laboratory. Serum was separated by centrifugation for 5 min at 2,000 rpm. Cloacal and environmental specimens were vortexed, and swabs were discarded. For the fecal/slurry specimens, we conducted an extra centrifugation for 5 min at 2,000 rpm to separate the mixture of virus and viral medium. Each type of specimen was split into 3 aliquots and frozen at –80°C until use.

We first screened all serum samples by hemagglutination inhibition (HI) assay (*20*), and samples with an HI titer ≥10 were tested by a microneutralization (MN) assay (*21*). Considering the prevalence of avian-lineage viruses in China and their availability, we used a human H7N9 isolate (A/Jiangsu/Wuxi05/2013), clade 2.3.4.4 H5N6 virus (A/chicken/Jiangsu/WXBING2/2014), clade 2.3.2.1c H5N1 virus (A/chicken/Jiangsu/WX927/2013), and Y280-like H9N2 virus (A/chicken/Jiangsu/WXWA021/2013) for HI and MN assays. We defined a seropositive result as an MN titer ≥80 for all tested viruses. Seroconversion was defined as detection of a ≥4-fold rise in MN antibody titer between initial serum sample and a paired second serum sample, with the second sample achieving a titer ≥80. Additional details for the HI and MN assays, PCR detection, viral isolation, sequencing of the cloacal and environmental samples, and the phylogenetic analysis of the AIVs we identified are available in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/7/17-2059-Techapp1.pdf). We deposited sequence data in the GISAID database (http://platform.gisaid.org; accession nos. EPI_ISL_277027–277050, 277052–277064, and 277093–277127).

Statistical Analysis

We calculated the proportion (and associated 95% CIs) of poultry workers, swine workers, and control participants who were seropositive or seroconverted. We estimated the incidence of seroconversion per 1,000 person-years (and associated 95% CIs) for participants with multiple longitudinal serum samples using the time between baseline and follow-up as their person-time contribution. We excluded participants who were seropositive at baseline. We analyzed categorical and continuous variables using the χ^2 or Fisher exact test and the Student *t*-test where necessary. Risk factors for virus infection (any seropositivity or seroconversion for each individual) were assessed only among participants with paired serum samples using logistic regression models after adjustment for sex and age group or variables with p values <0.05, summarized by odds ratios (ORs) with 95% CIs. Exact Poisson regression model was used to explore the effect of exposure on 1,000 person-year incidence in the cohorts, assessed by incidence rate ratios with 95% CIs. All tests were 2-sided with a 0.05 level of significance. Analyses were performed using SPSS software version 16.0 (SPSS, Chicago, IL, USA).

Results

Demographic Characteristics of Participants

In July 2013, we enrolled 1,995 participants: 511 poultry workers, 569 swine workers, and 915 general population controls. Of these original 1,995 participants, 1,137 were followed up at year 1 (July 2014), 892 at year 2 (July 2015), and 701 at year 3 (July 2016) (Figure 2). To compensate for the number of participants lost to follow-up, we enrolled an additional 866 participants in July 2014, 603 in July 2015, and 124 in July 2016 (Figure 2). New participants enrolled in 2014 were also followed in 2015 (396) and 2016 (339) and new participants enrolled in 2015 were followed in 2016 (479) (Figure 2). Poultry and swine workers tended to be older and less educated than controls (p<0.05), and swine workers comprised a significantly higher proportion of men among the 3 groups (online Technical Appendix Table 1).

Seroprevalence

Seroprevalence differed by group and over time (Table 1). The overall seroprevalence of H7N9, H9N2, and H5N1 viruses in poultry workers was significantly higher than in swine workers and controls (p<0.05). Of 964 enrolled poultry workers, 9 (0.93% [95% CI 0.43%–1.76%]) were seropositive for H7N9, 18 (1.87% [95% CI 1.11%–2.94%]) for H9N2, and 18 (1.87% [95% CI 1.11%–2.9%]) for H5N1 during the study period. In comparison, of 1,079 enrolled swine workers, only 2 (0.19% [95% CI 0.02%–0.67%]) were seropositive for H7N9 and 3 (0.28% [95% CI 0.06%–0.81%]) for H9N2. Similar seroprevalence was observed among the 1,545 enrolled controls. No poultry workers were found seropositive for H7N9 in the 2016 survey and for H5N1 virus in the 2014 and 2015 surveys. In addition, we observed a significant increase in seroprevalence of 3.46% for H5N1 virus among poultry workers in the 2016 survey, compared with the previous year's survey. No participants in any group were seropositive for H5N6 throughout the study.

Incidence of Seroconversion

During the study period, 30 participants seroconverted (Table 2). Among the poultry workers, 2 (0.43%) seroconverted for H7N9, 13 (2.78%) for H9N2, and 7 (1.5%) for H5N1 (Table 3), resulting in incidences of 1.27/1,000 person-years for H7N9, 8.28/1,000 person-years for H9N2, and 4.46/1,000 person-years for H5N1 (Table 4). Among

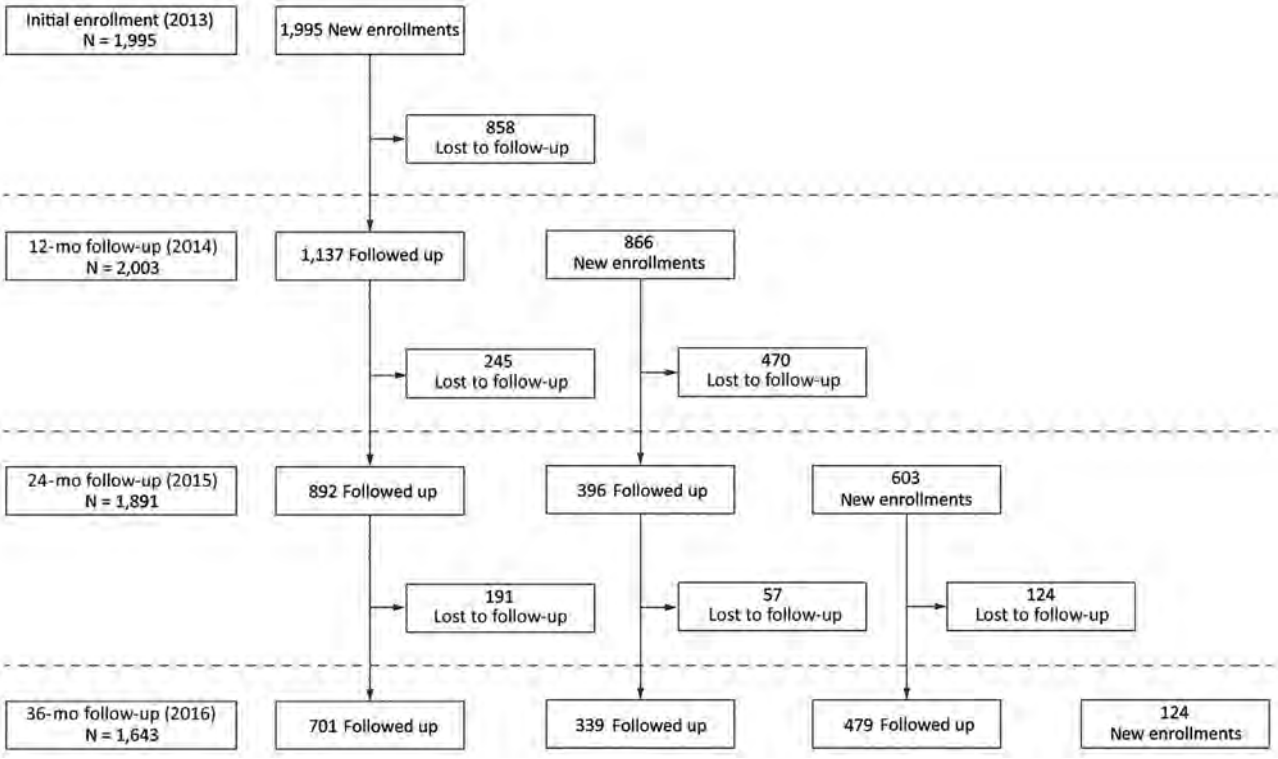

Figure 2. Flowchart for participant enrollment and follow-up in study of avian influenza A virus infection among workers at live poultry markets, Wuxi, Jiangsu Province, China, 2013–2016.

swine workers and controls, only 1 control seroconverted for the H7N9 virus, and 3 (0.58%) swine workers and 4 (0.39%) controls seroconverted for H9N2 (Table 3). Although the incidence among swine workers and controls was low or 0 for H7N9 and H5N1, the incidence of H9N2 was relatively high among swine workers (1.93/1,000 person-years) and controls (1.54/1,000 person-years) (Table 4). Poultry workers were more likely than controls to have infection with H9N2 (incidence rate ratio 5.36 [95% CI 1.65%–22.55%]) and H5N1, but seroconversion rates between the groups did not differ significantly for H7N9 (Table 4).

Table 1. Seroprevalence of microneutralization titers against influenza A(H7N9), A(H9N2), and A(H5N1) viruses in poultry workers, swine workers, and controls, eastern China, 2013–2016*

	No. seropositive/no. total (% [95% CI])			
Antigen/year	Poultry workers	Swine workers	Controls	p value
H7N9				
2013	3/511 (0.58 [0.12–1.71])	0/569 (0 [0–0.65])	2/915 (0.22 [0.03–0.79])	0.13
2014	3/533 (0.56 [0.12–1.64])	1/589 (0.17 [0–0.94])	1/881 (0.11 [0–0.63])	0.19
2015	3/535 (0.56 [0.12–1.63])	0/501 (0 [0–0.73])	0/855 (0 [0–0.43])	0.04
2016	0/491 (0 [0–0.75])	1/367 (0.27 [0.01–1.51])	1/785 (0.13 [0–0.71])	0.48
Overall†	9/964 (0.93 [0.43–1.76])	2/1,079 (0.19 [0.02–0.67])	4/1,545 (0.26 [0.07–0.66])	0.03
H9N2				
2013	1/511 (0.20 [0.01–1.09])	0/569 (0 [0–0.65])	2/915 (0.22 [0.03–0.79])	0.61
2014	2/533 (0.38 [0.05–1.35])	1/589 (0.17 [0–0.94])	1/881 (0.11 [0–0.63])	0.70
2015	11/535 (2.06 [1.03–3.65])	0/501 (0 [0–0.73])	4/855 (0.47 [0.13–1.19])	<0.001
2016	7/491 (1.43 [0.58–2.92])	3/367 (0.82 [0.17–2.37])	2/785 (0.25 [0.03–0.92])	0.05
Overall†	18/964 (1.87 [1.11–2.94])	3/1,079 (0.28 [0.06–0.81])	9/1,545 (0.58 [0.27–1.10])	<0.001
H5N1				
2013	1/511 (0.20 [0–1.09])	0/569 (0 [0–0.65])	0/915 (0 [0–0.40])	0.26
2014	0/533 (0 [0–0.69])	0/589 (0 [0–0.62])	0/881 (0 [0–0.42])	NA
2015	0/535 (0 [0–0.69])	0/501 (0 [0–0.73])	0/855 (0 [0–0.43])	NA
2016	17/491 (3.46 [2.03–5.49])	0/367 (0 [0–1.00])	0/785 (0 [0–0.47])	<0.001
Overall†	18/964 (1.87 [1.11–2.94])	0/1,079 (0 [0–0.34])	0/1,545 (0 [0–0.24])	<0.001

*NA, the statistics were not performed because of 0 in the 2 groups.
†The overall seroprevalence was calculated as the number of seropositive persons divided by the number of all new enrolled persons during the study period.

Table 2. Characteristics of poultry workers, swine workers, and controls with seroconversion of influenza A(H7N9), A(H9N2), and A(H5N1) viruses, eastern China, 2013–2016*

Virus, participant no.	Age, y/sex	Occupation	Chronic medical condition	MN titer 2013	2014	2015	2016
H7N9							
1	28/F	Chicken slaughtering	No	40	320	5	5
2	41/F	Chicken slaughtering	No	5	5	320	NA
3	63/F	Retired	No	20	80	NA	NA
H9N2							
4	48/F	Chicken backyard grower	No	5	5	80	40
5	28/M	Chicken raising	No	NA	5	80	80
6	51/F	Chicken raising	No	5	5	5	80
7	47/F	Chicken seller	No	5	20	80	40
8	47/M	Chicken seller	No	5	5	160	NA
9	46/M	Chicken seller	No	5	5	160	NA
10	51/M	Chicken seller	Chronic bronchitis	5	40	160	NA
11	49/M	Chicken/duck seller	Diabetes	NA	NA	20	80
12	59/F	Chicken/duck seller	No	5	5	80	320
13	39/F	Chicken/duck seller	No	5	NA	20	80
14	27/F	Chicken/goose seller	No	5	320	40	40
15	57/F	Chicken/pigeon slaughtering	No	5	40	80	5
16	52/F	Duck/goose seller	No	5	80	5	5
17	32/M	Pig slaughtering	No	5	5	5	80
18	52/M	Pig slaughtering	No	5	80	NA	5
19	26/M	Pork seller	No	5	5	5	160
20	40/M	Grocer, control	Chronic bronchitis	5	160	5	5
21	48/M	Grocer, control	No	5	5	80	5
22	38/M	Grocer, control	Diabetes	5	5	160	5
23	61/M	Retired, control	No	NA	5	5	80
H5N1							
24	39/F	Chicken/duck/goose seller	No	5	5	20	80
25	45/F	Chicken/duck/pigeon raising	No	20	10	40	80
26	48/M	Pigeon seller	No	10	10	10	80
27	60/F	Chicken/goose seller	No	10	5	40	80
28	55/F	Duck/goose seller	No	5	5	40	160
29	46/F	Chicken slaughtering	No	40	20	20	80
30	53/F	Chicken slaughtering	No	20	5	20	80

*MN, microneutralization; NA, the participant was not available in this year.

Risk Factors for AIV Infections

Poultry workers who performed selling had 4.25 (95% CI 1.20–25.32) times higher odds of H9N2 virus infection than did poultry workers who performed slaughtering (Table 5). Among poultry workers, female sex (adjusted OR 5.48 [95% CI 2.38–12.62]) and exposure to pigeons (adjusted OR 3.13 [95% CI 1.23–8.00]) were also significant risk factors for H5N1 virus seropositivity or seroconversion. Controls who were male (adjusted OR 8.75 [95% CI 1.09–70.45]) or had chronic respiratory disease (adjusted OR 7.24 [95% CI 1.42–37.00]) were more likely to be seropositive or to seroconvert for H9N2.

Diversity and Reassortment of AIVs at LPMs

During the study period, we collected and screened 3,121 samples from 9 LPMs for IAVs. A total of 466 (23.2%) of 2,010 cloacal swab samples, 145 (24.5%) of 590 environmental swab samples, and 115 (22.0%) of 521 fecal/slurry specimens were positive for influenza A (Figure 3, panel A). Single infection with H9, H7, and H5 subtypes was detected in 229 (31.5%), 27 (3.7%), and 25 (3.4%) of 726 AIV-positive specimens, respectively. Sequencing results of 45 isolated strains and 33 original specimens (online Technical Appendix Table 3) showed that 10 AIV subtypes were detected in LPMs (Figure 3, panel B). To further study the origin of these 10 subtypes, we performed a detailed phylogenetic analysis for all available gene segments (online Technical Appendix Figure). The analyses revealed multiple gene segment exchanges among and within subtypes or interspecies among those circulating in domestic and wild birds, resulting in new genetic constellations and reassortant viruses, which we have represented

Table 3. Seroconversion of microneutralization titers against influenza A(H7N9), A(H9N2), and A(H5N1) viruses in poultry workers, swine workers, and controls, eastern China, 2013–2016

Virus	No. seropositive/no. total (% [95% CI]) Poultry workers	Swine workers	Controls
H7N9	2/468 (0.43 [0.05–1.54])	0/514 (0 [0–0.72])	1/1030 (0.10 [0.00–0.54])
H9N2	13/468 (2.78 [1.48–4.70])	3/514 (0.58 [0.12–1.70])	4/1030 (0.39 [0.11–0.99])
H5N1	7/468 (1.50 [0.60–3.06])	0/514 (0 [0–0.72])	0/1030 (0 [0–0.36])

Table 4. Seroincidence of influenza A(H7N9), A(H9N2), and A(H5N1) viruses in in poultry workers, swine workers, and controls, eastern China, 2013–2016*

Antigen, participant category	Person-years	No. seroconversions	Incidence (95% CI)	IRR (95% CI)
H7N9				
Poultry workers	1,569	2	1.27 (0.15–4.60)	3.30 (0.17–194.48)
Swine workers	1,558	0	0 (0–2.36)	1.66 (0–64.73)
Controls	2,586	1	0.39 (0.01–2.15)	Reference
H9N2				
Poultry workers	1,569	13	8.28 (4.42–14.12)	5.36 (1.65–22.55)
Swine workers	1,558	3	1.93 (0.40–5.61)	1.24 (0.18–7.36)
Controls	2,586	4	1.54 (0.42–3.96)	Reference
H5N1				
Poultry workers	1,569	7	4.46 (1.80–9.17)	NA
Swine workers	1,558	0	0 (0–2.36)	NA
Controls	2,586	0	0 (0–1.43)	Reference

*Incidence is per 1,000 person-years. IRR, incidence rate ratio; NA, statistics not performed because of 0 in 2 groups.

schematically (Figure 4). Overall, 2 reassortment models were observed for these viruses. The 1 reassortment model mentioned only internal gene reassortment (Figure 4, panel A), such as the matrix (M) gene of 3 H5N1 (A/environment/Wuxi/4689/2015, A/environment/Wuxi/5068/2015, and A/environment/Wuxi/5081/2015) and 1 H5N2 (A/chicken/Wuxi/6462/2015) virus originating from Y280-like H9N2 viruses, the polymerase basic 2 gene of 1 H9N2 (A/chicken/Wuxi/6082/2015) virus from A/chicken/Zhejiang/7450/2015 H5N2-like virus, and all internal genes of 2 H3N8 (A/duck/Wuxi/7275/2016 and A/goose/Wuxi/7276/2016) viruses multireasserted from chicken or wild bird HxNy–like virus. The other reassortment model included multireassortment involving both the surface protein genes (hemagglutinin [HA], neuraminidase [NA], or both) and internal genes among the different subtypes or lineage and interspecies (Figure 4, panel B). The HA gene of 3 H5N2 viruses originated from clade 2.3.4 H5N2 (A/chicken/Wuhan/HAQL07/2014) or clade 7 H5N1 (A/chicken/Zhejiang/7450/2015)-like viruses and the HA gene of 3 H11N2 viruses from A/duck/Jiangxi/22537/2012-like H11N9 virus, the NA gene in all of them was originated from HxN2-like viruses. The 1 H3N8 virus (A/chicken/Wuxi/4859/2015) also had multireassortments that the HA and NA were respectively generated from the HA of Eurasian (A/duck/Jiangsu/26/2004) and North American lineage (A/pintail/Alberta/232/1992) H3N8-like virus, and the internal genes were reassorted with 6 subtypes circulating in ducks and wild birds. The HA of H1N2-like virus (A/Anseriformes/Anhui/L6/2014), the NA of clade 2.3.2.1c H5N1-like virus (A/chicken/Wuhan/HAQL07/2014), and the M gene of Y-280 lineage H9N2-like virus (A/chicken/

Table 5. Risk factors for testing seropositive or seroconverting against influenza A(H9N2) and A(H5N1) viruses among poultry workers and controls, eastern China, 2013–2016*

Risk factor, antigen	Total	Seropositive or seroconverted, no. (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Poultry worker				
H9N2	468	18 (3.85)		
Exposure behavior†				
Selling	181	13 (7.18)	4.68 (1.04–21.13)	4.25 (1.20–25.32)
Raising	198	3 (1.52)	0.93 (0.15–5.65)	1.12 (0.18–6.85)
Cleaning	134	1 (0.75)	0.46 (0.04–5.08)	0.22 (0.05–4.99)
Transporting	46	0	NA	NA
Slaughtering	123	2 (1.63)	Reference	Reference
H5N1	468	18 (3.85)		
Sex				
F	222	14 (6.31)	4.07 (1.32–12.56)	5.48 (2.38–12.62)
M	246	4 (1.63)	Reference	Reference
Exposure†				
Goose	49	5 (10.20)	3.24 (1.11–9.42)	2.64 (0.72–9.74)
Pigeon	66	6 (9.09)	2.85 (1.06–7.70)	3.13 (1.23–8.00)
Duck	104	7 (6.73)	2.06 (0.81–5.23)	1.87 (0.77–5,01)
Chicken	413	14 (3.39)	Reference	Reference
Controls, H9N2	1030	9 (0.87)		
Sex				
M	495	8 (1.62)	8.77 (1.10–70.39)	8.75 (1.09–70.45)
F	535	1 (0.19)	Reference	Reference
Chronic respiratory disease				
Yes	38	2 (5.26)	7.82 (1.57–38.96)	7.24 (1.42–37.00)
No	992	7 (0.71)	Reference	Reference

*OR, odds ratio; NA, statistics not performed because of 0 in 2 or 3 groups.
†Participants might be included in multiple categories.

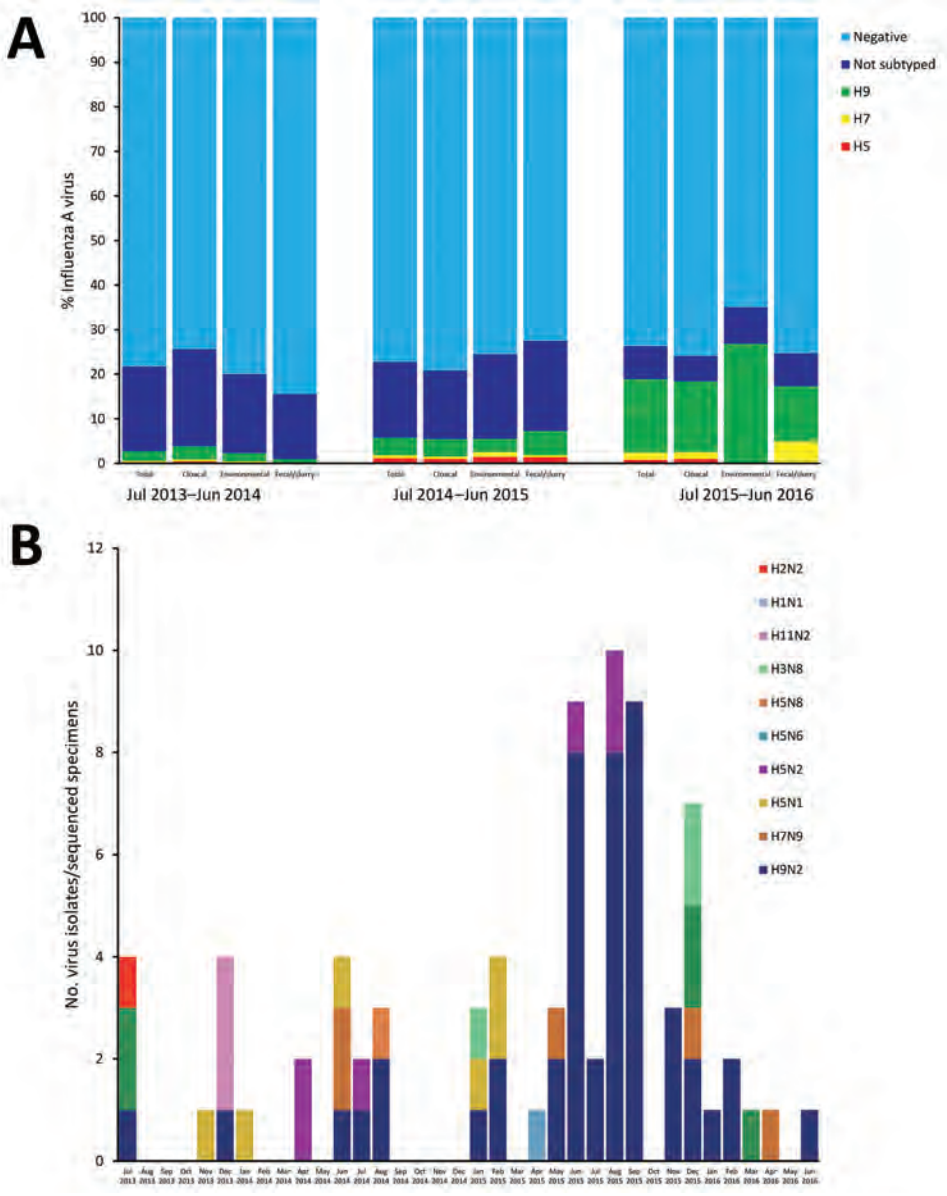

Figure 3. Influenza A virus detection in samples from live poultry markets, Wuxi, Jiangsu Province, China, 2013–2016. A) Proportion of H9, H7, and H5 subtype detection in cloacal swab, environmental swabs, and fecal/slurry samples; B) genetic classification and number of influenza isolates and sequenced specimens over time. Some could not be subtyped because of weakly positive laboratory results.

Shandong/wf0202/2012) reassorted and generated new H1N1 virus (A/chicken/Wuxi/5682/2015).

All H5 subtypes possessed a polybasic amino acid residue at the cleavage site (RERRRKR/GL), indicating they were highly pathogenic in chickens, whereas the other subtypes were low pathogenicity (online Technical Appendix Table 3). We detected the HA Q226L (H3 numbering) mutation in 4 H7N9 and all H9N2 viruses, indicating a binding ability to the human-like receptor. However, all subtypes had no polymerase basic 2 E627K and D701N mutations. All H7, H9, and H5 subtypes had the deletion in NA stalk associated with enhanced virulence in mice, as well as adaptation and transmission in poultry. All H9N2 viruses had oseltamivir resistance mutations of R292K in NA (N2 numbering), and adamantine resistance-associated mutation of S31N of M2 protein in 2 H5N1 and all H5N2, H1N1, H7N9, and H9N2 viruses.

Discussion

We estimated the seroprevalence and seroincidence of H7N9, H9N2, H5N1, and H5N6 viruses in an open cohort of poultry workers, swine workers, and the general population in Wuxi, Jiangsu Province, China. Poultry workers had relatively higher seroprevalence and seroincidence of H7N9, H9N2, and H5N1 than swine workers and the general population, although the overall seroprevalence and seroincidence was low. Active surveillance for AIVs revealed that 10 subtypes were circulating at LPMs, and

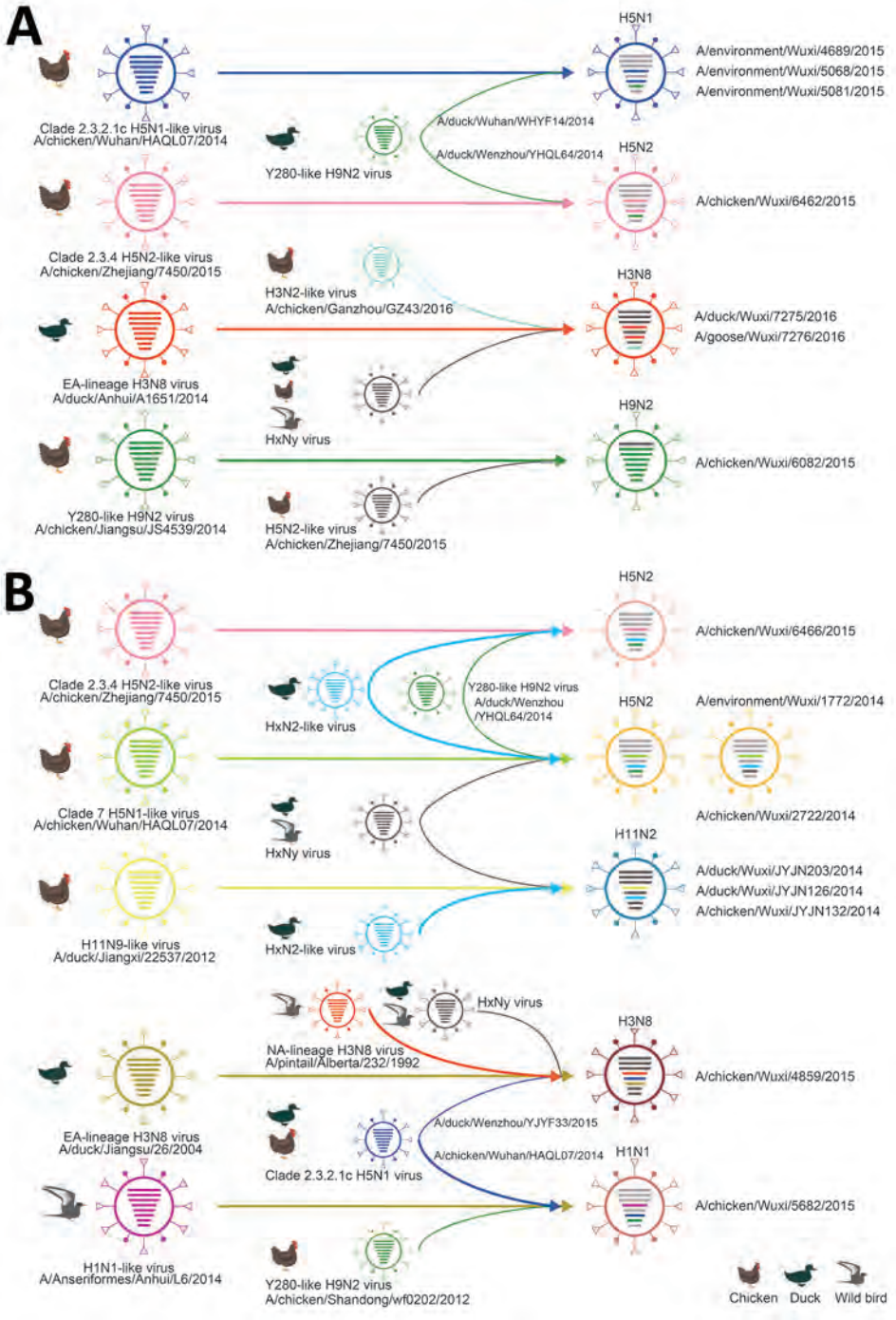

Figure 4. Probable genesis of reassortant influenza A viruses, Wuxi, Jiangsu Province, China, 2013–2016. A) Internal gene reassortment; B) hemagglutinin, neuraminidase, and internal gene exchanges. Virus particles are represented by ovals containing horizontal bars that represent the 8 gene segments (top to bottom: polymerase basic 2, polymerase basic 1, polymerase acidic, hemagglutinin, nucleoprotein, neuraminidase, matrix, and nonstructural); colors indicate sequence origin based on initial viruses shown at far left (gray bars indicate no sequence data available).

extensive gene segment reassorts occurred among and within subtype or interspecies that circulate in domestic poultry and wild birds.

Serologic evidence of human infection with H7N9 has previously been reported (*14*–*18*,*22*–*24*). In those studies, the seroprevalence ranged from 0% to 17.1%. In our study, a much lower seroprevalence of anti-H7N9 virus ranged from 0% to 0.56% during the enrollment and follow-up times in poultry workers. Our findings are similar to the 0.11% seroprevalence of MN titers ≥20 found in poultry workers in 6 provinces in China (*18*). In comparison with studies that did not perform MN testing, the proportion of elevated HI titers ≥20 in our study was also much lower (0%–2.83% in poultry workers). For example, 7.2%–14.9% of poultry workers in Shenzhen had HI titers ≥160 (*14*). Another study found that 1.6% of poultry workers with HI

titers ≥40 in Guangzhou (*22*); 2 studies in Zhejiang Province reported that 3.7% and 6.3% of poultry workers had HI titers ≥80 (*16*,*24*); a study in Taiwan reported 0% of poultry workers with HI titers ≥10 (*23*). In our study, swine workers and general population controls had an extremely low seroprevalence of the H7N9 virus, similar to the results of serologic studies in southern China (*14*–*16*). Our observed low seroprevalence is not surprising because the number of reported H7N9 cases and potential H7N9-positive markets in Wuxi was small during the study period. Differences in seroprevalence across studies also could be explained by differences between serologic assays because different tests might have marked sensitivity/specificity and high interstudy variability. Although the findings from our study and these early serologic studies reassuringly suggest that the number of undetected cases of H7N9 virus was low, close monitoring of transmission remains essential as the virus and epidemic continued to evolve.

Human infections with H9N2 virus have been reported since 1998, and concern about its pandemic potential has increased, especially in recent years. Because this virus always causes mild upper respiratory tract illness that is clinically indistinguishable from the symptoms of common influenza caused by seasonal human H1N1 and H3N2 viruses, the incidence of H9N2 infections might be underestimated. Previous studies in China (*25*–*28*) and other countries (*29*–*35*) estimated that seroprevalence ranged from 0.5% to 4.6% in poultry workers. Our results showed that poultry workers had an overall H9N2 seroprevalence of 1.87% and a seroincidence of 8.78/1,000 person-years, which is significantly higher than those of H7N9 and H5N1. We detected no significant serologic response at baseline, but the seropositive rate increased considerably during the next 3 follow-up points in poultry workers. This finding seems to be consistent with an increased prevalence (2.73% during July 2013–June 2014, 5.10% during July 2014–June 2015, and 22.22% during July 2015–June 2016) of H9N2 viruses detected in poultry at LPMs.

We also tested clade 2.3.2.1c H5N1 and clade 2.3.4.4 H5N6 viruses. Overall, the seroprevalence of H5N1 was low, and only poultry workers in 2016 had seropositive titers for a seroprevalence of 3.46%, which was similar to findings from studies conducted in southern China during the same period (*14*,*36*,*37*). Also, antibody levels were relatively low (the highest titer was 160), consistent with the low immunogenicity of H5N1 (*38*). Since the first H5N6 infections in humans reported in China in 2014, a total of 17 cases have been reported, but none of the participants in our study were seropositive or seroconverted during the study period. However, the circulation of H5N6 in LPMs and the continuous reassortment of their internal genes with Y280-like H9N2 virus remains a potential cause of human infections.

Our active surveillance data revealed a high diversity of AIVs at LPMs. We observed genetic evidence of extensive reassortment of viral genes among and within subtype, and the new viral genes were introduced from the wild bird gene pool to domestic poultry, which further enriched such diversity. Additional co-detections of H9N2 with H7N9, H5N1, or H5N6 might provide the potential conditions for intersubtype reassortment. Our data also showed that H9N2 was the dominant circulating subtype, showing a high prevalence of 31.5%. Furthermore, all or some of the internal genes of the viruses we identified were from the Y280-like H9N2 virus, such as H7N9, H5N1, H5N2, and H1N1. All H5 subtype viruses showed a polybasic cleavage site, indicating its high pathogenicity in poultry. Although no outbreaks of H5 subtype viruses were reported in Wuxi, the outbreaks of H5N1, H5N2, and H5N6 in poultry were reported in several cities of Jiangsu Province surrounding Wuxi (*39*). Because H9N2 is not highly pathogenic, the extent of infection in poultry and humans is likely to remain underappreciated. Interaction or reassortment between the prevailing human and avian influenza viruses is considered the most probable scenario for generating new pandemic strains. We also argue that almost anywhere in the world where LPMs exist, especially large LPMs with different poultry, disparate viruses could be mixed yielding new AIVs. These viruses can move quickly across large geographic areas and change rapidly. Hence, our findings support the conclusion that LPMs play a critical role in the continual emergence of new reassortant AIVs that can spread through poultry populations. Thus, influenza surveillance among wild bird and domestic poultry at LPMs should be strengthened.

Our study had several limitations. First, although our study provides serologic evidence of virus infection, we did not conduct surveillance for influenza-like illness among participants, which prevents us from identifying laboratory-confirmed human disease and obtaining evidence of direct transmission from poultry to humans. Second, because of possible waning of antibodies or lack of antibody response to AIVs during the 1-year follow-up period, we were unlikely to have detected all seroconversions during the study period; thus, our study might underestimate the seroincidence.

In conclusion, conducting surveillance for new influenza virus surveillance at LPMs, especially when the LPMs are large and can sustain virus transmission, and monitoring the poultry and poultry workers for the new AIV infections are critical. Despite overall low seroprevalence or seroincidence, poultry workers had a higher risk for infection than swine workers and controls. Thus, it seems prudent to encourage poultry workers to use personal protective equipment (e.g., masks and gloves) and to undergo educational programs to help them understand and prevent AIV transmission between humans and poultry.

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Epidemiology and Geographic Distribution of Blastomycosis, Histoplasmosis, and Coccidioidomycosis, Ontario, Canada, 1990–2015

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Learning Objectives

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

- Describe the epidemiology and geographic distribution of microbiology laboratory-confirmed cases of blastomycosis in Ontario, Canada, from 1990 to 2015, according to a case series
- Determine the epidemiology and geographic distribution of microbiology laboratory-confirmed cases of histoplasmosis and coccidioidomycosis in Ontario, Canada from 1990 to 2015, according to a case series
- Identify clinical and public health implications of the epidemiology and geographic distribution of microbiology laboratory-confirmed cases of blastomycosis, histoplasmosis, and coccidioidomycosis in Ontario, Canada, from 1990 to 2015, according to a case series

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Endemic mycoses represent a growing public health challenge in North America. We describe the epidemiology of 1,392 microbiology laboratory–confirmed cases of blastomycosis, histoplasmosis, and coccidioidomycosis in Ontario during 1990–2015. Blastomycosis was the most common infection (1,092 cases; incidence of 0.41 cases/100,000 population), followed by histoplasmosis (211 cases) and coccidioidomycosis (89 cases). Incidence of blastomycosis increased from 1995 to 2001 and has remained elevated, especially in the northwest region, incorporating several localized hotspots where disease incidence (10.9 cases/100,000 population) is 12.6 times greater than in any other region of the province. This retrospective study substantially increases the number of known endemic fungal infections reported in Canada, confirms Ontario as an important region of endemicity for blastomycosis and histoplasmosis, and provides an epidemiologic baseline for future disease surveillance. Clinicians should include blastomycosis and histoplasmosis in the differential diagnosis of antibiotic-refractory pneumonia in patients traveling to or residing in Ontario.

In North America, the endemic mycoses blastomycosis, histoplasmosis, and coccidioidomycosis are responsible for serious illness in immunocompetent and immunocompromised hosts ranging from asymptomatic, self-limiting illness to invasive, life-threatening disease (*1*,*2*). Infection occurs when a susceptible host inhales fungal spores from the surrounding environment (*2*). Thus, infections occur sporadically, with occasional point-source outbreaks in the localized geographic areas of endemicity defined by the natural habitat of *Blastomyces*, *Histoplasma*, and *Coccidioides* fungi (*2*).

Despite the potential severity of these infections, these diseases are reportable in only select states and provinces, providing only partial coverage of known regions of endemicity (*3*). The lack of mandatory public health reporting in most areas and the small number of epidemiologic studies make it difficult to understand the true burden of disease, which, in turn, contributes to a low clinical index of suspicion, especially outside endemic regions, leading to diagnostic delays and a consequent increase in illness and death (*2*,*4*). Several recent reports suggest increasing incidence and expanding geographic endemicity of the dimorphic fungal infections in North America (*1*,*4*–*10*). Additional shifts in prevalence and endemic range are expected as climate change alters ecosystems in North America (*11*). To address these knowledge gaps and concerns, several more comprehensive epidemiologic assessments have been performed recently in the United States (*12*–*17*).

Although often excluded from disease distribution maps of North America (*18*), the regions to which blastomycosis and histoplasmosis are endemic extend into Canada. Historically, blastomycosis has been considered endemic to Manitoba, northwestern Ontario, and Quebec (*19*–*22*) with the Kenora area of northwestern Ontario exhibiting the highest reported incidence of blastomycosis in the world (*4*–*6*,*23*). Before 1989, when mandatory reporting in Ontario was suspended, cases of blastomycosis were rare (1.8 cases/year) and thought to be acquired almost exclusively in the northwest region of the province (*24*). Since that time, the known blastomycosis-endemic range has expanded to include all of Ontario; provincial incidence increased until 2003 or later (*4*,*25*). A recent study in Quebec confirms the endemic status of blastomycosis (*26*); sporadic clusters of human and canine infections have occurred in Saskatchewan (*27*) and New Brunswick (*19*). Histoplasmosis is considered endemic to regions bordering the St. Lawrence River (*19*,*28*–*30*), especially Quebec (*19*,*31*,*32*); a single case cluster occurred in Alberta (*19*), but there are no recent epidemiologic reports from Ontario. Coccidioidomycosis is not considered endemic to Canada, but data on travel-related cases are outdated (*19*).

With approval from Research Ethics committees at Public Health Ontario and The Hospital for Sick Children, we describe the epidemiology of microbiology laboratory–confirmed cases of blastomycosis, histoplasmosis, and coccidioidomycosis in Ontario, Canada, during 1990–2015. When combined with studies from Manitoba (*21*) and Quebec (*26*,*32*), this study provides a more comprehensive picture of the incidence of blastomycosis and cases of histoplasmosis from mycosis-endemic regions in Canada to complement US studies.

Methods

Study Setting, Data Sources, and Case Definition

Ontario, Canada's most populous province (population of 13.4 million in 2016 [*33*]), is divided into 14 Local Health Integration Networks (LHINs) that provide health services for their respective populations (Figure 1). Because of the need for specialized expertise and containment level 3 laboratory facilities for manipulating *Histoplasma*, *Blastomyces,* and *Coccidioides* species, Public Health Ontario Laboratory (PHOL) is the only referral facility in the province for their handling and diagnosis. This centralization ensures a high level of provincewide case ascertainment for microbiology laboratory–confirmed human infections. We performed a retrospective review of PHOL data to detect cases of blastomycosis, histoplasmosis, and coccidioidomycosis. Inclusion criteria were positive culture, microscopy, or both for *Blastomyces dermatitidis/gilchristii* during January 1, 1995–December 31, 2015; for *Coccidioides immitis/posadasii* and *Histoplasma capsulatum* infections, the study period was January 1, 1990–December 31, 2015. Patients with ≥1 specimen submitted within a 6-month period were each counted as a single case. Demographic information included patient age,

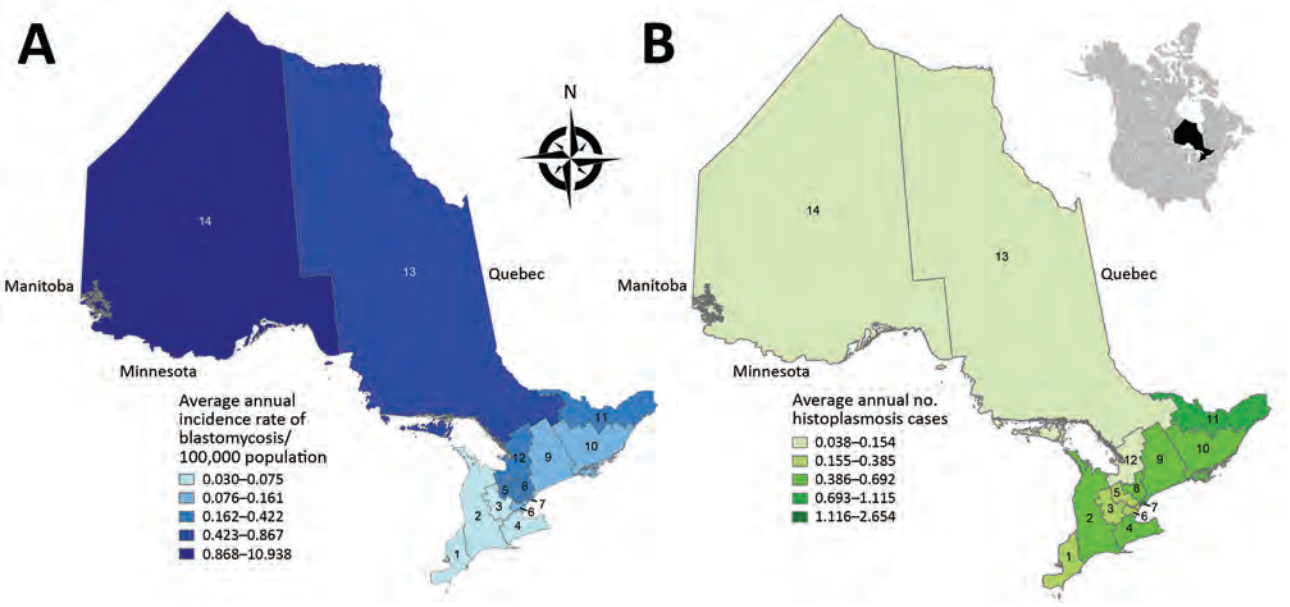

Figure 1. Geographic distribution of A) annualized incidence (no. cases/100,000 population) of blastomycosis (1995–2015) and B) no. cases of histoplasmosis (1990–2015) by Ontario Local Health Integration Network (LHIN), Ontario, Canada. 1, Erie St. Clair; 2, South West; 3, Waterloo Wellington; 4, Hamilton Niagara Haldimond Brant; 5, Central West; 6, Mississauga Halton; 7, Toronto Central; 8, Central; 9, Central East; 10, South East; 11, Champlain; 12, North Simcoe Muskoka; 13, North East; 14, North West. Incidence was calculated using LHIN-specific population denominators from Statistics Canada (*34*). Inset shows the location of Ontario within North America.

sex, and address (city, forward sortation area [FSA, first 3 characters of postal code]; sender address (institution, city, FSA); date of specimen receipt; and specimen type. When specific data were not available, we excluded cases from individual analyses requiring these data (Table 1). We assessed statistical significance by χ^2 test ($p \leq 0.05$ was statistically significant).

Descriptive Epidemiologic Analysis

We calculated annual and stratum-specific (age-, LHIN-, and regional group–specific) incidence (no. cases/100,000 population) for blastomycosis using population denominators from Statistics Canada extracted from the Ontario Ministry of Health and Long-Term Care: IntelliHealth Ontario on February 18, 2014, and January 15, 2016. We used population projections for 2014 and 2015 (*35*).

We examined temporal trends in disease occurrence by performing aggregated seasonal case counts based on date of specimen receipt (date of symptom onset was not available). We defined winter as December–February, spring as March–May, summer as June–August, and autumn as September–November (*36*). We assessed significance by χ^2 test (Bonferroni-corrected $p \leq 0.05$ was statistically significant).

Geographic Distribution, Spatial Statistics, and Hotspot Analysis

We examined the geographic distribution of blastomycosis and histoplasmosis by assigning each case to 1 of Ontario's 14 LHINs. We used the patient's home address, if known, to assign the case to a LHIN (blastomycosis n = 544, histoplasmosis n = 42). If the patient's home address was not known (blastomycosis n = 526, histoplasmosis n = 169), we used the sender's (i.e., hospital, physician's office, or community health center) FSA to assign cases to LHINs. Of 586 cases in which both patient's home FSA and sender's FSA were known, 89.3% (523/586) of the time they were the same, suggesting that sender's FSA is a usable surrogate for patient location. Patient and sender location were unknown for 22 cases of blastomycosis. We mapped annualized incidence rates of blastomycosis and number of cases of histoplasmosis across Ontario's 14 LHINs using ArcGIS version 10.4 software (ESRI Inc., Redlands, CA, USA). We obtained Ontario and LHIN boundary files from Statistics Canada (*34*).

To examine temporal and geographic trends for blastomycosis, we aggregated data from the LHINs into 5 larger regional groups by geographic continuity and similar incidence rates: Northwest (North West LHIN); Northeast (North East LHIN); South-central (Toronto, North Simcoe Muskoka, Central, and Central West LHINs); Southeast (Central East, South East, and Champlain LHINs); and Southwest (Erie St. Clair, South West, Waterloo Wellington, Hamilton Niagara Haldimand Brant, and Mississauga Halton LHINs). Because some of the LHINs had very few cases, we aggregated data to stabilize the variance from data with sparse cells. We applied the GENMOD procedure

Table 1. Characteristics of microbiology laboratory–confirmed blastomycosis, histoplasmosis, and coccidioidomycosis cases reported in Ontario, Canada, 1990–2015

Characteristic	No. (%) cases*		
	Blastomycosis, n = 1,092	Histoplasmosis, n = 211	Coccidioidomycosis, n = 89
Patient sex	n = 963	n = 180	n = 80
M	627 (65.1)	144 (80.0)	48 (60.0)
F	336 (34.9)	36 (20.0)	32 (40.0)
Patient age, y	n = 973	n = 158	n = 71
<19	126 (12.9)	2 (1.3)	0
20–29	119 (12.2)	11 (6.7)	1 (1.4)
30–39	167 (17.2)	28 (17.7)	7 (9.9)
40–49	201 (20.7)	32 (20.3)	7 (9.9)
50–59	175 (18.0)	47 (29.7)	22 (31.0)
60–69	90 (9.2)	22 (13.9)	21 (29.6)
≥70	95 (9.8)	16 (10.1)	13 (19.7)
Source of specimen isolation	n = 895	n = 202	n = 81
Respiratory	754 (84.2)	91 (45.0)	65 (80.2)
Skin, wound, subcutaneous tissue	77 (8.6)	14 (6.9)	5 (6.2)
Mucous membrane†	6 (0.67)	3 (1.5)	0
Bone, joint	14 (1.6)	2 (0.99)	3 (3.7)
Genitourinary	1 (0.11)	0	0
Gastrointestinal	2 (0.22)	7 (3.5)	0
CNS	6 (0.67)	7 (3.5)	0
Other‡	10 (1.1)	46 (22.8)	3 (3.7)
Multiple§	25 (2.8)	32 (15.8)	5 (6.2)

*Counts for blastomycosis are from 1995–2015 and for histoplasmosis and coccidioidomycosis from 1990–2015 data. We omitted cases for which age, sex, or source of specimen isolation were unknown from the calculations. n values by each category are also provided.
†Specimen types included ocular fluid, oral biopsy (tongue), nasal swab, and nasal biopsy.
‡Specimen types included bone marrow, lymph node tissue, blood, parathyroid gland tissue, and adrenal gland tissue and fluid.
§Specimens from ≥2 noncontiguous body sites received <6 months apart.

in SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA), to categorize data into the 5 geographic regions and 4 time intervals (1995–1999, 2000–2004, 2005–2009, 2010–2015). We fitted data to Poisson regression models with the logarithm of total population within each time interval and region used as an offset. We calculated incidence rate ratios (IRR) and 95% CIs by performing a series of pairwise contrast estimates between each regional group and time interval ($p \leq 0.05$ was statistically significant). We further investigated temporal changes within each regional group using 6 pairwise comparisons of annual incidence between each of the 4 time intervals with a Tukey-Kramer adjustment for multiple comparisons.

We conducted spatial analysis with clustering methods to identify hotspots of blastomycosis using Spatial Statistics Toolbox Getis-Ord Gi* statistic in ArcGIS version 10.4. We performed optimized hotspot analysis using case counts normalized with 2016 census subdivision data from Statistics Canada (*33*,*37*). We set polygons to Statistics Canada census subdivisions with polygons with "0" incidences included in the analysis. Statistically significant spatial clustering of higher than average (hotspot) and lower than average (coldspot) values were identified at CIs 90%, 95%, and 99% (1 – p value), signifying the intensity of the hotspot or coldspot. We restricted analysis to cases for which patient home city, FSA, or both were available (n = 544). We plotted individual cases by patient home city, FSA, or both, with circle size proportional to number of cases.

Results

We identified 1,392 laboratory-confirmed dimorphic fungal infections in Ontario during 1990–2015. Among these, blastomycosis was the most common (n = 1,092; 78.4%), followed by histoplasmosis (n = 211; 15.2%) and coccidioidomycosis (n = 89; 6.4%).

Blastomycosis

During the study period, a median of 62 cases/year (range 10–82 cases/year) of blastomycosis occurred; yearly incidence ranged from 0.09–0.60/100,000 population, with an overall annual incidence rate of 0.41/100,000 population (95% CI 0.31–0.52) (Figure 2). Men were more frequently infected than women ($p \leq 0.001$), and infection was most common in those 40–49 years of age (Table 1). Pediatric patients (≤19 years of age) represented 12.9% of cases; 2 cases were reported in infants ≤1 year old. *Blastomyces* fungus was most commonly isolated from respiratory specimens, followed by skin, wounds, subcutaneous tissue, and bone/joint (Table 1). We observed seasonal trends; significantly more cases were diagnosed in the autumn (Bonferroni-corrected p = 0.002) and winter (Bonferroni-corrected p = 0.024) than summer (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/7/17-2063-Techapp1.pdf).

The incidence of blastomycosis in Ontario increased from 0.09/100,000 population in 1995 to 0.52/100,000 population in 2001 and then remained elevated during 2001–2015 (0.48/100,000 population), peaking in 2009

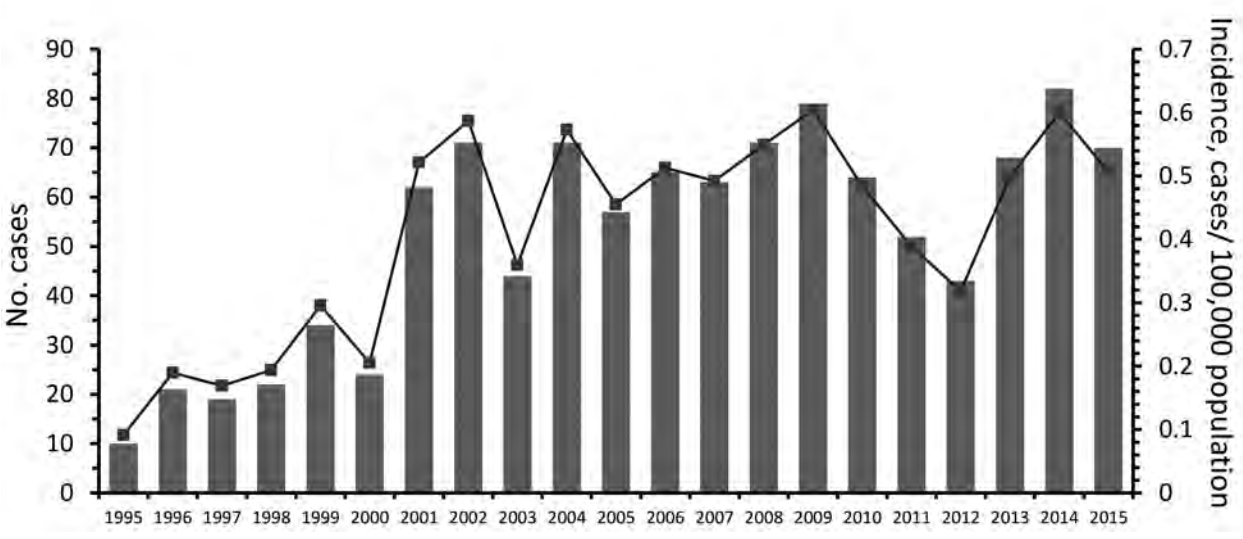

Figure 2. The number of cases (bars) and annual incidence (line) of microbiology laboratory–confirmed blastomycosis in Ontario, Canada, 1995–2015. Incidence was calculated using population denominators from Statistics Canada (*34,37*).

and 2014 with annual incidence rates of 0.60/100,000 population (Figure 2). This increase was statistically significant as indicated by Poisson regression IRRs comparing 1995–1999 versus 2000–2004, 2005–2009, and 2010–2015 (Table 2). Geographic regional analysis suggested that this increase was largely attributable to the Northwest region of the province, where there was also a statistically significant increase in blastomycosis during the same time intervals (Table 2; online Technical Appendix Figure 2). We detected no significant temporal trends in any of the other geographic regions.

The incidence of blastomycosis varied considerably across provincial LHINs (Figure 1, panel A). Disproportionately more cases of blastomycosis were from the North West LHIN (51.3%, n = 560), where the annualized incidence of 10.9/100,000 population (Figure 1, panel A) was 12.6 times greater than any other LHIN. Poisson regression analysis contrasting regional groups showed that the rate of infection was 12.8–105.2 times greater in the Northwest region compared with all other groups (Table 2). Several statistically significant hotspots (95%–99% CI) were identified in and around Kenora and Rainy River, Ontario, located in the Northwest region (online Technical Appendix Figure 3), consistent with the large number of cases in this area (Figure 3). Rates of disease were also significantly elevated in the Northeast region (0.87/100,000 population) compared with the 3 lower-incidence southern regions (Southeast, South-central, Southwest) (Table 2). We identified no statistically significant coldspots (online Technical Appendix Figure 3). In addition to the high number of cases in the Northwest and Northeast regions, we saw a substantial distribution of blastomycosis cases extending into the South-central region (including the Toronto area) during the study period (online Technical Appendix Figure 2; Figure 3).

Histoplasmosis

There were 211 cases of laboratory-confirmed cases of histoplasmosis in Ontario (1990–2015), but no year-on-year or seasonal trends were observed (Figure 4, panel A; online Technical Appendix Figure 1). We identified a median of 7.5 cases each year (range 3–13 cases/year). A diagnosis of histoplasmosis was more common in men than women ($p \leq 0.001$); the greatest proportion of cases occurred in the 50–59 year-old cohort, incorporating both sexes (47/158; 29.7%). Respiratory specimens represented almost half (45%) of the cases, followed by skin, wound, subcutaneous tissue (6.9%), bone marrow (8.9%), and lymph node tissue (7.4%) (Table 1). By geographic distribution, histoplasmosis cases were concentrated in the Toronto Central (69 cases), South East (18 cases), and Champlain (29 cases) LHINs (Figure 1, panel B).

Coccidioidomycosis

For 1990–2015, we detected 89 cases of coccidioidomycosis, a median of 2.5 cases/year (range 1–11 cases/year). We observed no year-on-year or seasonal trends in disease occurrence, yet case counts were notably higher in 1992, 2000, 2005, 2011, 2012, and 2015 (Figure 4, panel B; online Technical Appendix Figure 1). As observed for the other endemic mycoses, men were more frequently infected ($p \leq 0.001$) (Table 1). Median patient age was 59 years (range 24–90 years), and the greatest proportion of cases occurred in the 50–59 (22/71; 31.0%) and 60–69 (21/71; 29.6%) year-old cohorts. Respiratory specimens were the most common source of isolates (80.2%) (Table 1).

Table 2. Temporal and geographic trends of annual incidence and incidence rate ratios of blastomycosis in Ontario, Canada, 1990–2015, by province and region*

Geographic region	Years	Annual incidence	Poisson regression analysis, IRR (95% CI)†			
			Ontario			
			1995–1999	2000–2004	2005–2009	
Ontario	1995–1999	0.19				
	2000–2004	0.42	**3.62 (1.91–6.86)**			
	2005–2009	0.52	**4.46 (2.39–8.35)**	1.23 (0.82–1.84)		
	2010–2015	0.47	**3.87 (2.05–7.31)**	1.07 (0.71–1.62)	0.87 (0.58–1.29)	
			Northwest			
			1995–1999	2000–2004	2005–2009	
Northwest	1995–1999	1.91				
	2000–2004	14.60	**7.31 (2.87–18.64)**			
	2005–2009	15.49	**7.91 (3.11–20.10)**	1.07 (0.68–1.69)		
	2010–2015	11.90	**6.12 (2.37–15.82)**	0.83 (0.51–1.35)	0.77 (0.48–1.25)	
			Southwest	South-central	Southeast	Northeast
Southwest	1995–2015	0.05				
South-central	1995–2015	0.29	**3.09 (1.01–9.50)**			
Southeast	1995–2015	0.14	2.00 (0.62–6.49)	0.65 (0.28–1.52)		
Northeast	1995–2015	0.87	**8.24 (2.82–24.12)**	**2.66 (1.32–5.35)**	**4.12 (1.88–9.07)**	
Northwest	1995–2015	10.9	**105.21 (38.78–285.43)**	**33.97 (19.10–60.41)**	**52.68 (26.69–104.00)**	**12.76 (7.92–20.56)**

*Incidence is no. cases/100,000 population. IRR, incidence rate ratio.
†IRRs were derived from Poisson regression analysis showing pairwise contrasts of blastomycosis incidence rates between different time intervals and geographic regions. Bold type indicates statistically significant values (p<0.05).

Discussion

Our 26-year longitudinal study characterized the epidemiology of microbiologically confirmed cases of blastomycosis, histoplasmosis, and coccidioidomycosis in Ontario, Canada. Although we underestimated the true burden of these diseases by not capturing non–culture-based diagnoses (confirmed through serology, histopathology, or antigen testing), these data substantially increase the known number of cases of endemic fungal infections reported in Canada. Clinicians and public health officials need to be aware that Ontario represents an important region of endemicity for blastomycosis and histoplasmosis and should consider these infections in their differential diagnoses, especially in cases of pneumonia that fails to respond to empiric antimicrobial drugs, in patients residing in or traveling to Ontario, Canada.

Blastomycosis represents an increasingly substantial public health concern in Ontario. The annualized incidence, determined from microbiologically confirmed cases for the province (0.41 cases/100,000 population) (, is higher than that previously reported for 1994–2003 (0.3 cases/100,000 population) (*4*). The incidence within individual LHINs is also increasing. Morris et al. (*4*) noted that disease rates in Ontario increased from 1.8 cases/year during 1981–1989, when blastomycosis was a reportable disease in Ontario, to 59 cases/year in 2001–2003, when it was no longer reportable. We confirm a statistically significant increase in blastomycosis from the late 1990s (0.19 cases/100,000 population) to the early 2000s (0.42 cases/100,000 population) and further show that the incidence remained elevated until 2015 (0.52 cases/100,000 population for 2005–2009 and 0.47 cases/100,000 population for 2010–2015). Most of this effect was attributable to the ≈6- to 7-fold increase in incidence in northwestern Ontario during the corresponding time intervals. Laboratory practices for culture isolation and identification have not changed over the study period; however, enhanced public awareness in the late 1990s may have facilitated more diagnoses (*4*,*6*).

The provincial and North West LHIN rates of blastomycosis are probably underestimated because they do not include cases identified solely by histopathology or serology or those identified outside the province. A substantial number of cases from northwestern Ontario are diagnosed in the bordering province of Manitoba (59/143 Ontario cases, 41.3%, during 1988–1999) (*4*,*5*,*21*). Few cases are diagnosed by antigen testing, which is not performed in Ontario. For 2006–2015, Litvenjenko and Lunny reported 581 blastomycosis hospitalizations in Ontario (0.44 cases/100,000 population), which included cases identified by nonculture methods but not nonhospitalized patients (*23*). By comparison, we identified more microbiology laboratory–confirmed cases (n = 657) during the same time (0.50 cases/100,000 population), suggesting that laboratory counts at PHOL do provide a high degree of case ascertainment of blastomycosis in Ontario. Among the Canadian provinces of Manitoba, Ontario, and Quebec, which are endemic for blastomycosis, Manitoba reported the highest rate of 0.62 cases/100,000 population (1988–1999) (excluding Ontario residents treated in Manitoba [*21*]). Quebec reported a much lower overall rate of 0.13/100,000 population (1988–2011) (*26*).

Given the seriousness of blastomycosis and the consistently elevated incidence, we have advocated in the past for the reinstatement of mandatory disease reporting. Recent

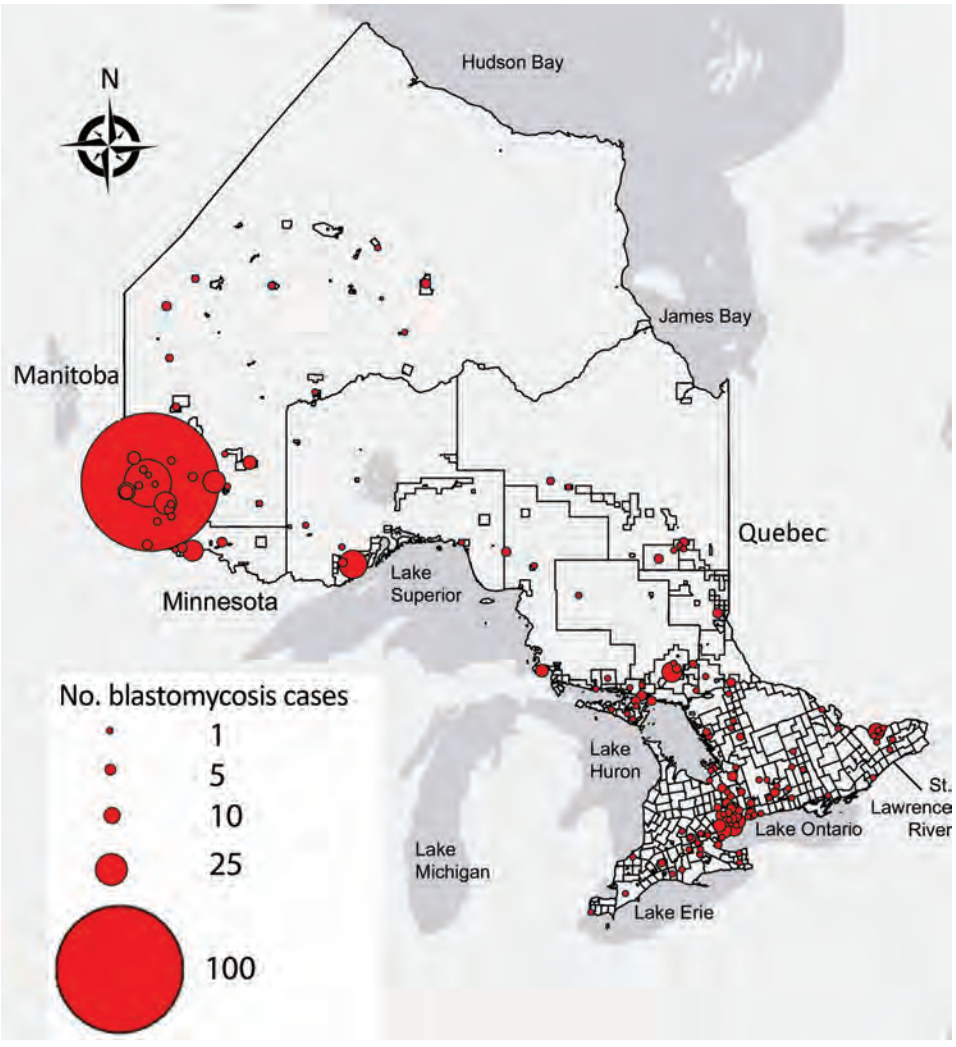

Figure 3. Geographic distribution of blastomycosis cases with known patient city and forward sortation area (first 3 characters of postal code) (n = 544) in Ontario, 1995–2015. Size of dot is proportional to number of cases at a given location.

legislative changes passed in December 2017 have designated blastomycosis as a communicable disease reportable to public health authorities in Ontario (*38*). Timely access to comprehensive surveillance data will allow for a more accurate assessment of disease incidence. It will enable public health officials to track changes in disease incidence or regions of endemicity caused by anthropogenic activities and climatic changes and disturbances (*1*,*11*), and to identify case clusters and point-source outbreaks. Mandatory disease reporting and surveillance will aid the diagnosis of unknown cases, enable prompt initiation of treatment to decrease illness and death (*2*,*7*,*39*), and provide support for targeted public health interventions, such as public awareness campaigns (e.g., health advisories for blastomycosis in Big Grassy First Nation and Manitoulin Island, Ontario) (*6*,*7*,*40*,*41*) and preventive measures for vulnerable groups.

This study reaffirms that the Northwest region of Ontario is highly endemic for blastomycosis with an increasing incidence of the disease over the study period. The North West LHIN incidence of 10.9 cases/100,000 population is substantially higher than the provincial rate of 0.41 cases/100,000 population. The Northwestern Health Unit (western half of the North West LHIN) has a hospitalization rate for blastomycosis of 35.0/100,000 population (*23*), whereas the Kenora area is reportedly hyperendemic with an incidence of 117.2 cases/100,000 population *(6)* and a hospitalization rate of 57.9/100,000 population (*23*). Our analysis also shows several hotspots of blastomycosis in and around the cities of Kenora and Rainy River, with a correspondingly high number of cases of blastomycosis in nearby northern counties of Minnesota (*42*). These hotspots should be interpreted as intersections between areas of human habitation and an ecologic niche in which the conditions promote fungal growth, liberation, and subsequent host infection. The Eagle River area of Wisconsin is a similar localized blastomycosis-hyperendemic region (100 cases/100,000 population) (*43*), with blastomycosis endemic to a much larger geographic area encompassing the US states

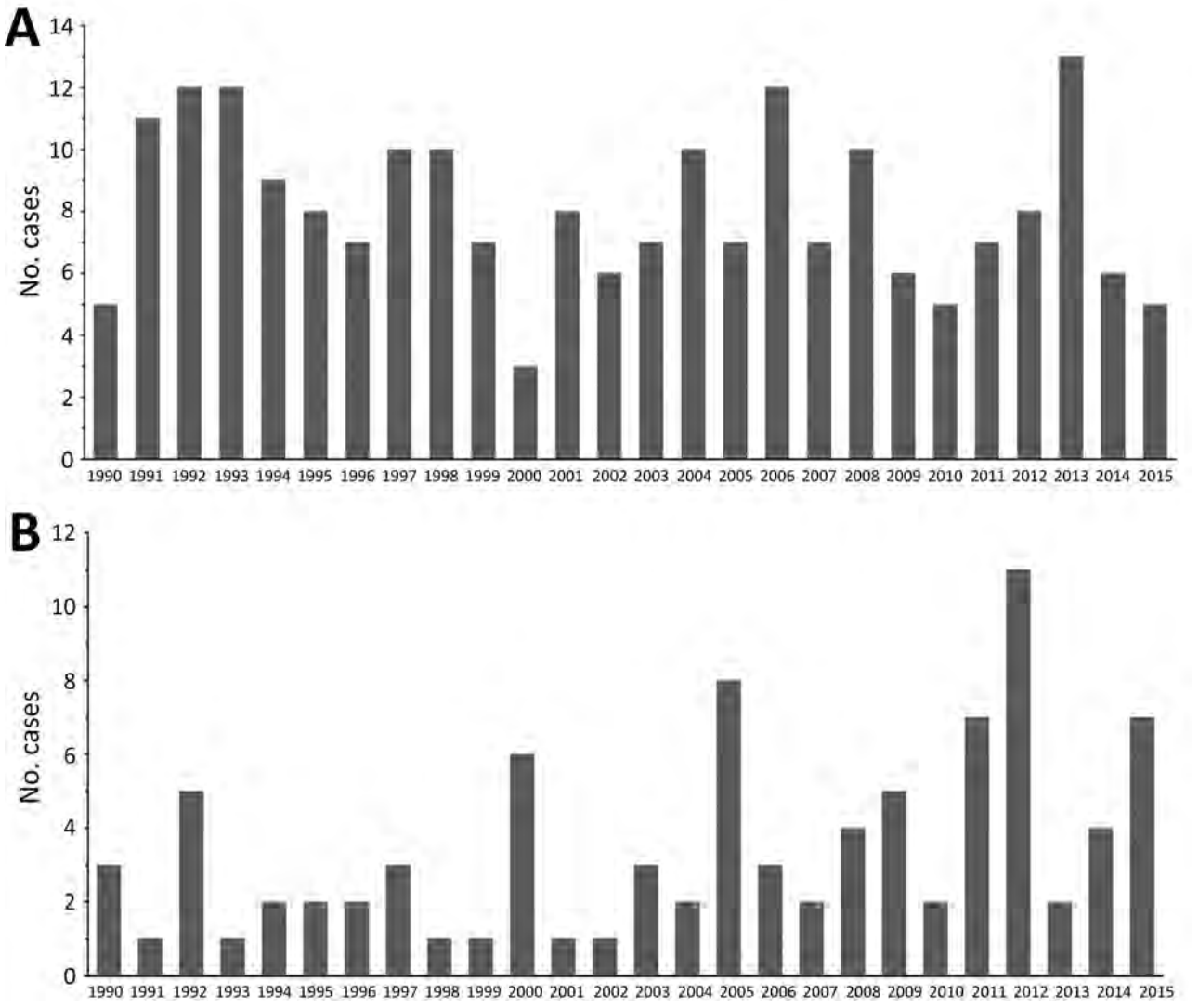

Figure 4. Number of cases of microbiology laboratory–confirmed A) histoplasmosis and B) coccidioidomycosis in Ontario, Canada, 1990–2015.

bordering the Mississippi and Ohio rivers (*14*). The Northeast region of Ontario had the second highest incidence (0.87 cases/100,000 population) in Ontario, followed by the South-central region, which includes Toronto (0.29 cases/100,000 population). Whereas some of the infections may have been acquired during travel to northwestern Ontario, physicians are increasingly encountering patients with blastomycosis who have not traveled to high-incidence locales (*25*,*44*,*45*), suggesting an increased, although statistically unsupported, environmental presence of *Blastomyces* spp. in the Northeast and South-central regions of the province.

Similar to other studies (*4*,*5*,*35*), we observed seasonality of blastomycosis. This finding suggests summer exposure followed by a variable incubation period of 30–45 days (up to 106 days) (*39*,*46*), resulting in diagnosis in the autumn and winter months.

There were 211 microbiology laboratory–confirmed histoplasmosis cases in Ontario from 1990–2015. *H. capsulatum* is endemic to the states along the Mississippi River basin and the regions bordering the St. Lawrence Seaway and Great Lakes River Drainage Basins (*12*,*15*,*19*,*30*). Whereas there are a few older reports of histoplasmosis in Ontario (*19*,*28*–*30*) and Quebec (*19*,*31*,*32*), this study reaffirms Ontario as an area of endemicity. Consistent with its known epidemiologic range, we observed the highest proportion of cases of histoplasmosis in the LHINs bordering the Great Lakes and the St. Lawrence Seaway. Given these findings, we recommend further study to determine the true incidence of histoplasmosis in Ontario; studies should incorporate not only microbiology laboratory–confirmed cases but also those identified by other common diagnostic modalities, such as serology, antigen

testing, and histopathology. Frequent isolation from nonrespiratory specimens (e.g., lymph tissue, nodes, and bone marrow) is consistent with lymphohematogenous spread during infection (*47*) but also suggests that pulmonary mycoses are underrepresented among culture-confirmed cases in Ontario, presumably because they are diagnosed by nonculture methods.

Coccidioidomycosis is not endemic to Canada, and any cases diagnosed in Canada are considered to have been acquired during travel to coccidioidomycosis-endemic areas (*19,48*) specifically the southwestern United States, northern Mexico, and parts of Central and South America (*2*). Although patient travel history was not included in this study, the low number of cases of coccidioidomycosis (n = 89) support this conclusion. Previous Canadian studies report only 2 cases in Ontario (*19,48*). We report 89 cases (2.5 cases/year), a substantial increase that may be caused by an increase in travel of retirees or others to areas endemic to or experiencing an increased incidence of disease (*2,13,15,17,19*). In Ontario, peaks in disease incidence for 2005, 2011, and 2015 mirrored those in California and Arizona (*13,49*). Thus, physicians should consider coccidioidomycosis as a potential cause of disease when treating patients with appropriate symptoms and a history of travel to the southwestern United States.

As with any retrospective study, limitations are inherent to the design. We did not capture symptomatic and mild self-limiting infections, which represent a large proportion of all infections (50%–90%, depending on the fungus) (*2*). Likewise, we did not include mycoses treated empirically without microscopy or culture proof, cases identified at autopsy that did not undergo culture (≈33% of CNS blastomycosis cases [*50*]), cases confirmed solely through histopathology or serology, or cases diagnosed outside Ontario (*21*). We did not genotype repeat isolates from the same patient to investigate persistence or reactivation of the disease. Overall, the numbers presented in this analysis most likely underestimate the true extent of these infections in Ontario. Even though patient demographics were missing for some cases, our results were akin to those reported in other jurisdictions (*14,15,17*). We calculated incidence on the basis of cases assigned to LHINs using patient home address FSA or hospital or physician FSA, which may or may not represent where the infection was acquired (*4*).

In conclusion, this work contributes substantially to our understanding of the geographic distribution and epidemiology of the dimorphic endemic mycoses in Ontario, Canada; however, many cases have likely been missed. The recent restoration of blastomycosis to the list of public health–reportable diseases will assist outbreak investigation, public health planning, and patient and physician education.

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About the Author

Ms. Brown holds an MSc from the University of Toronto, where her research focused on genetic analysis of *Blastomyces dermatitidis* and *B. gilchristii*. Her research interests include infectious disease epidemiology, and using genetic and phylogeographic methods to the study emerging pathogens.

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Registry Cohort Study to Determine Risk for Multiple Sclerosis after Vaccination for Pandemic Influenza A(H1N1) with Arepanrix, Manitoba, Canada

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To investigate a potential risk for multiple sclerosis (MS) after vaccination with Arepanrix, the GlaxoSmithKline AS03-adjuvanted influenza A(H1N1)pdm09 vaccine, we used the provincewide immunization registry for Manitoba, Canada, to match 341,347 persons vaccinated during the 2009 pandemic to 485,941 unvaccinated persons on age, sex, address, and a propensity score measuring the probability of vaccination. We used a previously validated algorithm to identify MS cases from provincial hospital, physician, and prescription drug claims databases. After 12 months of follow-up, the age-adjusted incidence rate of MS was 17.7 cases per 100,000 person-years in the Arepanrix cohort and 24.2 per 100,000 in the unvaccinated cohort. The corresponding adjusted hazard ratio was 0.9. We observed similar patterns when we measured incidence over the entire follow-up period. The AS03 adjuvant, a candidate for inclusion in future pandemic vaccines, does not appear to increase the short-term risk for MS when included in influenza vaccines.

Multiple sclerosis (MS) is a chronic debilitating disease of the central nervous system (CNS) that affects >2.5 million persons worldwide (*1*). Its etiology is unknown but most likely is due to complex interactions between genetic and environmental factors (*2*). A role for infectious agents and vaccines has been suggested (*2*), but concrete evidence is lacking (*3*–*6*).

Soon after the 2009 influenza A(H1N1) pandemic, a signal of increased incidence of MS was detected in a postlicensure record-linkage study among residents of 3 counties in Sweden who received Pandemrix (GlaxoSmithKline, Dresden, Germany), an inactivated monovalent AS03-adjuvanted influenza A(H1N1)pdm09 vaccine (*7*). Another study from Stockholm, Sweden, reported increased risk for paraesthesias, but not of MS, among persons vaccinated with Pandemrix (*8*). Neither study was designed to assess an association with MS, and neither used validated algorithms for identifying MS from administrative databases. Because AS03, an adjuvant system containing α-tocopherol and squalene in an oil-in-water emulsion (*9*), is likely to be used in future pandemic vaccines, the European Medicine Agency mandated a study to evaluate the relationship between use of the GlaxoSmithKline AS03-adjuvanted pandemic vaccines and MS.

We assessed whether use of another AS03-adjuvanted A(H1N1)pdm09 vaccine, Arepanrix (GlaxoSmithKline, Quebec City, QC, Canada), was associated with increased risk for incident MS in Manitoba, Canada. Because of a combination of genetic and environmental factors, the prevalence of MS varies geographically (*2*). Canada is a high-prevalence region for MS. Within Canada, the prevalence of MS is particularly high in central and western provinces, such as Manitoba (*10*,*11*). Our secondary objective was to assess whether administration of Arepanrix was associated with increased risk for CNS demyelinating events that do not ultimately lead to MS (hereafter other demyelinating conditions).

Methods

Design and Data Sources

Manitoba Health is a government agency that provides publicly funded universal healthcare to virtually all of Manitoba's 1.3 million residents. Insured services include hospital, physician, and preventive services, including vaccinations. All provided services are recorded in centralized electronic databases that can be linked using a unique lifetime personal health identification number (PHIN). A population registry tracks addresses and dates of birth,

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insurance coverage, and death for all insured persons. We analyzed population-based cohorts assembled by linking Manitoba Health's vaccine registry with hospital, physician, and prescription claim databases, all part of a comprehensive repository of administrative and clinical databases housed at the Manitoba Centre for Health Policy (*12*). The Manitoba Immunization Monitoring System (MIMS) is a population-based provincewide registry of virtually all vaccines administered to Manitoba residents since 1988 (*13*). Vaccine type and date of vaccination are captured through direct data entry for vaccines administered by public health staff (who administered most influenza vaccines during the pandemic) or using physician claims data for vaccines administered by physicians (*13*).

Since 1971, the Hospital Abstracts Database recorded all hospital admissions in the province, including diagnoses and treatments coded using the International Classification of Diseases (ICD), Tenth Revision, and the Canadian Classification of Health Interventions (*12*). The Medical Services Database, also in operation since 1971, captures physician services including tariff codes for each service provided and a single ICD, Ninth Revision, diagnosis (*12*). The provincial Drug Program Information Network captures all out-of-hospital prescriptions dispensed in Manitoba since 1995 (*14*).

The study was approved by the Research Ethics Board of the University of Manitoba and the governmental Health Information Privacy Committee and registered with ClinicalTrials.gov (NCT02367222). Because this study was an European Medicine Agency regulatory requirement, patients were not involved in the development of research questions, study design, or conduct.

Study Population

Anyone ≥6 months of age who was registered with Manitoba Health during September 15, 2009–March 15, 2010, when virtually all pandemic vaccines were administered, was eligible for inclusion in the study. We excluded participants who had <1 year of insurance coverage before enrollment (insufficient historical data) or ≥1 physician or hospitalization records for any demyelinating condition before enrollment.

Determination of Vaccination Status

We obtained information about the receipt of the pandemic influenza, seasonal influenza, and other vaccines during and before the 2009–10 season from MIMS. Manitoba's routine vaccination schedule includes seasonal trivalent inactivated influenza vaccines (TIVs); during the study period, vaccines used were were Fluviral (GlaxoSmithKline) and Vaxigrip (Sanofi Pasteur, Lyon, France). Most pandemic vaccines were administered during a mass immunization campaign that began October 26, 2009 (*15*). Like elsewhere in Canada, Arepanrix was used to vaccinate adults and children >6 months of age. Later, 2 unadjuvanted vaccines, from GlaxoSmithKline and CSL Limited (Parkville, VIC, Australia), were offered to pregnant women and children ≥10 years of age. As recommended by the World Health Organization, all vaccines contained 3.75 μg (per 0.5 mL) of hemagglutinin from an A/California/7/2009 (H1N1)v–like strain (X-179A). Because of limited supplies at campaign start, healthcare workers, Aboriginal persons, pregnant women, children 6–60 months of age, persons <65 years with chronic medical conditions, and all immunocompromised persons were prioritized (*15*).

To assemble study cohorts, we used a high-dimensional propensity score (PS) algorithm to calculate a PS for each eligible participant (*16*). PS is the conditional probability of receiving an intervention, an influenza A(H1N1)pdm09 vaccine in this case, given the value of a set of confounders (*17*). Use of PS in observational studies enables forming more comparable study groups by limiting comparisons to persons who had the same probability of receiving the intervention (*17*). This approach is particularly suitable for postlicensure studies of drug and vaccine safety in which the outcomes are rare, limiting the utility of conventional multivariable adjustment methods, but the intervention and confounder data are rich. The availability of vaccination status for the whole population (from MIMS) facilitated development and testing of the score.

We computed PS as the probability of receiving an influenza A(H1N1)pdm09 vaccine predicted by a logistic regression model that included vaccine receipt as the dependent variable and >400 independent variables including demographic information (e.g., socioeconomic status), coexisting illnesses, healthcare use (e.g., hospitalizations or physician visits), prescription drug use, and prior vaccinations. We matched each vaccinated person with a randomly selected unvaccinated person with the closest PS and the same age, sex, and neighborhood of residence.

Study Endpoints

The primary endpoint was incidence of MS within 12 months after the index date. We defined the index date as the date of vaccination for vaccinated persons or the date of vaccination of the matched vaccinated person for unvaccinated persons. Secondary endpoints were incidence of MS until the end of follow-up (December 31, 2012) and of other demyelinating conditions within 12 months after the index date. We identified all endpoints by linking with Manitoba Health's hospital, physician, and prescription claims databases. We used a previously validated algorithm, based on chart reviews, as well as separate medical record reviews and self-administered questionnaires, to identify cases (*10*). A case of MS was defined as ≥3 hospital, physician, or prescription claims for MS by an individual person. In

validation studies in Manitoba and Nova Scotia (Canada), this definition had a positive predictive value of 80%–93% and a negative predictive value of 98% (*18*). The date of MS diagnosis was the date of the first medical contact for MS. Other demyelinating conditions were defined by ≥1 hospitalizations or ≥2 physician claims ≥30 days apart for any of the following: optic neuritis, acute transverse myelitis, demyelinating disease of CNS unspecified, other acute disseminated demyelination, or neuromyelitis optica (provided there was no subsequent MS diagnosis). We considered a case incident if no previous physician or hospitalization records indicated a diagnosis of any demyelinating condition going back to 1971.

Covariates

Based on their postal codes, we assigned participants to a neighborhood of residence (neighborhood clusters within the capital city of Winnipeg and health districts in the rest of the province). We linked postal codes to 2006 Canadian census data to determine household income (quintiles) measured at the level of Census Dissemination areas. We used previously validated algorithms, based on the frequency of certain ICD codes, to identify various chronic diseases and health conditions including pregnancy (*19*–*21*).

Statistical Analysis

For each endpoint, we calculated crude and age-standardized incidence rates (ASRs) and ratios (ARRs). We conducted survival analyses, measuring time-to-onset from the index date to the diagnosis date. Persons were censored on the earliest of study end date, loss to follow-up (because of death or immigration), or subsequent receipt of a different vaccine (because cases identified afterward might have resulted from the more recent vaccine). Two influenza vaccines given on the same day, typically an A(H1N1)pdm09 vaccine and a 2009–10 TIV, were considered as 1 episode. However, in analyses stratified by vaccine type, we grouped these episodes separately as the "concurrent A(H1N1)pdm09 vaccine/TIV" cohort and compared the incidence of MS in this group with that among persons who received only 1 vaccine: the "A(H1N1)pdm09 vaccine alone" cohort or the "TIV alone" cohort.

We estimated hazards ratios and corresponding 95% CIs associated with the receipt of an A(H1N1)pdm09 vaccine using Cox proportional hazard models with stratification on the matched pairs (to account for matching) (*22*). We verified the proportional hazards assumption using graphical and formal methods (*23*). We looked for effect modification with the receipt of the 2008–09 TIV, testing for interactions between A(H1N1)pdm09 vaccine and TIV terms using a likelihood ratio test with a liberal threshold for statistical significance ($p<0.15$). We also completed exploratory analyses to examine the association between unadjuvanted A(H1N1)pdm09 vaccines and MS. We could not adequately complete a planned subgroup analysis by age group and history of autoimmune diseases because of small MS numbers in most subgroups. Based on 341,000 vaccinated persons and MS incidence rate of 23 cases/100,000 persons among 485,000 nonvaccinated persons, our analysis had 95% power to detect a 20% increase in risk and 75% power to detect a 10% increase in risk, assuming a 2-sided $\alpha = 0.05$ (*24*).

Results

A total of 341,347 (29%) persons received ≥1 doses of an A(H1N1)pdm09 vaccine during the enrollment period. Of these, 278,131 (57%) received an A(H1N1)pdm09 vaccine only, 144,594 (30%) received a TIV only, and 63,216 (13%) received both. Almost all (96%) persons who received an A(H1N1)pdm09 vaccine received the adjuvanted Arepanrix either alone (78%) or in addition to a TIV (18%).

Although subcohorts might appear different when their aggregate characteristics are compared (Table 1), we based the actual analysis on the matched pairs (of vaccinated and unvaccinated matches) who were generally similar. As expected, children and younger adults dominated (54%) the A(H1N1)pdm09 vaccine group, whereas older (≥55) adults (78%) and persons with chronic illnesses (30%) dominated the TIV group. There were more pregnant women in the vaccinated group, representing >50% of those who received the unadjuvanted A(H1N1)pdm09 vaccine. Vaccinated persons were more likely to have previously received the 2008–09 TIV and ≥1 pneumococcal vaccines.

By the end of the first year of follow-up, a total of 106 incident MS cases had been diagnosed among the unvaccinated cohort (Table 2), corresponding to an ASR of 24.2 (95% CI 20.1–28.3)/100,000 person-years, compared with 69 cases and an ASR of 20.2 (95% CI 15.4–24.9)/100,000 person-years among persons who received any influenza vaccine. The ASR was lower for persons who received Arepanrix (17.7 [95% CI 14.1–21.2]/100,000 person-years), corresponding to an ARR of 0.7 [95% CI 0.3–1.7]. The rate was similar for the unadjuvanted vaccine cohort. The ASR was higher for persons who received the 2009–10 TIV alone (36.8 [95% CI 25.0–48.6]/100,000 person-years) than for those who did not (ARR 1.5 [95% CI 0.3–6.8]). We observed no increase in risk for persons who received the TIV and A(H1N1)pdm09 vaccine concurrently. Regardless of vaccine type, ARRs calculated over the entire period (median of 3 years) were consistent with lack of association with vaccine administration.

After 1 year of follow-up, only 27 persons among the unvaccinated cohort met the case definition for having other demyelinating conditions, corresponding to an ASR of 6.9 (95% CI 2.6–11.1)/100,000 person-years, compared with 17 cases and an ASR of 4.7 (95% CI 0.0–10.6)/100,000 person-years

Table 1. Cohort characteristics by vaccination status, Manitoba, Canada, 2009–2012*

	No. (%) persons					
	Adjuvanted A(H1N1)pdm09		Unadjuvanted A(H1N1)pdm09			
Variable	Alone	And TIV	Alone	And TIV	TIV alone	Unvaccinated
Total	267,539 (100)	61,239 (100)	10,592 (100)	1,977 (100)	144,594 (100)	485,941 (100)
Age group, y						
≤14	83,097 (31.1)	10,206 (16.7)	1,245 (11.8)	109 (5.5)	4,915 (3.4)	99,463 (20.5)
15–34	62,261 (23.3)	12,637 (20.6)	4,717 (44.5)	679 (34.3)	7,094 (4.9)	92,008 (18.9)
35–44	38,401 (14.4)	9,272 (15.1)	1,835 (17.3)	363 (18.4)	6,463 (4.5)	51,795 (10.7)
45–54	39,958 (14.9)	12,018 (19.6)	1,452 (13.7)	389 (19.7)	12,696 (8.8)	74,454 (15.3)
≥55	43,822 (16.4)	17,106 (27.9)	1,343 (12.7)	437 (22.1)	113,426 (78.4)	168,221 (34.6)
Sex						
F	144,461 (54.0)	31,081 (50.8)	7,352 (69.4)	1,194 (60.4)	82,868 (57.3)	266,956 (54.9)
Urban residence	146,256 (54.7)	41,916 (68.4)	6,202 (58.6)	1,604 (81.1)	96,605 (66.8)	292,583 (60.2)
Income quintile						
Q1 (lowest)	53,269 (19.9)	9,766 (15.9)	1,803 (17.0)	279 (14.1)	27,899 (19.3)	92,147 (19.0)
Q2	47,036 (17.6)	11,066 (18.1)	1,833 (17.3)	355 (18.0)	27,593 (19.1)	91,214 (18.8)
Q3	48,257 (18.0)	10,976 (17.9)	1,766 (16.7)	358 (18.1)	28,037 (19.4)	91,542 (18.8)
Q4	50,592 (18.9)	12,454 (20.3)	2,373 (22.4)	447 (22.6)	27,167 (18.8)	95,379 (19.6)
Q5 (highest)	62,320 (23.3)	15,602 (25.5)	2,613 (24.7)	503 (25.4)	25,103 (17.4)	101,411 (20.9)
Cannot be calculated	6,065 (2.3)	1,375 (2.2)	204 (1.9)	35 (1.8)	8,795 (6.1)	14,248 (2.9)
Immunosuppressed	11,541 (4.3)	4,028 (6.6)	322 (3.0)	78 (3.9)	20,990 (14.5)	33,561 (6.9)
Autoimmune diseases	6,680 (2.5)	2,669 (4.4)	197 (1.9)	45 (2.3)	10,179 (7.0)	17,661 (3.6)
Any chronic diseases	18486 (6.9)	7,485 (12.2)	364 (3.4)	96 (4.9)	42,909 (29.7)	65,158 (13.4)
Pregnant, % of all 15–49-year-old females	2,184 (3.1)	355 (2.4)	2,857 (50.6)	330 (40.4)	816 (7.4)	5,160 (4.9)
High priority for A(H1N1)pdm09 vaccine	142,909 (53.4)	25,652 (41.9)	6,200 (58.5)	683 (34.5)	59,165 (40.9)	230,948 (47.5)
High priority for TIV	42,207 (15.8)	12,958 (21.2)	3,231 (30.5)	419 (21.2)	105,557 (73.0)	158,565 (32.6)
Received 2008–09 TIV	41,896 (15.7)	22,231 (36.3)	1,237 (11.7)	542 (27.4)	109,107 (75.5)	42,071 (8.7)
Received pneumococcal vaccine	49,203 (18.4)	11,554 (18.9)	286 (2.7)	64 (3.2)	87,552 (60.6)	86,261 (17.8)

*A(H1N1)pdm09, pandemic influenza A(H1N1) strain; Q, quintile; TIV, trivalent influenza vaccine.

among the vaccinated cohort (Table 3). Generally, ARRs calculated over this period were consistent with lack of an association with vaccine administration. Findings were similar over the longer follow-up period, except for persons who received an unadjuvanted vaccine where the ASR was higher (7.4 [95% CI 0.0–18.0]/100,000 person-years), but because of the small number of cases (<6), the corresponding ARR (2.1) was imprecise (95% CI 0.1–39.9).

In Cox models adjusted for matching (model A), we found no evidence of an association between MS and the receipt of any vaccine (Table 4). For instance, the hazard ratio for receipt of Arepanrix alone was 0.9 (95% CI 0.6–1.4) and did not appreciably change with further adjustment for receipt of the 2008–09 TIV (model B). We observed similar patterns when we measured disease occurrence over the entire follow-up period (Table 4).

In Cox models, we found no evidence of increased risk for other demyelinating conditions with the receipt of a pandemic vaccine (Table 4). The receipt of either TIV alone or concurrently with the adjuvanted pandemic vaccine was associated with an increased risk for these conditions and the association persisted after adjusting for receipt of the 2008–09 TIV and when repeated for the entire study period. However, although these findings were consistent, none of these associations was statistically significant or precise because few cases were diagnosed among these groups.

Discussion

In this large population-based registry study, we found no evidence of an association between the adjuvanted influenza A(H1N1)pdm09 vaccine used in Canada and the incidence of MS or that of other acquired CNS demyelinating disorders. These findings are largely consistent with limited prior work regarding the A(H1N1)pdm09 vaccine and other influenza vaccines.

Few studies have examined the association between the A(H1N1)pdm09 vaccine and occurrence of MS. In published (mostly manufacturer-sponsored) randomized controlled trials conducted during the pandemic, there were no reports of clinically significant adverse events (including MS) of the different pandemic vaccine formulations (*25*). These findings are reassuring, but these trials might have not been large enough to detect a small increase in risk.

Similarly, vaccine adverse events surveillance systems in Europe and the United States did not detect increased risk for MS with pandemic vaccine use (*26*,*27*). No increased risk was found in an analysis of the European EudraVigilance database, which tracked reports of suspected autoimmune disorders after use of either adjuvanted (including Pandemrix) or unadjuvanted A(H1N1)pdm09 vaccines (*26*). Analyses of the US Vaccine Adverse Event Reporting System reached a similar conclusion (*27*).

Table 2. Crude and age-standardized rates of incident multiple sclerosis and influenza vaccination status, Manitoba, Canada, 2009–2012*

Vaccination status	No. events	Rate (95% CI)		Rate ratio (95% CI)	
		Crude	Age-standardized	Crude	Age-adjusted
1 year after index date					
Unvaccinated	106	23.2 (19.2–28.0)	24.2 (20.1–28.3)	1	1
Vaccinated, A(H1N1) pdm09/TIV	69	19.1 (15.1–24.2)	20.2 (15.4–24.9)	0.8 (0.6–1.1)	0.8 (0.3–2.2)
A(H1N1)pdm09 alone	43	17.6 (13.1–23.8)	17.7 (14.1–21.2)	0.8 (0.5–1.1)	0.7 (0.3–1.7)
Concurrent A(H1N1)pdm09/TIV	12	20.3 (11.5–35.7)	19.4 (8.6–30.2)	0.9 (0.5–1.6)	0.8 (0.1–5.0)
TIV alone	14	24.3 (14.4–41.1)	36.8 (25.0–48.6)	1.0 (0.6–1.8)	1.5 (0.3–6.8)
Adjuvanted A(H1N1)pdm09 alone	40	17.1 (12.5–23.3)	17.4 (13.8–21.1)	0.7 (0.5–1.1)	0.7 (0.3–1.7)
Concurrent adjuvanted A(H1N1)pdm09/TIV	11	19.2 (10.6–34.7)	18.3 (7.3–29.3)	0.8 (0.4–1.5)	0.8 (0.1–5.1)
Unadjuvanted A(H1N1)pdm09 alone	s	s	18.6 (8.8–28.3)	1.3 (0.4–4.2)	0.8 (0.1–4.2)
Concurrent unadjuvanted A(H1N1)pdm09/TIV	s	s	37.9 (13.8–62.0)	2.3 (0.3–16.4)	1.6 (0.1–26.6)
Entire follow-up period					
Unvaccinated	188	15.6 (13.5–18.0)	16.0 (13.5–18.5)	1	1
Vaccinated, A(H1N1)pdm09/TIV	132	15.1 (12.7–17.9)	15.4 (12.4–18.4)	1.0 (0.8–1.2)	1.0 (0.5–1.9)
A(H1N1)pdm09 alone	82	14.6 (11.7–18.1)	14.9 (9.3–20.5)	0.9 (0.7–1.2)	0.9 (0.3–2.8)
Concurrent A(H1N1)pdm09/TIV	33	20.1 (14.3–28.2)	18.2 (11.8–24.6)	1.3 (0.9–1.9)	1.1 (0.4–3.6)
TIV alone	17	11.4 (7.1–18.4)	16.6 (9.5–23.8)	0.7 (0.4–1.2)	1.0 (0.3–3.9)
Adjuvanted A(H1N1)pdm09 alone	78	14.5 (11.6–18.0)	15.0 (9.3–20.7)	0.9 (0.7–1.2)	0.9 (0.3–2.9)
Concurrent adjuvanted A(H1N1)pdm09/TIV	32	20.1 (14.2–28.4)	18.4 (11.9–24.9)	1.3 (0.9–1.9)	1.1 (0.4–3.7)
Unadjuvanted A(H1N1)pdm09 alone	s	s	9.7 (3.6–15.8)	1.1 (0.4–2.9)	0.6 (0.1–2.6)
Unadjuvanted A(H1N1)pdm09/TIV	s	s	13.1 (0.0–27.2)	1.2 (0.2–8.5)	0.8 (0.0–13.5)

*Rates are per 100,000 person-years. A(H1N1)pdm09, pandemic influenza A(H1N1) strain; s, suppressed because of small sample size (n = 1–5) in accordance with the requirements of the data custodian; TIV, trivalent influenza vaccine.

A large retrospective record-linkage study from Sweden reported increased risk for paresthesias, but not of MS, among persons vaccinated with Pandemrix (*8*,*28*). Among persons with a high risk for influenza complications who were mostly vaccinated in the first 45 days of the campaign (healthcare workers, children, pregnant women, and persons with chronic diseases), the risk for MS was 1.17 (95% CI 0.82–1.66), and the risk estimates were highest within 6 weeks after vaccination (1.35 [95% CI 0.68–2.67]). There was no similar increase of risk among other groups. The authors attributed the excess risk among high-risk groups to possible confounding by underlying co-morbidity and vaccine indication.

Other AS03-adjuvanted influenza vaccines (e.g., avian influenza [H5N1] vaccines), as well as vaccines based on other oil-in-water adjuvants (e.g., MF59), were found in several randomized controlled trials to be more reactogenic than unadjuvanted TIVs. However, no serious adverse events, including MS, were reported (*29*). Finally, no evidence exists to support a link with use of unadjuvanted TIVs. In a systematic review, the authors identified 4 observational studies; the pooled risk ratio of developing MS

Table 3. Crude and age-standardized rates of incident demyelinating conditions not ultimately diagnosed as multiple sclerosis and influenza vaccination status, Manitoba, Canada, 2009–2012*

Vaccination status	No. events	Rate (95% CI)		Rate ratio (95% CI)	
		Crude	Age-standardized	Crude	Age-adjusted
1 year after index date					
Unvaccinated	27	5.9 (4.1–8.6)	6.9 (2.6–11.1)	1	1
Vaccinated, A(H1N1)pdm09/TIV	17	4.7 (2.9–7.6)	4.7 (0.0–10.6)	0.8 (0.4–1.5)	0.7 (0.1–6.4)
A(H1N1) pdm09 alone	s	s	5.6 (0.0–13.3)	0.8 (0.4–1.6)	0.8 (0.1–10.6)
Concurrent A(H1N1) pdm09/TIV	s	s	7.8 (0.0–17.0)	1.4 (0.6–3.7)	1.1 (0.1–15.2)
TIV alone	0	0	NA	NA	NA
Adjuvanted A(H1N1)pdm09 alone	11	4.7 (2.6–8.5)	5.4 (0.0–13.1)	0.8 (0.4–1.6)	0.8 (0.1–10.8)
Concurrent adjuvanted A(H1N1)pdm09/TIV	s	s	8.0 (0.0–17.3)	1.5 (0.6–3.8)	1.2 (0.1–15.5)
Unadjuvanted A(H1N1)pdm09 alone	s	s	6.2 (0.0–16.0)	1.7 (0.2–12.8)	0.9 (0.0–18.1)
Unadjuvanted A(H1N1)pdm09/TIV	0	0	NA	NA	NA
Entire follow-up period					
Unvaccinated	40	3.3 (2.4–4.5)	3.6 (0.8–6.3)	1	1
Vaccinated, A(H1N1)pdm09/TIV	25	2.9 (1.9–4.2)	2.9 (0.0–7.1)	0.9 (0.5–1.4)	0.8 (0.1–6.2)
A(H1N1)pdm09 alone	17	3.0 (1.9–4.9)	3.5 (0.0–9.1)	0.9 (0.5–1.6)	1.0 (0.1–10.3)
Concurrent A(H1N1)pdm09/TIV	s	s	3.8 (0.0–10.7)	1.1 (0.5–2.6)	1.1 (0.1–15.6)
TIV alone	s	s	0.4 (0.0–2.1)	0.4 (0.1–1.7)	0.1 (0.0–1.0)
Adjuvanted A(H1N1)pdm09 alone	15	2.8 (1.7–4.6)	3.2 (0.0–8.9)	0.8 (0.5–1.5)	0.9 (0.1–10.5)
Concurrent adjuvanted A(H1N1)pdm09/TIV	6	3.8 (1.7–8.4)	4.0 (0.0–10.9)	1.1 (0.5–2.7)	1.1 (0.1–16.1)
Unadjuvanted A(H1N1) alone	s	s	7.4 (0.0–18.0)	2.5 (0.6–10.5)	2.1 (0.1–39.9)
Unadjuvanted A(H1N1)pdm09/TIV	0	0	NA	NA	NA

*Rates are per 100,000 person-years. A(H1N1)pdm09, pandemic influenza A(H1N1) strain; NA, cannot be estimated; s, suppressed because of small sample size (n = 1–5) in accordance with the requirements of the data custodian; TIV, trivalent influenza vaccine.

Table 4. Association between influenza vaccination and incidence of multiple sclerosis and demyelinating conditions not ultimately diagnosed as multiple sclerosis, Manitoba, Canada, 2009–2012*

	Multiple sclerosis, HR (95% CI)		Other demyelinating conditions, HR (95% CI)	
Vaccination status	Model A	Model B	Model A	Model B
1 Year after index date				
Unvaccinated	Ref	Ref	Ref	Ref
Vaccinated, A(H1N1)pdm09/TIV	0.9 (0.6–1.2)	0.9 (0.6–1.3)	0.6 (0.3–1.2)	0.6 (0.3–1.2)
A(H1N1) pdm09 alone	0.9 (0.6–1.3)	0.9 (0.6–1.4)	0.5 (0.3–1.1)	0.5 (0.3–1.2)
Concurrent A(H1N1) pdm09/TIV	0.6 (0.3–1.3)	0.6 (0.3–1.4)	2.0 (0.4–10.9)	2.3 (0.4–15)
TIV alone	1.1 (0.5–2.3)	1.2 (0.5–2.9)	NA	NA
Adjuvanted A(H1N1)pdm09 alone	0.9 (0.6–1.4)	0.9 (0.6–1.5)	0.5 (0.2–1.1)	0.5 (0.2–1.1)
Concurrent adjuvanted A(H1N1)pdm09/TIV	0.6 (0.3–1.3)	0.6 (0.3–1.3)	2.0 (0.4–10.9)	2.3 (0.4–14.8)
Unadjuvanted A(H1N1)pdm09 alone	0.8 (0.2–3.4)	0.8 (0.2–3.5)	1.0 (0.1–15.9)	1.0 (0.1–15.9)
Unadjuvanted A(H1N1)pdm09/TIV	1.0 (0.1–15.9)	1.1 (0.1–18.0)	NA	NA
Entire follow-up period				
Unvaccinated	Ref	Ref	Ref	Ref
Vaccinated, A(H1N1)pdm09/TIV	1.0 (0.8–1.3)	1.1 (0.8–1.5)	0.7 (0.4–1.1)	0.7 (0.4–1.2)
A(H1N1) pdm09 alone	1.0 (0.8–1.4)	1.1 (0.8–1.5)	0.5 (0.3–1.0)	0.6 (0.3–1.1)
Concurrent A(H1N1) pdm09/TIV	1.1 (0.6–1.8)	1.2 (0.7–2)	1.7 (0.4–7)	2.1 (0.4–10.2)
TIV alone	0.9 (0.5–1.9)	1.2 (0.6–2.5)	1.0 (0.1–7.1)	1.5 (0.1–16.1)
Adjuvanted A(H1N1)pdm09 alone	1.0 (0.8–1.4)	1.1 (0.8–1.5)	0.5 (0.3–1.0)	0.5 (0.3–1.1)
Concurrent adjuvanted A(H1N1)pdm09/TIV	1.1 (0.6–1.8)	1.2 (0.7–2.0)	1.7 (0.4–7.0)	2.1 (0.4–10.1)
Unadjuvanted A(H1N1)pdm09 alone	0.8 (0.2–3.0)	0.8 (0.2–3.1)	1.0 (0.1–15.9)	1.0 (0.1–15.9)
Unadjuvanted A(H1N1)pdm09/TIV	1.0 (0.1–15.9)	1.2 (0.1–19.8)	NA	NA

*Model A estimates were adjusted for matching variables (propensity scores, age, sex, and area of residence). Model B estimates also were adjusted for receipt of the 2008–09 TIV. A(H1N1)pdm09, pandemic influenza A(H1N1) strain; HR, hazard ratio; NA, cannot be estimated; ref, referent; TIV, trivalent influenza vaccine; TIV.

after influenza vaccination was 0.97 (95% CI 0.77–1.23) with little evidence of heterogeneity (p = 0.368) (*30*).

We did not evaluate the association between A(H1N1) pdm09 vaccines and relapses in persons with established MS. The evidence for this association has been inconsistent. In several small randomized controlled trials, there was no increased incidence of relapses after A(H1N1) pdm09 vaccination (*31*,*32*). Observational studies provided conflicting findings, probably because of limitations in study design, such as small sample sizes and selection biases (*33*,*34*). Earlier studies and systematic reviews have not found any evidence of an increased risk for relapse with use of TIVs and other vaccines (*35*,*36*).

Evidence is scarce for an association between A(H1N1) pdm09 vaccination and other CNS demyelinating disorders. In a review of published case series, postmarketing surveillance data, and observational studies, a diagnosis of optic neuritis was not associated with influenza vaccination (*37*). Although 13 cases were reported after influenza vaccination, no association was found in 2 case–control studies (*37*). A more recent case–control study did not find an association between vaccine use in general and subsequent CNS demyelinating disorders (*38*).

We had access to accurate records of hospitalization, physician utilization, vaccination, and prescriptions for the entire population, which reduced the possibilities of selection bias and differential misclassification of exposures and outcomes and ensured that unvaccinated persons were truly enrolled and their healthcare use was captured. However, some variables might have been measured with error. The ascertainment of MS cases is most likely incomplete because initial symptoms might not be recognized as demyelinating, and diagnostic delays occur, although these have declined substantially over time in Manitoba (*10*) and elsewhere, such that most MS is diagnosed <1 year from onset (*39*). However, the incidence rate of MS for unvaccinated persons in this study was comparable with rates measured in similarly young populations in earlier studies from Manitoba and elsewhere (*10*,*40*). Furthermore, underascertainment is probably nondifferential with respect to A(H1N1)pdm09 vaccination because knowledge of vaccination status is unlikely to have directly influenced the way MS was diagnosed or coded, suggesting that our relative risk estimates of the association are likely to be accurate, even though our absolute MS incidence rates might be underestimated.

We did not have information about putative lifestyle and environmental risk factors. We attempted to adjust for these factors by matching on age, sex, region of residence, and PS. Matching on region reduces the likelihood of confounding by ethnicity because ethnic minorities tend to cluster in communities, even in the province's large urban centers. PS reduces confounding by measured (e.g., access to healthcare) and unmeasured (e.g., smoking) confounders because of the inclusion of proxy conditions (e.g., smoking-related diseases) in the PS calculation. Furthermore, the vaccinated and unvaccinated cohorts were comparable, indicating a reasonable performance of the matching procedure. However, residual confounding remains possible.

Although the relatively large sample size permitted calculation of reasonably precise estimates, small numbers in some subgroup analyses limited the precision of estimates. Generalizability of the findings to other populations depends on their geographic location, ethnic composition, and access to the pandemic vaccine products. Manitoba's population tends to be typical of many western populations, especially those in northern high-latitude countries, in terms of MS incidence and ethnic composition and even with the timing and epidemiology of the 2009 pandemic and the nature of the public health response to the pandemic. Finally, our findings might not be applicable to a future pandemic if the causative virus has a drastically different risk profile. However, we found no evidence that influenza virus antigenicity or pathogenicity influences the safety of commonly used adjuvant. Thus, our findings about the safety of the AS03 adjuvant are likely to hold true.

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The analysis dataset for this study can be directly requested from the data custodians (Manitoba Health and Manitoba Centre for Health Policy).

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Pneumococcal Meningitis in Adults after Introduction of PCV7 and PCV13, Israel, July 2009–June 2015[1]

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The indirect effect of pneumococcal conjugate vaccine on adult pneumococcal meningitis has not been thoroughly investigated. We present data from active surveillance on pneumococcal meningitis in adults in Israel occurring during July 2009–June 2015. Pneumococcal meningitis was diagnosed for 221 patients, 9.4% of all invasive pneumococcal disease (IPD) cases. Although overall IPD incidence decreased during the study period, meningitis increased nonsignificantly from 0.66 to 0.85 cases/100,000 population. Incidence of vaccine type (VT) pneumococcal meningitis (VT13) decreased by 70%, but non-VT13 pneumococcal meningitis increased from 0.32 to 0.75 cases/100,000 population (incident rate ratio 2.35, 95% CI 1.27–4.35). Pneumococcal meningitis patients were younger and healthier than nonmeningitis IPD patients, and 20.2% had a history of previous head surgery or cerebrospinal fluid leak compared with <2.0% of nonmeningitis patients (p<0.0001). Non-VT13 types that rarely cause IPD (15B/C, 6C, 23A, 23B, 24F) seem to be emerging as common causes of meningitis.

Streptococcus pneumoniae is the leading cause of bacterial meningitis for persons of all ages (*1*). Pneumococcal meningitis is a relatively rare but the most severe form of invasive pneumococcal disease (IPD). Untreated pneumococcal meningitis usually leads to death, and even with optimal treatment, mortality rates are high and disease is severe with frequent long-term sequelae (*1*,*2*).

Since the introduction of pneumococcal conjugate vaccines (PCVs) into the national immunization plans (NIPs) for children in different countries, IPD incidence has declined, not only among children but also among unvaccinated adult populations through herd (indirect) protection (*3*–*6*). Despite non-VT strains nearly completely replacing VT strains as the causative agents of invasive nasopharynx disease, this replacement by non-VT strains was only partial in both the pediatric and adult populations, presumably because of the lower invasive potential of most non-VT strains (*7*). However, a higher magnitude replacement of the serotypes associated with invasive disease was observed among immunocompromised and elderly populations than among the rest of the general population (*4*,*8*). Although several studies have assessed the effect of PCV on meningitis in children (*9*–*11*), data on the indirect effects on adult pneumococcal meningitis are scarce. The available data mostly address the effect of the 7-valent PCV (PCV7) (*12*,*13*) or PCV10 on pneumococcal meningitis in adults (*14*), and only 2 studies addressed the effect of PCV13 (*15*,*16*).

In Israel, pneumococcal polysaccharide vaccine 23 (PPSV23) has been part of the NIP for adults for many years; the constant coverage rate is >70% for adults ≥65 years of age. Administration of PCV7 at 2, 4, and 12 months of age and a catch-up plan was introduced to the NIP for children in July 2009, resulting in >70% vaccine

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[1]Some results from this study were presented at the 10th International Symposium on Pneumococci and Pneumococcal Diseases, June 26–30, 2016, Glasgow, Scotland.

[2]Additional members of the Israeli Adult Invasive Pneumococcal Disease Group are listed at the end of this article.

coverage (≥2 doses) for children <2 years of age within 1 year of implementation (*17*). Starting in October 2010, administration of PCV13 began to replace that of PCV7, and immunization coverage rapidly reached ≈95% for 2 doses and ≈90% for 3 doses (*18*). We previously described the indirect effect of PCV on the adult population in Israel and reported an ≈20% decrease of overall IPD incidence 4 years after the introduction of PCV7 and 2.5 years after the introduction of PCV13; we also reported that the proportion of pneumococcal meningitis cases among all IPD cases increased and that incidence did not decrease as did other IPDs (*4*). In this article, we assess meningitis IPD and nonmeningitis IPD incidence and the change in associated serotypes in adults 6 years after PCV7 introduction.

Materials and Methods

Ethics Statement

This study was conducted after protocols were approved by the Sheba Medical Center Institutional Review Board (Ramat Gan, Israel) and the Soroka University Medical Center Institutional Review Board (Beersheba, Israel). Because this study was a retrospective observational study, the institutional review boards waived the need for written informed consent, so informed consent was not obtained from participants. Therefore, all patient records and information were deidentified before analysis.

Study Period and Population

The period evaluated was July 1, 2009–June 30, 2015, a 6-year period starting when PCV7 was introduced into the NIP for children. We included all culture-confirmed IPD patients of the adult (≥18 years of age) population in Israel (n = 5,029,600 in 2009; n = 5,504,900 in 2015; http://www.cbs.gov.il).

Surveillance System and Study Design

To ensure both a high rate of reporting and data collection from the medical records, a large research network named the Israeli Adult Invasive Pneumococcal Disease (IAIPD) group was established. This group includes 2 researchers from each of the 27 acute care hospitals of Israel: a researcher from the microbiology laboratory and an infectious disease physician devoted to following the IPD patients and collecting the required data, as described previously (*4*).

In Israel, all invasive *S. pneumoniae* isolates are required by law to be reported and sent to the Ministry of Health reference laboratory (Jerusalem, Israel), and several different methods were used by clinical staff members to collect these bacterial isolates before submission. In addition to this passive surveillance, active surveillance was performed involving a capture–recapture method, in which the IAIPD representative at each of the 27 laboratories performing blood and cerebrospinal fluid (CSF) cultures reported all invasive *S. pneumoniae* isolates on a weekly basis and transported them to study headquarters (Pediatric Infectious Disease Laboratory, Soroka University Medical Center), as described previously (*18*).

IAIPD investigators retrospectively collected data for every laboratory-identified case from the medical files. Data were available from 24 of the 27 centers, constituting 90.9% of all IPD cases that were identified. Data collected included sociodemographic data (sex, age, place of birth, city of residence); medical history, including concurrent medical conditions and IPD-predisposing medical conditions; substance abuse; smoking history; influenza in the days preceding hospitalization; and vaccination history for influenza and pneumococcal pneumonia. The Centers for Disease Control and Prevention definition for IPD-predisposing medical conditions, on which vaccination recommendations are based (https://www.cdc.gov/vaccines/vpd/pneumo/downloads/pneumo-vaccine-timing.pdf), was used to divide adults into 3 categories: 1) high-risk patients, defined as patients having chronic renal failure, HIV, medically induced or innate immunodeficiencies, asplenia, hematologic or metastatic malignancies, CSF leak, or prior neurosurgery; 2) at-risk patients, defined as patients with diabetes mellitus, congestive heart failure, chronic lung disease, cirrhosis, or an addiction to alcohol; and 3) not at-risk patients, defined as those who were not recognized as having a predisposing medical condition. According to the NIP for adults in Israel, it is recommended that high-risk patients be vaccinated with PCV13 and PPSV23 and at-risk patients with PPSV23. We also collected data on in-hospital complications: septic shock, need for ventilation, disability as determined by transition to a long-term care facility, and concurrent medical conditions.

Case Definition

We defined IPD cases on the basis of positive pneumococcal cultures and did not include PCR testing or antigen detection, as described previously (*4*). We defined pneumococcal meningitis patients as those with an *S. pneumoniae*–positive CSF culture or those with an *S. pneumoniae*–positive blood culture who were also given a clinical diagnosis of meningitis by the treating physician (i.e., given a discharge diagnosis code of meningitis).

Vaccination Policy and Vaccine Uptake

PCV7 was licensed in Israel in 2007 and was introduced into the NIP for children in July 2009 with a 2-, 4-, and 12-month schedule and a 2-dose catch-up plan for all children <2 years of age at the time of introduction. The methods used to evaluate vaccine uptake initiated in July 2009 are described elsewhere (*18*).

Laboratory Testing

Susceptibility testing of isolates was performed at the local laboratory of each medical center. All centers assessed susceptibility to penicillin and ceftriaxone, including MIC determination by using ETEST (bioMérieux, Marcy l'Etoile, France), following the Clinical and Laboratory Standards Institute guidelines (http://www.facm.ucl.ac.be/intranet/CLSI/CLSI-2017-M100-S27.pdf). Serotyping was performed with all viable isolates at the headquarters laboratory by using the Quellung reaction (Staten Serum Institute, Copenhagen, Denmark).

Statistical Analyses

We determined the denominators for calculating the incidence and mortality rates by using data from the Israeli Central Bureau of Statistics (http://www.cbs.gov.il). We calculated incidence rate ratios (IRRs) by dividing the incidence rate (IR) of year 6 by the IR of year 1. We calculated 95% Poisson CIs for IRs and IRRs by following the method of Greenland and Rothman (*19*). We performed a Poisson regression to test the trend in IRs over the 6-year study. We used multivariate logistic models to determine the predictors of death among IPD patients and the predictors of meningitis development among meningitis patients who were not at risk for IPD. In the pneumococcal meningitis mortality rate model, we included the following variables: age, sex, predisposing medical conditions, nonhematologic metastatic malignancies, smoking history, and source of infection. In the models tested to define predictors of meningitis development among not at-risk patients, we included the following variables: age, concurrent medical conditions (other than those known to be predisposing for IPD), and serotype. We grouped serotypes by vaccine coverage in 1 model and specifically tested serogroup 23 in another model. We presented the model with the higher goodness-of-fit (model with serogroup 23 serving as the predictor). We included predictor variables with p values <0.2 in the univariate analysis in the respective multivariate model. We calculated adjusted odds ratios, 95% CIs, and adjusted p values and used SAS 9.4 software (SAS Institute, Cary, NC, USA). We calculated the Simpson diversity index to assess the change in diversity of serotypes in the bacterial population (*20*).

Results

During the 6-year study period, 2,579 IPD cases occurred, but 234 were excluded because of a lack of medical file data; 221 pneumococcal meningitis diagnoses in persons ≥18 years of age were reported, constituting 9.4% of all the IPD cases with diagnosis data available (n = 2,345). Of the 221 patients with pneumococcal meningitis diagnoses, *S. pneumoniae* was isolated from both CSF and blood cultures in 99 (44.8%) episodes, blood cultures only in 89 (40.3%), and CSF only in 33 (14.9%).

The mean annual incidence of pneumococcal meningitis was 0.77 cases/100,000 population, rising from 0.66 cases/100,000 population in the first year to 0.85 cases/100,000 population in the last study year, representing a 29% nonsignificant increase (IRR 1.29, 95% CI 0.81–2.06) (Figure 1; online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/7/17-0721-Techapp1.pdf). Over the same period, the incidence of nonmeningitis IPD decreased significantly by 26.7%, from 8.49 cases/100,000 population to 6.22 cases/100,000 population (IRR 0.73, 95% CI 0.63–0.85).

Characteristics of Pneumococcal Meningitis Patients

Similar to other IPD patients, 75% of meningitis IPD patients were of Jewish ethnicity, and ≈50% were men (similar to the distribution in the general population) **(**Table 1**)**. Patients with pneumococcal meningitis were significantly younger than IPD patients without meningitis (p<0.0001). Moreover, concurrent medical conditions were less frequent among the meningitis IPD patients than the nonmeningitis IPD patients; 87.3% of meningitis patients and 92.8% of nonmeningitis patients (p = 0.004) had any concurrent medical condition, and 58.7% of meningitis patients and 68.6% of nonmeningitis patients (p = 0.003) were at risk or at high risk for IPD (had concurrent medical conditions for which PPSV23 is recommended). The most common high-risk concurrent medical condition among the pneumococcal meningitis patients, occurring in 20.2% of the population, was previous neurosurgery or CSF leak, which only occurred in 2.0% of the nonmeningitis IPD patients (p<0.0001).

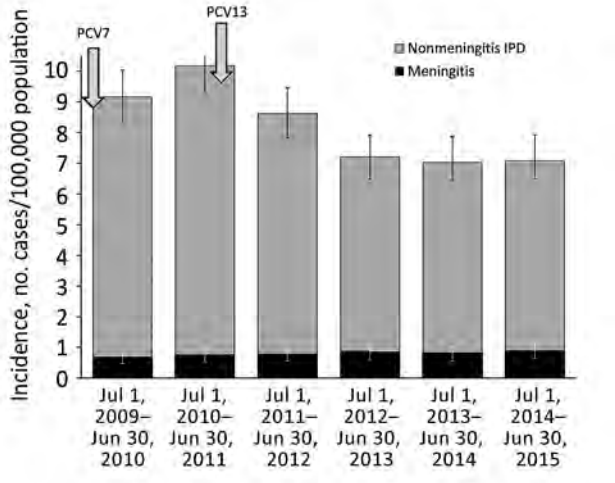

Figure 1. Incidence of meningitis and nonmeningitis IPD in patients ≥18 years of age, Israel, July 1, 2009–June 30, 2015. Introduction of PCV7 and PCV13 into the pediatric national immunization plan is depicted with arrows; 95% Poisson CIs are depicted for overall IPD and meningitis IPD. IPD, invasive pneumococcal disease; PCV, pneumococcal conjugate vaccine.

Table 1. Univariate analysis of the characteristics of patients with meningitis IPD and nonmeningitis IPD by IPD source, Israel, July 1, 2009–June 30, 2015*

Variable	Meningitis IPD, n = 221	Nonmeningitis IPD, n = 2,124†				p value§
		Pneumonia	Bacteremia, no source	IPD, rare types‡	All	
Population with full data available	213	1,596	301	203	2,124	
Sex¶						
M	104 (48.6)	890 (55.8)	171 (56.8)	108 (53.2)	1,182 (55.7)	**0.047**
F	110 (51.4)	704 (44.2)	130 (43.2)	95 (46.8)	940 (44.3)	
Jewish ethnicity¶	166 (75.5)	1,157 (72.6)	221 (73.7)	154 (75.9)	1,551 (73.0)	0.46
Age, y¶						**<0.0001**
Mean	57.68	64.83	61.83	64.03	64.37	
Median (range)	61.47 (18.2–91.3)	66.53 (18.1–105.0)	65.45 (18.1–98.3)	66.91 (18.1–97.8)	66.35 (18.1–105.0)	
18–49	72 (32.6)	368 (23.1)	74 (24.6)	45 (22.2)	491 (23.1)	
50–64	68 (30.8)	382 (23.9)	75 (24.9)	47 (23.2)	510 (24.0)	
≥65	81 (36.7)	846 (53.0)	152 (50.5)	111 (54.7)	1,123 (52.9)	
Concurrent medical conditions						
Any	186 (87.3)	1,481 (92.8)	287 (95.4)	189 (93.1)	1,972 (92.8)	**0.004**
High risk and at risk	130 (61.0)	1,093 (68.5)	233 (77.4)	156 (76.49)	1,495 (70.4)	**0.005**
High risk	81 (38.0)	645 (40.4)	179 (59.5)	107 (52.7)	939 (44.2)	0.083
Any immunosuppression	16 (7.5)	187 (11.7)	61 (20.3)	35 (17.2)	285 (13.4)	**0.014**
Bone marrow transplantation	4 (1.9)	42 (2.6)	17 (5.7)	12 (5.9)	71 (3.3)	0.248
HIV	0	33 (2.1)	3 (1.0)	3 (1.5)	39 (1.8)	**0.046**
Hematologic malignancy	19 (8.9)	208 (13.0)	68 (22.6)	41 (20.2)	320 (15.1)	**0.015**
Metastatic cancer	3 (1.4)	73 (4.6)	34 (11.3)	8 (3.9)	116 (5.5)	**0.010**
Asplenia	9 (4.2)	26 (1.6)	18 (6.0)	14 (6.9)	58 (2.7)	0.213
Chronic renal failure	14 (6.6)	277 (17.4)	46 (15.3)	35 (17.2)	363 (17.1)	**<0.0001**
Previous neurosurgery, CSF leak	43 (20.2)	29 (1.8)	6 (2.0)	7 (3.5)	42 (2.0)	**<0.0001**
At risk	49 (23.0)	448 (28.1)	54 (17.9)	49 (24.1)	556 (26.2)	0.314
Congestive heart failure	13 (6.1)	270 (16.9)	38 (12.6)	36 (17.7)	350 (16.5)	**<0.0001**
Chronic lung disease	20 (9.4)	358 (22.5)	35 (11.6)	29 (14.3)	425 (20.0)	**0.0002**
Cirrhosis	2 (0.9)	29 (1.8)	16 (5.3)	17 (8.4)	62 (2.9)	0.091
Diabetes mellitus	47 (22.1)	437 (27.4)	87 (28.9)	54 (26.6)	584 (27.5)	0.089
Alcohol abuse	3 (1.4)	51 (3.2)	10 (3.3)	8 (3.9)	69 (3.3)	0.138
Healthy	27 (12.7)	115 (7.2)	14 (4.7)	14 (6.9)	152 (7.2)	**0.004**

*Values are no. (%) except as indicated. CSF, cerebospinal fluid; IPD, invasive pneumococcal disease.
†The source of 24 nonmeningitis IPD cases was not reported, so these cases were only included in the "All" column.
‡These IPD cases occurred after endocarditis, sinusitis, mastoiditis, peritonitis, endometritis, osteomyelitis, cellulitis, or septic arthritis; a few resulted from abscesses in various locations.
§p value of meningitis vs. nonmeningitis IPD cases. Bold indicates significance.
¶The denominators are the total number of cases with information available pertaining to the relevant variable.

Outcomes

Only in-hospital outcomes were available. The crude overall case-fatality rate was 15.5% for meningitis IPD patients and 23.0% for nonmeningitis IPD patients (p = 0.0123) (Table 2). After adjusting for age, risk group, and VT in a multivariate logistic model, we found that the mortality rate of patients with pneumococcal meningitis did not differ from that of patients with nonmeningitis IPD (Table 3). Other outcomes were worse for pneumococcal meningitis patients; compared with nonmeningitis IPD patients, meningitis IPD patients were more frequently hospitalized in the intensive care unit, more frequently required mechanical ventilation, and had longer lengths of hospital stay (median 6 days for nonmeningitis IPD vs. 15 days for meningitis IPD; Table 2). In addition, nearly 20% of meningitis IPD patients were discharged to long-term care facilities compared with 7% of nonmeningitis IPD patients.

Antimicrobial Drug Resistance

Among the 221 isolates from meningitis IPD patients, 211 were susceptible to penicillin and 180 were susceptible to ceftriaxone. Isolates resistant to penicillin (MIC >0.06 μg/mL) were isolated from 25.1% of all meningitis patients. Ceftriaxone nonsusceptibility (MIC ≥1 μg/mL) emerged during the study years, from 2.1% (n = 1) in the first 2 years to >8% (n = 6) in the last 2 years (Figure 2). The single ceftriaxone-nonsusceptible meningitis isolate found during the first 2 years was serotype 14, but in the last 2 years, most ceftriaxone-nonsusceptible isolates were serotypes 19A and 23F. A single isolate of serotype 23F was highly resistant to ceftriaxone (MIC 2 μg/mL) and penicillin (MIC 8 μg/mL). The proportion of isolates from nonmeningitis IPD patients resistant to ceftriaxone, with a MIC ≥1 μg/mL (≈4%), did not change over the course of the study. Serotypes 14 and 19A each accounted for nearly 30% of the isolates, and 19F for 12%. All isolates were susceptible to vancomycin.

Table 2. Univariate analysis of clinical outcomes of meningitis versus nonmeningitis IPD patients, Israel, July 1, 2009–June 30, 2015*

Category	Pneumococcal meningitis, n = 221	Nonmeningitis IPD, n = 2,124	p value
Population with full data available	213	2,123	
Overall no. deaths (case-fatality rate)	33 (15.5)	488 (23.0)	0.0123
ICU admission	115 (54.0)	322 (15.2)	<0.0001
Ventilation required	91 (42.7)	405 (19.1)	<0.0001
Mean LOS, d†	21.0	10.5	<0.0001
Percentile LOS, by days, %			
5	55	33	
10	38.5	21	
25	21	11	
50	15	6	
75	12	4	
90	10	2	
Sepsis†	10/180 (5.6)	170/1,635 (10.4)	0.0391
Discharged to long-term care facility†	34/173 (19.7)	104/1,490 (7.0)	<0.0001

*Values are no. (%) except as indicated. ICU, intensive care unit; IPD, invasive pneumococcal disease; LOS, length of stay.
†Outcome assessed only for those who were discharged (deaths were excluded).

Table 3. Multivariate logistic model for predictors of death among IPD patients, Israel, July 1, 2009–June 30, 2015*

Variable	Adjusted OR (95% CI)	Adjusted p value
Age, y		
18–49		Referent
50–64	2.064 (1.39–3.06)	0.0003
≥65	4.847 (3.42–6.87)	<0.0001
Risk group		
Not at risk		Referent
At risk	1.338 (0.99–1.80)	0.0561
High risk	1.609 (1.23–2.11)	0.0006
Serotype by VT		
VT7		Referent
VT13–7	0.903 (0.65–1.25)	0.539
Non-VT13	1.119 (0.84–1.50)	0.449
Nonmeningitis IPD		Referent
Pneumococcal meningitis	0.747 (0.50–1.11)	0.153

*IPD, invasive pneumococcal disease; OR, odds ratio; VT, vaccine type.

Serotype Dynamics

Although overall pneumococcal meningitis incidence did not decrease, meningitis caused by VT13 serotypes decreased by 70% (IRR 0.297, 95% CI 0.11–0.82), similar to the decrease in VT13 nonmeningitis IPD cases. VT7 serotypes were totally eliminated by the sixth study year (Figure 3). The PCV13 serotypes 3, 7F, and 19A caused meningitis in the sixth study year and constituted 11.9% of all isolates that year. The diversity of the serotypes causing pneumococcal meningitis did not change substantially over the 6-year study; the Simpson diversity index was 0.951–0.974. To assess the dynamics of the serotypes commonly causing pneumococcal meningitis, we compared the serotype distribution of the first 2 study years (July 1, 2009–June 30, 2011; pre-PCV13 period) with that of the last 2 study years (July 1, 2013–June 30, 2015) (Figure 4). The dynamics of PCV13 serotype 3 contrasted with those of the other PCV13 serotypes; while the proportion and incidence of the common VT13 serotypes 6A, 23F, 19A, and 7F decreased, serotype 3 slightly increased. Despite the significant decrease in the incidence of VT13 strains and the elimination of VT7 strains, the overall incidence of pneumococcal meningitis increased because of a significant emergence of non-VT13 serotypes (IRR 2.352, 95% CI 1.27–4.35).

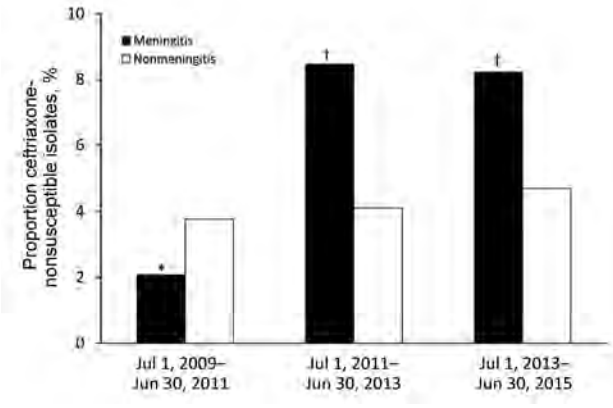

Figure 2. Proportion ceftriaxone-nonsusceptible isolates among all *Streptococcus pneumoniae* isolates acquired from patients with invasive pneumococcal disease, by 2-year period, Israel, July 1, 2009–June 30, 2015. Ceftriaxone-nonsusceptible isolates were those that could grow at a concentration above ceftriaxone's MIC (≥1 μg/mL). *In 2009–2011, the 1 ceftriaxone-nonsusceptible isolate was serotype 14. †In 2011–2013, the 5 ceftriaxone-nonsusceptible isolates included 2 of serotype 19F and 1 each of serotypes 19A, 34, and 23B. ‡In 2013–2015, the ceftriaxone-nonsusceptible isolates were serotypes 23F (n = 2) and 19A (n = 3), and 1 was not typeable.

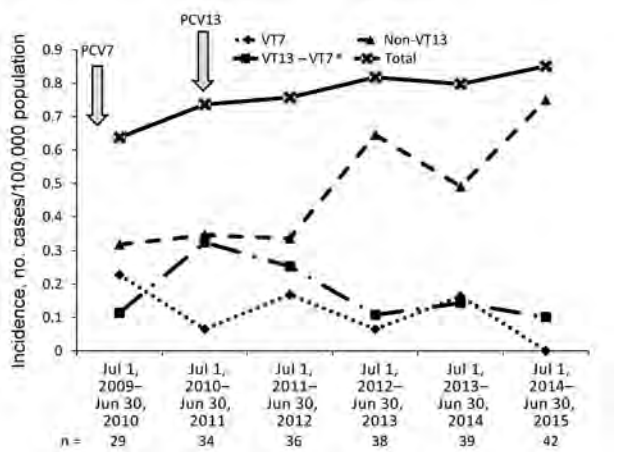

Figure 3. Incidence of pneumococcal meningitis in patients ≥18 years of age, by VT, Israel, July 1, 2009–June 30, 2015. The total number of cases per year are shown, and the introductions of PCV7 and PCV13 into the pediatric national immunization plan are depicted with arrows. *Serotypes included in the VT13 vaccine but not in the VT7 vaccine. PCV, pneumococcal conjugate vaccine; VT, vaccine type.

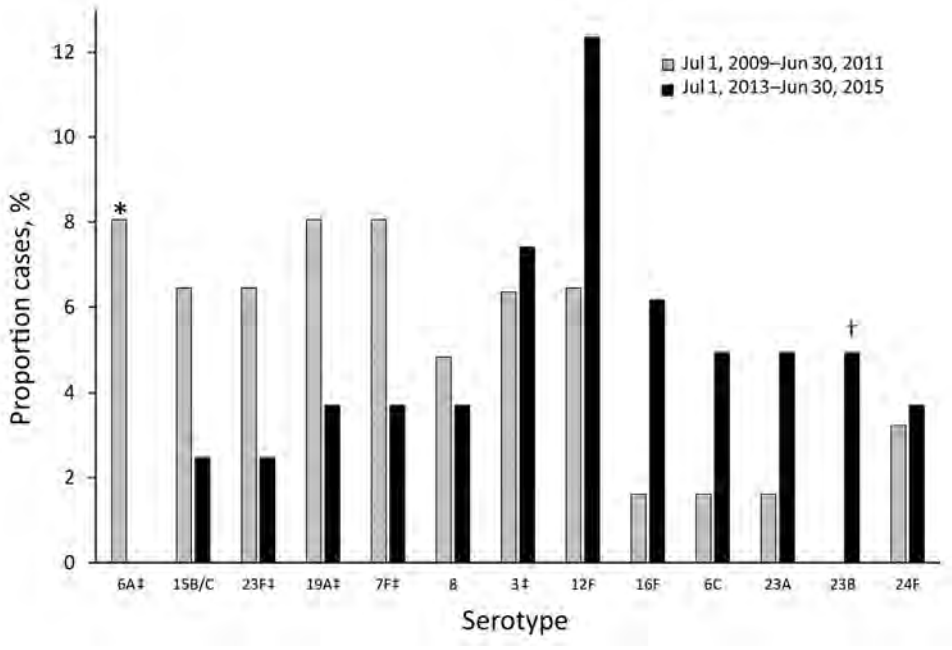

Figure 4. Comparison of serotypes causing pneumococcal meningitis during the first and last 2-year periods of study, Israel, July 1, 2009–June 30, 2011, and July 1, 2013–June 30, 2015. Only common serotypes (those occurring in >5% of cases in either the first 2-year period [n = 62] or last 2-year period [n = 81]) were included. *p<0.05; †p<0.1; ‡serotypes covered by pneumococcal conjugate vaccine 13.

The common emerging non-VT serotypes were 12F, 16F, 6C, 23A, 23B, and 24F.

Certain serotypes made up a significantly higher proportion of meningitis IPD than nonmeningitis IPD cases, particularly 23A (p = 0.0018) and 23B (p = 0.0001) but also 24F (p = 0.054), 23F (p = 0.072), 15B/C (p = 0.035), and 6C (p = 0.084) (all but 23F being non-VT13 serotypes) (Figure 5; online Technical Appendix Table 2). Other serotypes were commonly isolated from patients with nonmeningitis IPD and rarely detected in patients with meningitis IPD, particularly serotypes 14 (p = 0.093), 1 (p = 0.0002), 5 (p = 0.0013), and 9V (p = 0.045).

Predictors of Pneumococcal Meningitis

To elucidate unrecognized risks or predictors for pneumococcal meningitis beyond neurosurgery or CSF leak, we separately assessed all patients with IPD categorized as not at risk for IPD (i.e., without any recognized concurrent medical condition for which PPSV23 is recommended). First, we performed a univariate analysis comparing characteristics of the not at-risk patients with pneumococcal meningitis with those of the not at-risk patients with nonmeningitis IPD (online Technical Appendix Table 3). The medical conditions trauma, lipid disorders, and chronic or recurrent infections were more frequently reported for the meningitis IPD not at-risk population than the nonmeningitis IPD not at-risk population. Smoking and dementia were less frequently reported for the meningitis IPD patient population. To determine independent predictors for pneumococcal meningitis among the not at-risk patient population, we performed a multivariate logistic analysis. In general, having any concurrent medical condition was associated with nonmeningitis IPD, and the particular medical conditions lipid disorder, chronic or recurrent infections, and previous trauma were independently associated with meningitis IPD. Many of the chronic or recurrent infections reported included otitis media or sinusitis, but the numbers of these cases were too small for greater resolution (online Technical Appendix Table 3). Because we observed that certain serotypes were particularly associated with pneumococcal meningitis, we tested several models to determine whether particular serotypes predicted meningitis IPD in the not at-risk group. The best-fit model found that serogroup 23 was an independent predictor of pneumococcal meningitis in this patient population; adjusted odds ratio was 5.43 (95% CI 2.01–14.70) (Table 4).

Discussion

In our nationwide study, we assessed the dynamics of the incidence of pneumococcal meningitis in adults after sequential implementation of PCV7 and PCV13 in children. Our data demonstrate that some features of pneumococcal meningitis differ significantly from those of nonmeningitis IPD. The pneumococcal meningitis patient population was younger and had less frequent and different concurrent medical conditions. Outcomes also differed, with meningitis IPD patients having a more frequent need for ventilation, intensive care treatment, and treatment in long-term care facilities after discharge and a longer length of hospital stay. Yet mortality rates and case-fatality rates did not differ between the meningitis and nonmeningitis IPD populations. Although this finding seems surprising, a decrease in the pneumococcal meningitis case-fatality rate with adjuvant corticosteroid treatment was reported in a multi-

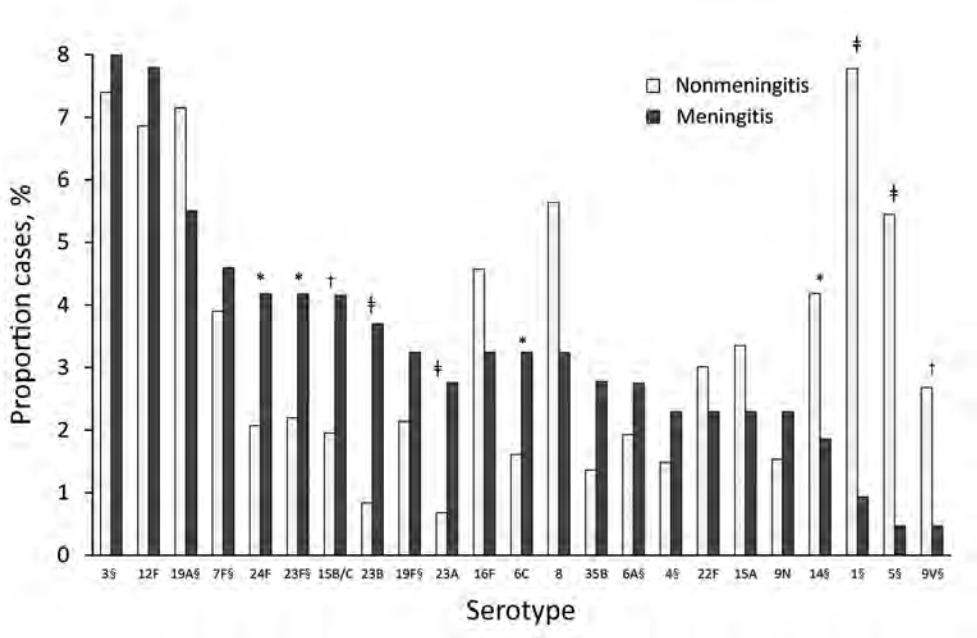

Figure 5. Serotypes associated with meningitis and nonmeningitis invasive pneumococcal disease, Israel, July 1, 2009–June 30, 2015. Only major serotypes (those totaling >3% of all *Streptococcus pneumoniae* isolates from all study years) were included. *p<0.1; †p<0.05; ‡p<0.005; §serotypes covered by pneumococcal conjugate vaccine 13.

center study in Europe (*21*). Although we do not know the proportion of patients in our study treated with corticosteroids, this treatment is part of routine practice for treating meningitis patients in Israel.

Ceftriaxone nonsusceptibility increased during the study period. This finding was unexpected because the only nonsusceptible isolate in the pre-PCV13 era was VT7 serotype 14. Antimicrobial drug recommendations did not change in a way that could have stimulated the emergence of ceftriaxone-nonsusceptible isolates. The emerging ceftriaxone-nonsusceptible isolates belonged mainly to serogroups 19 and 23 or non-VT13 serotypes (34 and an unencapsulated isolate [nontypeable]). Previous studies reported initial declines in rates of IPD with antimicrobial drug–resistant *S. pneumoniae* after the introduction of PCV7 in children and adults. These declines were attributed to the reduction of VT7 serotypes, which constituted most of the resistant strains. This observation of increased antimicrobial drug resistance is particularly worrisome; with ceftriaxone-nonsusceptible isolates totaling 8.3% of meningitis IPD cases, adding vancomycin to first-line therapy (pending culture results) might be necessary. Most of the isolates not susceptible to ceftriaxone were from serotypes covered by PCV13; thus, the ceftriaxone-nonsusceptible VT13 strains might be eventually eliminated through vaccination efforts, and these high nonsusceptibility rates might eventually decline with time. Although the European Society of Clinical Microbiology and Infectious Diseases and the Infectious Diseases Society of America guidelines (*22*,*23*) suggest adding vancomycin if a high prevalence of nonsusceptibility to penicillins and cephalosporins is observed, the guidelines do not specify at which resistance prevalence the policy should be enacted. Our results indicate the need for continued surveillance of ceftriaxone nonsusceptibility rates among pneumococcal meningitis cases.

The effect of the PCV NIP for children on pneumococcal meningitis in adults has been studied infrequently and mostly in the pre-PCV13 era. A review summarizing the effect of PCV7 on pneumococcal meningitis incidence in Europe and the United States reported a large variation in the effects, ranging from a 137% increase to a 77% decrease; the effect on VT7 pneumococcal meningitis incidence was also diverse (ranging from a 43% increase to an 87% decrease) (*24*). Studies in the United States and the Netherlands reported reductions in overall pneumococcal meningitis in adults after PCV7 or PCV10 introduction (*13*,*14*). Data on the effects of PCV13 on pneumococcal meningitis are scarce and include only data on pediatric populations; results differed according to geographic region. No significant change in pediatric pneumococcal meningitis rates was reported in 2 studies (*9*,*10*), and a decrease in pneumococcal meningitis in children <2 years of age was reported in others (*22*,*25*).

We show that in contrast to the significant indirect effect observed on overall IPD and particularly bacteremic pneumococcal pneumonia (*4*,*26*,*27*), adult pneumococcal meningitis incidence did not decrease after PCV implementation. We show that the reason for this is an increase of pneumococcal meningitis incidence caused by non-VT13 serotypes. A significant replacement in carriage of less invasive non-VT13 serotypes in children occurred after the PCV13 introduction; these strains had higher rates of

Table 4. Multivariate logistic model for predictors of meningitis among patients not at risk for IPD, Israel, July 1, 2009–June 30, 2015*

Variable	Adjusted OR (95% CI)	Adjusted p value
Age, y		
18–49		Referent
50–64	2.13 (1.16–3.91)	0.015
≥65	1.90 (1.02–3.55)	0.042
Concurrent medical conditions		
None		Referent
Any†	0.48 (0.27–0.85)	0.011
No history of previous trauma		Referent
Previous trauma	9.78 (2.24–42.75)	0.002
No lipid disorder		Referent
Lipid disorder	2.73 (1.19–6.24)	0.017
No history of infections		Referent
Recurrent or chronic infections‡	12.31 (3.86–39.22)	<0.0001
No history of dementia		Referent
Dementia	0.12 (0.02–0.91)	0.040
Serotype		
Non-23		Referent
23	5.43 (2.01–14.70)	0.0009

*Patients not at risk for IPD were defined as those without any recognized concurrent medical condition for which PPSV23 administration is recommended. IPD, invasive pneumococcal disease; OR, odds ratio; PPSV23, pneumococcal polysaccharide vaccine 23.
†Any concurrent medical condition, excluding those for which PPSV23 is recommended. Patients with these conditions were not included in the not at-risk group.
‡Various types of recurrent or chronic infections, including chronic otitis media, sinusitis, recurrent cellulitis, chronic osteomyelitis, and tuberculosis.

transmission to adults (*7*). But why these non-VT serotypes cause more meningitis than nonmeningitis IPD is unclear. Our data suggest roles of both the host and the pathogen; a higher proportion of patients with meningitis IPD had previous neurosurgery operations, CSF leak, and history of trauma, during which direct penetration of carried bacteria could potentially occur and explain the greater replacement with non-PCV13 serotypes. We also found that odds of pneumococcal meningitis development was higher among patients with a lipid disorder and lower among patients with dementia. We have no explanations for these findings. The latter observation could potentially be explained by classification bias, if less diagnostic efforts were taken to understand the status of patients with dementia.

We also show that independent of concurrent medical conditions, several serotypes, mostly non-VT13 serotypes, were significantly associated with meningitis, suggesting a specific role of the pathogen in patients with concurrent medical conditions. Serotypes 6C and 23 are either directly covered by PCV13 or indirectly covered through cross-protection from antibodies developed against serotypes 6B and 23F. The only previous study of the indirect effects of PCV13 childhood vaccination on adult pneumococcal meningitis reported similar results as ours; however, these results were based on a small sample size (<20 cases of pneumococcal meningitis) (*16*).

This study has several limitations. First, data from the pre-PCV7 era were not collected. Second, antimicrobial drug susceptibility testing was performed in different laboratories and variations in testing might have occurred. Third, meningitis cases that were diagnosed on the basis of PCR without culturing were not included, and thus, meningitis rates might be underestimated. Last, this study has the limitations of a retrospective study; thus, only data available from the electronic file were collected.

In conclusion, this nationwide study indicates that PCV7 and PCV13 childhood vaccination indirectly affected the incidence of adult pneumococcal meningitis. We report the elimination of the occurrence of VT7 serotypes among pneumococcal meningitis patients within 6 years after PCV7 implementation and 4.5 years after PCV13 implementation. Yet we report no decrease in overall IPD meningitis cases because of the significant emergence of several non-VT13 strains, particularly serotypes 6C, 12F, 16F, 23A, and 23B.

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Reemergence of *Reston ebolavirus* in Cynomolgus Monkeys, the Philippines, 2015

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In August 2015, a nonhuman primate facility south of Manila, the Philippines, noted unusual deaths of 6 cynomolgus monkeys (*Macaca fascicularis*), characterized by generalized rashes, inappetence, or sudden death. We identified *Reston ebolavirus* (RESTV) infection in monkeys by using serologic and molecular assays. We isolated viruses in tissues from infected monkeys and determined viral genome sequences. RESTV found in the 2015 outbreak is genetically closer to 1 of the 4 RESTVs that caused the 2008 outbreak among swine. Eight macaques, including 2 also infected with RESTV, tested positive for measles. Concurrently, the measles virus was circulating throughout the Philippines, indicating that the infection of the macaques may be a reverse zoonosis. Improved biosecurity measures will minimize the public health risk, as well as limit the introduction of disease and vectors.

Reston ebolavirus (RESTV) was discovered after an outbreak of hemorrhagic disease in cynomolgus macaques in a primate research facility in Reston, Virginia, USA in 1989 that had imported macaques from the Philippines (*1*). Subclinical infections in humans in the facility were determined through diagnostic testing. Other outbreaks of RESTV epizootics were identified in Sienna, Italy in 1992 (*2*); Alice, Texas, USA in 1993 (*3*); and 2 outbreaks in the Philippines in 1996; all 4 outbreaks involved purpose-bred cynomolgus macaques (*Macaca fascicularis*) attributed to a single nonhuman primate (NHP) facility in the Philippines (*4*). Until the 2015 outbreak described here, no outbreaks of RESTV had occurred in the Philippines since 1997; subsequently, the government permanently closed the facility.

The last known occurrence of RESTV epizootic in the Philippines was during 2008–2009 and affected 2 piggeries on the island of Luzon, 1 of the 3 major islands in the country. The disease was discovered as a co-infection with porcine reproductive and respiratory syndrome virus (PRRSV), also prevalent at that time (*5*). After this outbreak, Jayme et al. undertook a search for a possible reservoir in bats by using low levels of viral RNA detected in the microbat *Miniopterus schreibersii* (*6*).

As part of the established process for testing of macaques in the quarantine facility, animals that are sick or die are routinely tested for the presence of RESTV infection. In August 2015, six monkeys that were in the last stage of quarantine died suddenly; their bodies were submitted for testing. Although there are many fruit-bearing trees in the facility, the building was constructed in such a way that fruit bats could not make contact with the monkeys. However, rats were observed entering the cages of individual primates in the facility.

At the same time, an outbreak of measles virus (MV) was occurring among humans nationwide. During the first 6 months of 2015, there were 2,231 reported cases, of which 534 were laboratory-confirmed (*7*).

In this study, we describe the serologic and molecular detection of RESTV and MV from macaques in the quarantine facility in the Philippines in 2015, and demonstrate genetic characterization of the isolated RESTV.

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Materials and Methods

Facility

The size of the monkey quarantine facility, located in the province of Southern Luzon, is ≈3,000 km^2. The 174 monkeys sampled were housed in 2 separate buildings that are equipped with individual stainless steel squeeze cages measuring ≈58 × 48 × 77 cm^3, arranged in 4 rows: the cages in the first and second rows, and those in the third and fourth

rows face each other. Each building has its own anteroom and is surrounded by large windows that have screens and welded wire to protect the monkeys from vermin and prevent monkey escapes. Each cage is equipped with a lock and squeeze-back mechanism. There were separate personnel assigned to each building and each were required to wear individual personal protective equipment (undergarments, coveralls, mask, caps, goggles, socks, and boots) and to shower when entering and exiting the animal buildings. Materials, such as those used during animal care procedures, as well as cleaning implements, were not shared between buildings. We used new sterile disposable syringes with needles for each monkey and for each procedure. We disposed of used syringes and needles in dedicated containers in each building and disposed of them through a government-accredited waste contractor.

Samples

Both antigen and antibody detection methods were used in the laboratory investigation of the epizootic occurrence. We collected a total of 174 samples from the facility for RESTV IgG and MV IgM screening. Blood samples were centrifuged on site and serum samples were transferred to labeled cryovials and transported through a cold chain. The serum samples were heat-inactivated at 56°C for 1 h. Spleen, liver, and lymph nodes from 4 deceased monkeys were also collected and transported in the same manner as the sera and tested for RESTV by using molecular assays.

Serum samples from macaques in the 2 breeding facilities located in Oriental Mindoro and Rizal that supplied the macaques to the quarantine facility were also tested for RESTV antibodies, as were 71 personnel in the facilities.

RESTV Serologic Analysis

Indirect ELISA testing was used following the protocols of either the Centers for Disease Control and Prevention (CDC) (*8*) or the protocol of the National Institute of Infectious Diseases (Musashimurayama, Tokyo, Japan) reagents (*9*). Briefly, for the CDC protocol, the upper half of the ELISA plate (Falcon, Avenel, NJ, USA) was coated with gamma-irradiated antigens obtained from a RESTV-infected cell suspension, and the lower half with those obtained from a non-infected cell suspension. We added test samples to the wells, diluted 4-fold starting at 1:100. We used mouse anti-human IgG with horseradish peroxidase (Accurate Chemical and Scientific Company, Westbury, NY, USA), diluted at 1:4,000, as a secondary antibody. We added 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid substrate (Kirkegaard-Perry Laboratories, Gaithersburg, MD, USA) at the last step for visualization of the antigen-antibody reaction. The optical density value was recorded at 415 nm by using an ELISA plate reader (ThermoFisher Scientific, Carlsbad, CA, USA). Samples were considered reactive if the adjusted optical density (OD) was ≥0.95. For the National Institute of Infectious Diseases protocol, the upper half of the ELISA plate was coated with RESTV recombinant nucleoprotein (NP) tagged with glutathione S transferase, expressed in *Escherichia coli* at ≈100 ng/well, and the lower half with negative control glutathione S transferase antigen. Goat anti-human IgG conjugated with Novex horseradish peroxidase (ThermoFisher) diluted at 2 μg/mL was used as a secondary antibody. Samples were considered reactive if the sample showed an OD ≥0.56 at 1:100 dilution, or 0.23 at 1:400 dilution.

We retested all serologically reactive samples by using immunofluorescent assay (IFA) as described by Ikegami et al. (*10*). In brief, serum samples were 2-fold serially diluted in phosphate-buffered saline (PBS) from 1:10 to 1:640. Diluted serum (20 μL) was loaded onto each well of the IFA slide (14 wells; AR Brown Co., Ltd., Toyo, Japan) containing HeLa cells expressing RESTV recombinant NP. The slides were incubated for 1 h at 37°C and washed 3 times with PBS. Invitrogen Fluorescein isothiocyanate-labeled antibody against human IgG (ThermoFisher) diluted in PBS at 1:200 was added to each well and incubated for 1 h at 37°C. After washing with PBS, the slides were examined for the staining pattern under an immunofluorescent microscope (Nikon, Chiyoda, Japan) and their reactions were recorded.

In addition, we tested all reactive serum samples for antibodies against RESTV glycoprotein in a Luminex assay (Luminex Corporation, Austin, TX, USA). Briefly, Luminex beads coated with RESTV glycoprotein (Bead #35) were blocked in 2% skim milk Tween and phosphate-buffered saline (TPBS) for 30 min at room temperature in the dark with shaking in a flat-bottom microtiter plate. We washed the plate twice with TPBS by using a magnetic plate washer (BioPlex Pro II Wash Station; Bio-Rad, Hercules, CA, USA). Serum diluted (1:100, 100 μL) in TPBS was added and incubated for 30 minutes, as stated before. The plate was washed and 100 μL of a mixture of biotinylated Protein A (1:500)/biotinylated Protein G (1:250) (ThermoFisher Scientific, Brisbane, Queensland, Australia) was added to each well and incubated as described above. The plate was washed again, then 100 μL of streptavidin–phycoerythrin was added (1:1,000; Thermo Scientific, Brisbane, Queensland, Australia), and the plate was incubated as before. Samples were assayed on the BioPlex machine (Bio-Rad) and the median fluorescence intensity read for 100 beads.

Measles Serologic Analysis

The 174 serum samples from macaques in the primate quarantine facility were tested for MV antibody by Enzygnost Measles Anti-IgM ELISA (Siemens Healthcare

Diagnostics, Marburg, Hesse, Germany). We diluted the serum samples in rheumatoid factor adsorbent to remove inhibitors that might interfere with the reaction. We used the ELISA by following the manufacturer's instructions. A sample was determined to be negative if the corrected OD at a wavelength of 450 nm with a reference at a wavelength of 635 nm was ≤0.100, equivocal if it was 0.101–0.199, and positive for MV IgM if it was ≥0.200.

Molecular Detection of RESTV

We isolated total RNA from lymph node, liver, and spleen samples of infected animals by using a combined method of TRIzol inactivation and QIAGEN RNeasy Mini Kit (QIAGEN, Copenhagen, Denmark) RNA isolation as prescribed by CDC (*11*). We amplified the RESTV viral genome by targeting the NP gene developed by Sanchez et al. (*12*). The reaction was completed in a 20-μL reaction mixture containing 4 μL of RNA, 0.2 mmol/L of each dNTP, 1.6 mmol/LM $MgSO_4$, 0.8 μL of SuperScript III RT/Platinum Taq enzyme mix (Invitrogen), and 0.5 μmol/L each of the primers RES-NP1 and RES-NP2. Thermocycling conditions were set to RT at 50°C for 30 min, inactivation and initial denaturation at 95°C for 3 min, then 45 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, then ending with a final extension step of 72°C for 5 min. Visualization of DNA bands was performed following electrophoresis in 2% agarose gels. Amplification of the latent gene from tissues was also undertaken by using the method adapted from Zhai et al. (*13*) using the Superscript Platinum Taq One-Step RT-PCR kit to generate 611-bp PCR products.

We then purified the PCR products and sequenced them with the BigDye Terminator v3.1 (ThermoFisher Scientific, Waltham, MA, USA) and analyzed in a 3500 Series Genetic Analyzer (Applied Biosystems, Foster City, CA). Assembled sequencing results were subjected to BLAST alignment (https://www.ncbi.nlm.nih.gov/BLAST), which confirmed the identity of the sequences as RESTV.

Molecular Detection of Measles Virus

A TaqMan RT-PCR assay, distributed by CDC (*14*), was performed to detect MV RNA from macaques in the quarantine facility. Amplification of the MV RNA from sample DrpZ2-10B-G was undertaken using the HEN_RES_MOR primers described by Tong et al. (*15*) and produced a product of the expected size, which was sequenced.

RESTV Isolation from Tissues

We attempted virus isolation in the liver; spleen; axillary lymph nodes (from DrpZ1-103A-K, DrpZ5-2B-F, and DrpL7-7D-A), and axillary, cervical, inguinal, and mesenteric lymph nodes (from DrpZ2-10B-G). Tissues were homogenized in PBS for 30 s with silicon carbide beads, centrifuged for 5 min, and the supernatant was added to a flask of semiconfluent Vero C1008 cells in PC4 containment at the CSIRO Australia Animal Health Laboratory, Victoria, Melbourne, Australia. For detection of virus replication, we performed 3 blind passages, monitored for CPE, and used a real-time PCR specific for NP of RESTV (J.S. Towner and P. Rollin, US Centers for Disease Control and Prevention, pers. comm., 2009 May 1).

Next-Generation Sequencing of Isolates

We performed next-generation sequencing on the supernatant from passage 2 of the isolates from the axillary lymph nodes following TRIzol purification of RNA by using Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA, USA). RNA was transcribed to cDNA by using random primers and SuperScript III reverse transcription (ThermoFisher SuperScript III First-strand Synthesis System), and then cDNA was prepared with Klenow (Promega, Madison, WI, USA). We used the Illumina Nextera XT library preparation kit to prepare dual barcoded libraries for 150-bp paired-end sequencing on the MiSeq system (Illumina, Scoresby, Victoria, Australia).

We assembled the genome sequence of DrpZ5-2B-F (GenBank accession no. MF540570) and DrpZ2-10B-G (GenBank accession no. MF540571) by using RESTV genome GenBank accession no. FJ621585.1 as a reference. We generated phylogenetic trees (neighbor-joining method) from the full-length sequence (Figure 1) by using MEGA 6 software (https:/www./megasoftware.net).

Results

Serologic Analysis

The RESTV ELISA showed that 10 of 174 samples (5.74%) from macaques were reactive for RESTV IgG antibodies (Table 1), all of which had an IFA titer >640 (Figure 2). These samples were also reactive in a Luminex assay, confirming the presence of IgG against RESTV. Of the 174 serum samples, 8 (4.59%) were reactive for MV IgM in the ELISA; 1 of the macaques (Identification no. DrpZ1-26D-B) was serologically positive for RESTV and MV antibodies (Table 1). Among those serologically positive for only MV IgM, 1 macaque (identification no.: DrpZ2-10B-G) showed a positive result for a RESTV PCR in an autopsy sample (Table 2). The data indicated that among 174 macaques, 2 (1.1%) had a history of infection with RESTV and MV.

Of the macaques from the 2 breeding facilities located in Oriental Mindoro and Rizal that supplied the animals to the quarantine facility, 10% were tested, as were personnel from both facilities. None of these personnel or macaques had detectable antibodies to RESTV.

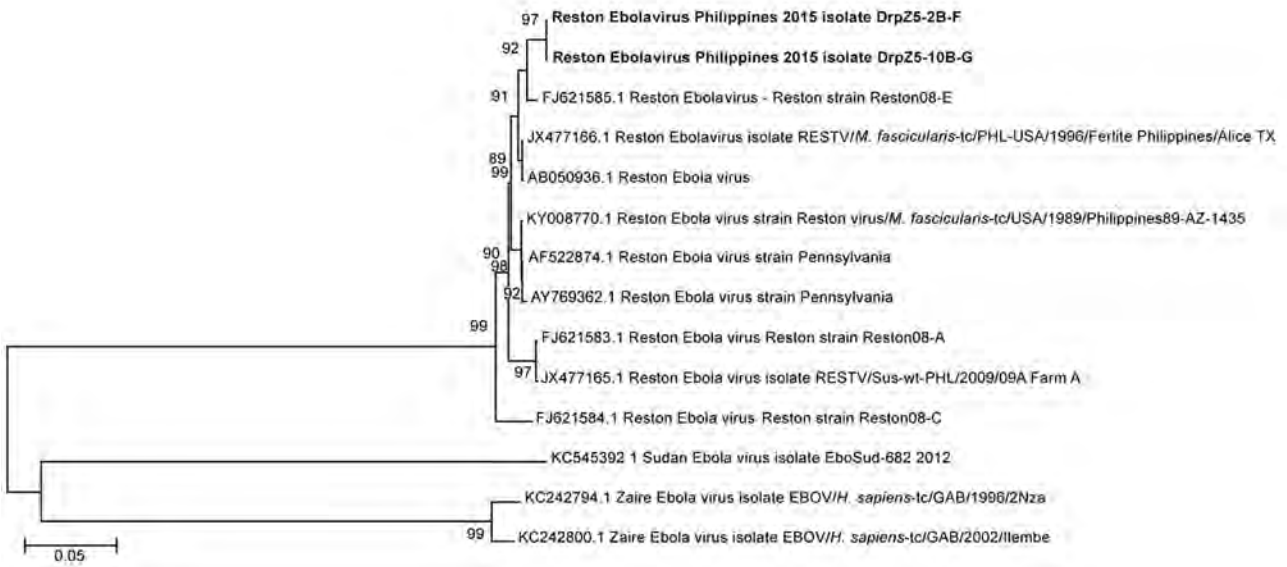

Figure 1. Phylogenetic tree (neighbor-joining) of the full genomes of ebola viruses and comparison to the Reston 2015 viruses DrpZ52BF (GenBank accession no. MF540570) and DrpZ210BG (GenBank accession no. MF540571) produced by using MEGA 6 software (https://www.megasoftware.net). Bold text indicates the genomes being sequenced. Numbers along branches indicate bootstrap values. Scale bar indicates nucleotide substitutions per site.

Virus Isolation and Molecular Analyses

RESTV was successfully isolated in Vero C1008 cells from the inguinal and axillary lymph nodes of DrpZ5-2B-F and the axillary lymph node of DrpZ2-10B-G (Table 2). Initial amplification of a 337-bp product of the partial NP gene from the liver, spleen, and lymph node tissue samples in 4 NHPs confirmed the presence of RESTV. Further amplification and sequencing of the partial L gene along with real-time detection further confirmed the RESTV infection. In all cases, Blast N revealed that the RESTV in this outbreak

Table 1. *Reston ebolavirus* antibody-positive results in 174 cynomolgus macaque samples, the Philippines, August 2015*

Monkey ID	Date collected	Serologic analysis	
		RESTV IgG	MV IgM
Drp6bL-27K-G	18	+	–
DrpL5–29D-B	18	+	–
DrpL7–7D-A†	27	+	–
DrpZ3–34C-E	27	+	–
DrpZ1–26D-B	27	+	+
DrpZ18–32B-E	27	+	–
DrpL3–3D-C	27	+	–
2DrpZ4–45C-F	27	+	–
13116B	27	+	–
DrpZ18–24B-B	27	+	–
DrpZ2–10B-G‡	27	–	+
DrpZ7–22A-F	27	–	+
DrpZ5–30D-A	27	–	+
DrpZ7–33A-I	27	–	+
DrpZ9–29A-I	27	–	+
2DrpZ5–36C-C	27	–	+
DrpZ8–12B-E	27	–	+

*ID, identification number; MV, measles virus; RESTV, *Reston ebolavirus*.
†An autopsy sample of DrpL7–7D-A was positive for *Reston ebolavirus* PCR.
‡An autopsy sample of DrpZ2-10B-G was positive for both RESTV and MV PCR.

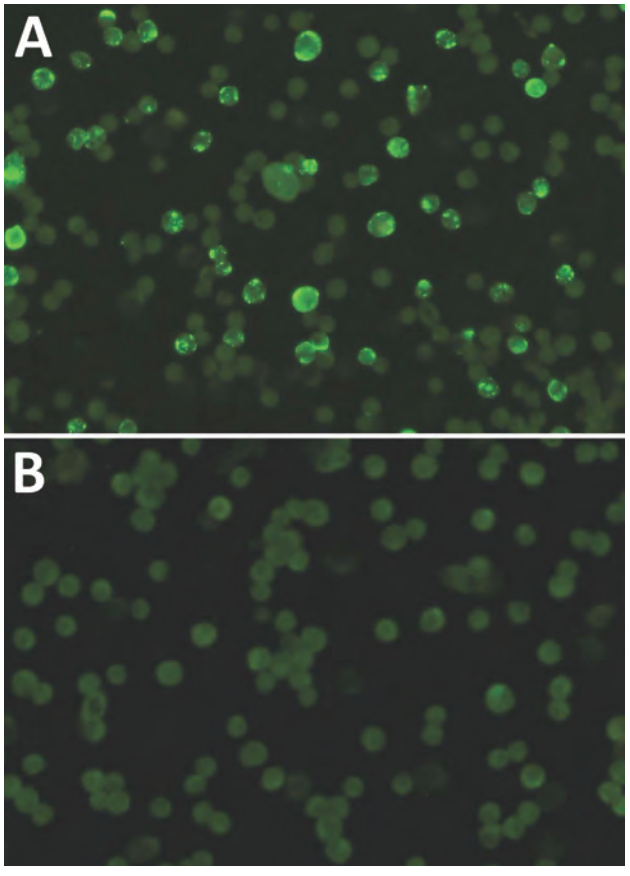

Figure 2. Immunofluorescence assay results of infected monkey serum A) characterized by granular staining pattern of HeLa cells and B) noninfected monkey serum. Original magnification ×400.

Table 2. Samples from cynomolgus macaques submitted for isolation of *Reston ebolavirus*, the Philippines, 2015*

Monkey ID	Sample type	Date collected	Isolation	RESTV PCR NP/L	RESTV PCR GenBank accession nos., NP/L	MV PCR L	MV PCR GenBank accession no.
DrpZ1-103A-K	Liver	Aug 27	–	+/+	MG431944/MG431953	ND	ND
	Spleen	Aug 27	–	+/+	MG431945/MG431954	ND	ND
	Axillary lymph node	Sep 5	–	+/+	MG431952/MG431961	ND	ND
DrpZ5-2B-F	Cervical lymph node	Sep 5	–	+/+	MG431946/MG431955	ND	ND
	Axillary lymph node	Sep 5	+	+/+	MG431947/MG431956	ND	ND
	Inguinal lymph node	Sep 5	+	+/+	MG431948/MG431957	ND	ND
	Mesenteric lymph node	Sep 5	–	+/+	MG431949/MG431958	ND	ND
DrpZ2-10B-G†	Axillary lymph node	Sep 5	+	+/+	MG431950/MG431959	+	MF496232
DrpL7-7D-A‡	Axillary lymph node	Sep 5	–	+/+	MG431951/MG431960	ND	ND

*L, L gene; MV, measles virus; ND, not done; NP, nucleoprotein; RESTV, *Reston ebolavirus*; +, positive; –, negative.
†Serum sample of DrpZ2-10B-G was RESTV IgG–/MV IgM+.
‡Serum sample of DrpL7-7D-A was RESTV IgG+/MV IgM–.

was most similar to the virus from the 2008 outbreak in swine (GenBank accession no. FJ624585.1) rather than the 1996 outbreak in NHPs.

The comparison of the whole genome sequencing of the 2 isolates DrpZ5-2B-F and DrpZ2-10B-G showed that there were 3 nucleotide differences. The first variation noted was in the NP gene (position 837 of the genome) of DrpZ5-2B-F, which resulted in a non-conservative amino acid change of a Thr (ACG) to a Lys (AAG) when compared with DrpZ2-10B-G and other RESTV isolates (Table 3). The change was also observed for all 4 specimen types of DrpZ5-2B-F (15–009–012), confirming that the change was not caused by passaging of the virus in cell culture. The second change noted was at position 4393 of the genome in the noncoding region between virus capsid proteins 35 and 40, and resulted in an adenosine for DrpZ5-2B-F and a guanine for DrpZ2-10B-G. The third variation was at position 10787 of the genome, and resulted in an amino acid change at position 162 of the VP24 protein from an Asn (AAC), which is common to all RESTV strains, to a Lys (AAA) for DrpZ5-2B-F. Therefore, DrpZ5-2B-F had 2 unique changes compared with other RESTV isolates. Both isolates showed 98% similarity to their closest RESTV strain (GenBank accession no. FJ621585.1).

Because among 4 macaques that had a positive result by RESTV PCR, 1 (ID: DrpZ2–10B-G) was serologically positive for MV (Table 1), we subjected a lymph node sample of this macaque to testing for MV by TaqMan RT-PCR to confirm the dual infection. As a result, we detected the MV genome, indicating that a dual infection occurred in this macaque (Table 2). Amplification of the partial L gene of MV RNA from sample DrpZ2–10B-G, followed by sequencing of the product and a BlastN of the sequence, revealed that the MV belonged to genotype B3, which had caused a large outbreak in the Philippines in 2014 (*16*) (Figure 3). MV RNA was also detected in 6 other macaques in the quarantine facility by using TaqMan RT-PCR.

Discussion

In 2015, 19 years after the last known epizootic occurrence of RESTV in macaques in the Philippines, we detected and confirmed the incidence of RESTV in macaques in a primate facility south of Manila, by serologic and molecular testing. In spite of the long hiatus, RESTV was found in a controlled environment in which monkeys are systematically housed to avoid spread of diseases and to which no wild monkeys have been introduced. Personnel in the facility had no evidence of infection because no RESTV antibodies were detected.

We observed rats in cages in the primate facility that housed the primates being tested, indicating the potential for small animals to gain access to the facility. A recent study identified the microbat *Miniopterus schreibersii* as a possible reservoir of RESTV (*6*); therefore, this bat species and similar ones of this size may be the source of infection in the quarantine facility. If this is the case, improved biosecurity measures are warranted to limit the introduction of disease. However, we do not claim the bat species as the direct source of infection in 2015 outbreak. Because the facility building has its own anteroom with welded wire window screens, there is little likelihood that bats entered the facility.

Dual infections of RESTV and simian hemorrhagic fever virus (SHFV) in cynomologus monkeys have been reported in a facility in Reston, Virginia, and SHFV is

Table 3. Nucleotide differences between 2 *Reston ebolavirus* isolates identified in the Philippines, 2015, compared with a reference sequence*

Genome position	Reference sequence NC_004161.1	40 DrpZ5-2B-F	43 DrpZ2-10B-G	Amino acid position
837	C Thr	A Lys	C Thr	NP (125)
4393	A	A	G	Noncoding (no amino acid change)
10787	C Asn	A Lys	C Asn	VP24 (162)

*NP, nucleoprotein; VP, virus protein.

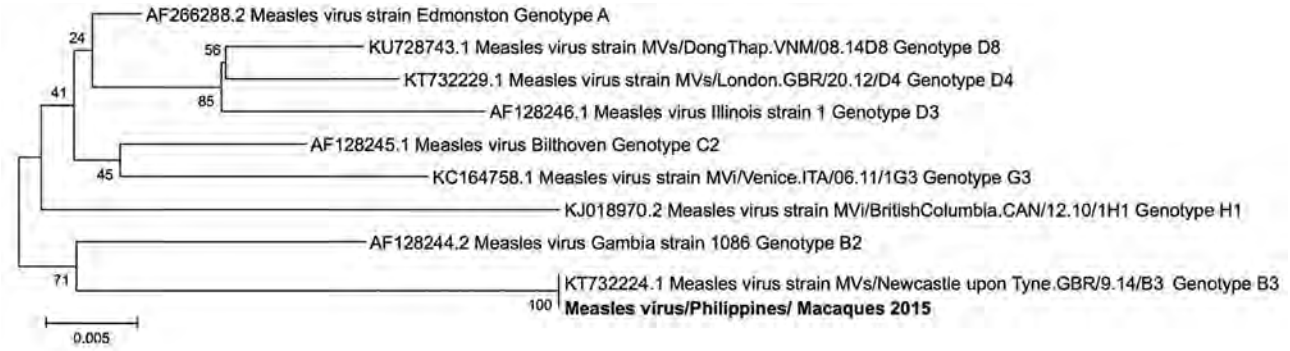

Figure 3. Phylogenetic tree (neighbor-joining) of the partial L gene (418 nt) of measles virus (GenBank accession no. MF496232) detected in macaques in 2015, produced by using MEGA 6 software (https://www.megasoftware.net). Numbers along branches indicate bootstrap values. Scale bar indicates nucleotide substitutions per site. Bold text indicates measles virus strain isolated in Philippines.

the suspected causal agent for mortality in monkeys (*17*). Dual infections of RESTV and PRRSV in swine have been identified in the Philippines (*5*) and in Shanghai, China (*18*). In these cases, all of the RESTV-positive swine were coinfected with PRRSV. In contrast, we found in this study that 1 (ID: DrpZ1–26D-B) of the 10 macaques positive for RESTV antibody was also positive for MV antibody. Furthermore, another macaque (ID: DrpZ2–10B-G) was confirmed to have dual infection of RESTV and MV by using PCR. The results show similarities with dual infections such as SHFV and RESTV in macaques (*17*), or RESTV and PRRSV infections in swine (*5*). However, MV was not detected among most macaques positive for RESTV that died from the disease. Also, it remains unclear whether the MV infection supports an increase in RESTV replication in macaques. We found that 8 macaques had antibodies against MV, and 1 was MV PCR positive. Considering the risk for human-to-primate transmission (*19*,*20*), there is a possibility that MV infection in macaques is associated with human MV outbreak in the Philippines, although further studies are required to identify the mode of transmission of MV infection in macaques.

The RESTV sequences obtained were most similar to Reston-08-E from the Philippines 2008 outbreak in swine (*5*) (Figure 1). There were 3 nucleotide variations between the viral isolates that were sequenced, 2 of which in isolate DrpZ5–2B-F resulted in nonconservative changes in the NP and VP24 proteins that were unique when compared to all of the RESTV isolates sequenced. Because of the similarity with other Ebola viruses and the virus' ability to infect humans, there is a concern that RESTV could mutate during passage through animals like macaques and cause an epidemic of disease in humans. Because it could mutate to pose health consequences for humans, continued surveillance is required to reduce the risk of transmitting Reston Ebola virus.

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Global Distribution of Human Protoparvoviruses

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Development of next-generation sequencing and metagenomics has revolutionized detection of novel viruses. Among these viruses are 3 human protoparvoviruses: bufavirus, tusavirus, and cutavirus. These viruses have been detected in feces of children with diarrhea. In addition, cutavirus has been detected in skin biopsy specimens of cutaneous T-cell lymphoma patients in France and in 1 melanoma patient in Denmark. We studied seroprevalences of IgG against bufavirus, tusavirus, and cutavirus in various populations (n = 840), and found a striking geographic difference in prevalence of bufavirus IgG. Although prevalence was low in adult populations in Finland (1.9%) and the United States (3.6%), bufavirus IgG was highly prevalent in populations in Iraq (84.8%), Iran (56.1%), and Kenya (72.3%). Conversely, cutavirus IgG showed evenly low prevalences (0%–5.6%) in all cohorts, and tusavirus IgG was not detected. These results provide new insights on the global distribution and endemic areas of protoparvoviruses.

Parvoviruses are small, nonenveloped, single-stranded DNA viruses that infect a wide variety of animals ranging from insects and shrimp to birds and mammals. Human parvoviruses belong to 4 genera: *Erythroparvovirus*, *Bocaparvovirus*, *Tetraparvovirus*, and *Dependoparvovirus* (*1*). The recently described bufavirus, tusavirus, and cutavirus are the first members of the genus *Protoparvovirus* found in humans. All 3 viruses were identified by next-generation sequencing and metagenomics in feces of children with diarrhea: bufavirus from Burkina Faso in 2012, tusavirus from Tunisia in 2014, and cutavirus from Brazil and Botswana in 2016 (*2–4*). In addition, cutavirus was detected by in silico analysis of existing next-generation sequencing libraries and by PCR of malignant skin tissues of patients in France with cutaneous T-cell lymphoma (*4*).

To date, 3 genotypes of bufavirus have been detected, and bufavirus DNA has been detected in 1 nasal swab specimen of a child in Finland and in ≤4% of fecal samples from patients with diarrhea in Africa, Europe, and Asia (*2*,*5–13*). Recently, a bufavirus 3 sequence was reported in a fecal sample in Peru, which expanded the geographic locations where bufavirus has been detected (*14*). All of these studies have reported DNA sequences of either bufavirus 1 or 3; bufavirus 2 DNA has been detected only in 1 child in Burkina Faso. Humans have been shown to have IgG against all 3 bufavirus genotypes, which also seem to represent distinct serotypes (*12*).

Although the seroprevalence of bufavirus in Finland was found to be low (3.1% in children and in adults born in Finland), the presence of bufavirus IgG in 5/12 adults originating from Asia suggested that the bufavirus prevalence might be higher in other continents. Furthermore, the strong IgG responses indicate that these 3 viruses might cause systemic infections similar to other known human parvoviruses, such as human parvovirus B19, human bocaviruses, and human parvovirus 4 (*15*). In addition to bufavirus in humans, several animal species, including nonhuman primates, shrews, bats, rats, swine, and fur seals, have been shown to be infected with specific bufavirus-like viruses (*16–23*).

Conversely, tusavirus DNA has been detected only in feces of 1 child in Tunisia (*3*). In addition, 1 of 228 children in Finland showed a low-level IgG response (*12*). However, these findings are scarce, and more studies are needed to determine whether tusavirus is truly a human virus.

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Cutavirus is the newest member of the human parvoviruses. This virus was originally detected in feces of children with diarrhea in 2016; cutavirus DNA was also detected in cancerous tissues of 4/17 patients in France with cutaneous T-cell lymphoma (*4*). Although test results for other skin cancer types and healthy skin examined in this study were negative for cutavirus, 1 melanoma patient in Denmark was shown to have cutavirus DNA in malignant skin (*24*).

The etiologic roles of bufavirus, tusavirus, and cutavirus in human disease remain uncertain and more studies are needed. In this study, we developed a new cutavirus IgG enzyme immunoassay (EIA) and combined it with our existing IgG EIA panel of bufavirus genotypes 1–3 and tusavirus. We then analyzed 6 human populations on 4 continents for IgG against these 3 protoparvoviruses. We included veterinarians from Finland (n = 324) to assess the possible contribution of human–animal contact; adults from the United States (n = 84), Iraq (n = 99), and Iran (n = 107); and adults (n = 119) and children (n = 107) from Kenya to identify age-related and geographic distributions of these emerging viruses in humans.

Materials and Methods

Study Cohorts

The cohorts included in the study were from 5 countries on 4 continents. The study and all sampling were conducted in accordance with relevant guidelines and regulations.

For the veterinary cohort from Finland, we obtained serum samples from 324 healthy adult volunteers (Table 1). Samples were collected from participants at the national Annual Veterinary Congress in 2009 in Helsinki, Finland (*25*). Most (82%) of the volunteers were veterinarians, veterinary students, or veterinary nurses, and 92% completed an electronic questionnaire to obtain background information. Written informed consent was obtained from all study participants, and the study was approved by the Ethics Committee of Helsinki University Central Hospital.

For the cohort from the United States, we obtained serum samples from 84 healthy blood donors at Blood Systems Research Institute (San Francisco, CA, USA) (Table 1). The samples were collected during April 2009 in 2 locations (Arizona [n = 40] and Mississippi [n = 44]). Under US human and health service regulations, the study of preexisting, deidentified samples is not classified as human subject research.

For the cohort from Iraq, we obtained serum samples from 99 healthy adults (Table 1) to assess exposure of the population to various virus infections in Nasiriyah, Dhi Qar, in southern Iraq (*26*). Written informed consent was obtained from all study participants, and the study was approved by the Ethics Committees of Medical Sciences at Basrah University and the Al-Hussein Teaching Hospital.

For the cohort from Iran, we obtained serum samples from 107 healthy adults (Table 1) at the Hamadan Blood Transfusion Organization (Hamadan, Iran). Informed consent was waived for analysis of these deidentified blood donor samples, and the study was approved by the Ethics Committee of Hamadan University of Medical Sciences.

For the cohort from Kenya, we obtained serum samples from 107 children and 119 adults who had a febrile illness of unknown cause and had visited health clinics in Mwatate, Voi, or Wundanyi in Taita Taveta County in southern Kenya (Table 1). A questionnaire

Table 1. Characteristics of cohorts used in study of global distribution of human protoparvoviruses*

Cohort	No. persons	Health status	Mean age, y (range)	No. (%) male:female; unknown	Time of sample collection	Other features
Finland	324	Constitutionally healthy	40.2 (19–79)	45 (13.9):279 (86.1)	2009 Oct	Adults: veterinarians
United States	84	Constitutionally healthy	41.3 (18–72)	64 (76.2):20 (23.8)	2009 Apr	Adults: blood donors
Iraq	99	Constitutionally healthy	39.7 (18–60)	71 (71.4):28 (28.3)	2013 Nov–Dec	Adults: medical staff, blood donors, and university students
Iran	107	Constitutionally healthy	42.2 (18–77)	50 (46.7):57 (53.3)	2015–2016	Adults: blood donors
Kenya, children	107	Febrile at time of sampling (mean temperature 38.6°C, range 37.5°C–40.4°C)	6.9 (0.5–17.8)	59 (55.1):43 (40.2); 5 (4.7)†	2016 Apr–Aug	Children: includes 9 HIV+ (8 receiving HAART)
Kenya, adults	119	Febrile at time of sampling (mean temperature 38.9°C, range 37.5°C–39.8°C)	43.3 (18.2–88.3)	42 (35.3):76 (63.9); 1 (0.8)†	2016 Apr–Nov	Adults: includes 38 HIV+ (35 receiving HAART)

*HAART, highly active antiretroviral therapy; +, positive.
†Sex was not specified in the questionnaire.

to obtain background information and symptoms was completed by all patients, and written informed consent was obtained from all study participants or guardians of children. The study was approved by the Kenyatta National Hospital–University of Nairobi Ethics and Research Committee.

Serologic Analysis

Cutavirus Capsid Protein 2 Virus-Like Particles

To analyze serum samples for IgG against all human protoparvoviruses, we included cutavirus in an in-house EIA panel for bufavirus genotypes 1–3 and tusavirus (*12*). The virus capsid protein 2 (VP2) gene for cutavirus was cloned from the original DNA extract from feces of the cutavirus DNA–positive child from Brazil (Br337) (*4*) by using primers VP2 fwd Br337 *Bam*HI (5′-TAggatccATGTCAGAACCAGCTAATGATAC-3′) and VP2 rev Br337 *Sal*I (5′-CTCgtcgacTTACAATGTGTAGTTTGGTAGACA-3′) (restriction sites are indicated by lowercase letters).

The obtained VP2 (GenBank accession no. MH127919) was used to create a recombinant baculovirus with the Bac-to-Bac system (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and cutavirus VP2 virus-like particles (VLPs) were expressed and purified as described for bufavirus VP2 VLPs and tusavirus VP2 VLPs (*12*).

Combined Bufavirus 1–3/Tusavirus–Cutavirus IgG EIA

We analyzed all serum samples by using the combined bufavirus 1–3/tusavirus–cutavirus IgG EIAs, with insect cell lysate as a control antigen, as described, but included cutavirus VLP antigen in separate wells (*12*). In brief, we applied biotinylated antigens (VP2 VLPs, 80 ng/well) or cell lysate control to streptavidin-coated plates. After incubation and postcoating, we applied serum diluted 1:200 to each well. To detect bound IgG, we used horseradish peroxidase–conjugated antihuman IgG as the secondary antibody and 3,3′,5,5′-tetramethylbenzidine (Dako, Santa Clara, CA, USA) as the substrate.

We measured optical densities (ODs) at 450 nm (Multiskan EX; Thermo Fischer Scientific, Pittsburgh, PA, USA) and subtracted blank ODs from test ODs to get the final OD. We confirmed all samples with an OD ≥0.1 by using a competition assay, as described (*12*,*27*). In the competition assay, serum antibodies were blocked separately with 3 unbiotinylated antigens in solution: the same (homologous) antigen as in the EIA, the heterologous antigen of the phylogenetically closest protoparvovirus, and the heterologous antigen of a more distant protoparvovirus, before repeating the EIAs. A sample was considered IgG positive when full homologous blocking but no (or partial) heterologous blocking occurred, as described (*12*).

Statistical Analysis

We performed statistical analysis by using 2 × 2 tables and test statistics (mid p-exact value) in OpenEpi software

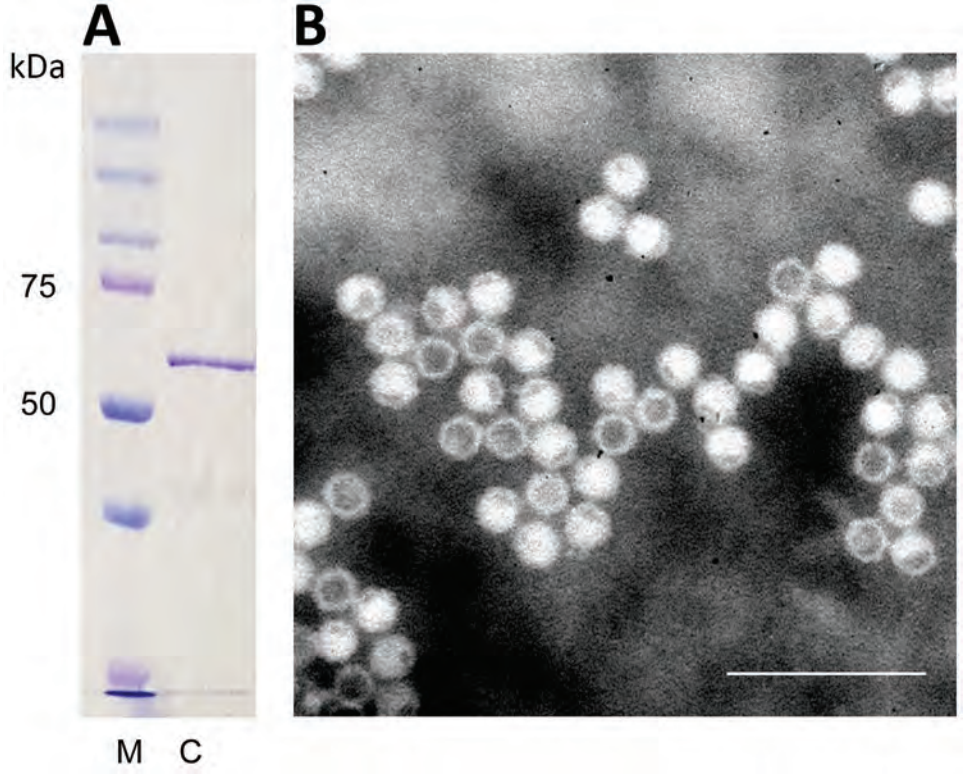

Figure 1. Identification of cutavirus from human serum samples. A) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of virus capsid protein 2. Lane M, protein size marker; lane C, cutavirus. B) Electron micrograph of cutavirus virus-like particles. Scale bar indicates 100 nm.

(https://www.OpenEpi.com). A 2-tailed p value <0.05 was considered statistically significant.

Results

Cutavirus IgG EIA and Cross-Reactivity of IgG

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis identified cutavirus VP2s of expected size (≈64 kDa) (Figure 1, panel A). Electron microscopy identified parvovirus-like VLPs with a diameter of ≈25 nm (Figure 1, panel B).

In the cutavirus IgG EIA, it was evident that bufavirus 2 and cutavirus cross-react: in all samples with an OD >0.5 for bufavirus 2 or cutavirus, a reaction was always observed with the other antigen. In samples showing weak reactivity (OD 0.1–0.5) in cutavirus or bufavirus 2 IgG EIAs, both cross-reactivities and single-specific reactivities were observed. In the competition assay, the specific reactivity of bufavirus or cutavirus was blocked completely only by homologous antigen, whereas cross-reactive reactions were blocked by homologous and heterologous antigens. In several instances, heterologous antigen slightly reduced the specific EIA reactivity; however, this reduction was much less than that caused by homologous antigen.

In cohorts from the Middle East and Africa that showed high prevalences of bufavirus IgG, some cross-reactivity was also observed among the 3 bufavirus genotypes, mostly for samples with higher ODs. However, the competition assay used for all positive samples could distinguish genotype-specific reactivity for correct interpretation. Tusavirus IgG did not cross-react with bufavirus IgGs or cutavirus IgG.

Bufavirus IgG in Adults

Bufavirus IgG was rare among veterinarians from Finland and blood donors from the United States: only 1.9% of the veterinarians and 3.6% of the blood donors had bufavirus IgG (Table 2). In these cohorts, each bufavirus IgG–positive person had antibodies against only 1 bufavirus genotype. No indications of specific animal contact being associated with bufavirus seropositivity were found when we compared background information for bufavirus IgG–positive and bufavirus IgG–negative veterinarians. For blood donors from the United States, all 3 bufavirus IgG–positive samples were from Mississippi. However, all samples from Arizona were negative for bufavirus IgG. The most commonly detected genotype was bufavirus 1 in Finland and bufavirus 3 in the United States (Table 2).

In striking contrast to adults from the United States and Finland, including our previous results for students and staff members from Finland (*12*), bufavirus IgG was common in Iraq, Iran, and Kenya, for which 84.8%, 56.1%, and 72.3%, respectively, of adult populations had IgG against ≥1 bufavirus genotypes (Table 2). In the Middle East, bufavirus 1 was the most common type, whereas in Kenya, bufavirus 3 was the predominant genotype (Table 2). Bufavirus 2 was the second most prevalent bufavirus in all 3 high-prevalence countries. In Iraq, we found that 30 (30.3%) of 99 persons had antibodies against 2 bufavirus genotypes, and 7 (7.0%) of 99 persons had antibodies against all 3 bufavirus genotypes. In adults from Kenya, we found similar prevalences: 30 (25.2%) of 119 persons had antibodies against 2 bufavirus genotypes, and 6 (5.0%) of 119 persons had antibodies against all 3 bufavirus genotypes. However, in Iran, we found that double or triple prevalences were lower: 14 (13.1%) of 107 persons had antibodies against 2 bufavirus genotypes, and 2 (1.9%) of 107 persons had antibodies against all 3 bufavirus genotypes.

In Kenya, HIV-positive patients had a similar bufavirus IgG prevalence as the rest of the cohort: 78.9% (30/38) in HIV-positive adults (mean age 46.3 years, range 27–85 years) vs. 69.1% (56/81) in HIV-negative adults (mean age 41.9 years, range 18–88 years) (p = 0.275). When we compared only the 38 HIV-positive persons and 60 HIV-negative persons within the same age range (27–85 years), bufavirus seroprevalences were even more similar: 79% for HIV-positive persons and 75% for HIV-negative persons (p = 0.669). However, possible undiagnosed cases of infection with HIV and unequal numbers could affect the accuracy of this comparison.

When the adult cohorts were analyzed more closely and persons were divided by age into equal-sized groups

Table 2. Seroprevalence of IgG against protoparvoviruses in different population cohorts*

Cohort	No. persons	Any bufavirus IgG†	IgG against bufavirus genotypes 1	2‡	3	Tusavirus IgG	Cutavirus IgG‡
Finland, healthy adults (veterinarians)	324	6 (1.9)	4 (1.2)	1 (0.3)§	1 (0.3)	0	16 (4.9)§
United States, healthy adults	84	3 (3.6)	0	0	3 (3.6)	0	0
Iraq, healthy adults	99	84 (84.8)	80 (80.8)	33 (33.3)¶	15 (15.2)	0	1 (1.0)¶
Iran, healthy adults	107	60 (56.1)	55 (51.4)	17 (17.9)	6 (5.6)	0	6 (5.6)
Kenya, febrile children <18 y of age	107	22 (20.6)	3 (2.8)	4 (3.7)	20 (18.7)	0	2 (1.9)
Kenya, febrile adults	119	86 (72.3)	31 (26.1)	43 (36.1)#	56 (47.1)	0	5 (4.2)#

*Values are no. (%) unless otherwise noted..
†Includes persons that were IgG+ against ≥1 bufavirus genotypes.
‡Unclear bufavirus 2 and cutavirus blocking results were observed for 1§ (0.3%), 2¶ (2.0%), and 3# (2.5%) persons. These values are not included in overall seroprevalence calculations.

of <40 years of age and ≥40 years of age, we found that for younger adults in Iran, bufavirus seroprevalence was lower than that for older adults (21/54 [38.9%] vs. 39/53 [73.6%]; p = 0.0003). However, a similar distinction was not observed for adults from Iraq or Kenya (Table 3). For veterinarians from Finland and adults from the United States, we found that younger adults also had a lower bufavirus seroprevalence, albeit without statistical power, because of the low overall prevalence of bufavirus IgG in these countries. When we divided the cohorts into persons <30 years of age and ≥30 years of age, a similar trend was also observed for adults in Kenya (14/35 [56.0%] vs. 70/92 [76.1%]; p = 0.0595). This trend was not observed for persons in Iraq.

Bufavirus IgG in Children in Kenya

In Kenya, the bufavirus IgG prevalence in children was significantly lower than that in adults (20.6% in children <18 years of age vs. 72.3% in adults; p<0.0001), but we observed similar proportions of bufavirus and a predominance of bufavirus 3 in both adults and children. (Table 2). When we divided the cohort of children into those <5 years of age and those 5–17 years of age, the prevalence of bufavirus IgG by age increased from 12% to 28.1% (Figure 2).

Cutavirus and Tusavirus IgG in Adults and Children

The prevalence of cutavirus IgG was generally low for all groups, ranging from 1.0% in Iraq to 5.6% in Iran, and cutavirus IgG was not detected in adults in the United States (Table 2). In veterinarians in Finland, cutavirus IgG (4.9%) was more common than bufavirus IgG (1.9%) (p = 0.032). Two adults from Kenya and 1 veterinarian from Finland had both cutavirus IgG and bufavirus 2 IgG in their samples, which showed that these 2 antigenically similar viruses can infect the same person and elicit specific immune responses against each virus. However, for 6 patients (1 in Finland, 2 in Iraq, and 3 in Kenya), we could not determine whether the reactivity detected was specific for bufavirus 2, cutavirus, or both. These results were not included in the final prevalence calculations (Table 2). Tusavirus IgG was not detected in any cohort (Table 2).

Discussion

During the current decade, several new parvoviruses have been detected, mostly because of the development of NGS methods. Bufavirus, tusavirus, and cutavirus are the newest of these viruses detected in human samples (*2*–*4*). Bufavirus has been associated with gastroenteritis, and cutavirus is being studied for its relationship to skin cancers (*4,8*–*10,12,24*). However, studies that attempted to detect Bufavirus, tusavirus, and cutavirus DNA in any sample type or virus antibodies in serum samples have been infrequent (*13*).

We found high (50%–85%) seroprevalences of bufavirus IgG in cohorts from the Middle East and Africa, which indicated that bufavirus infections are endemic to these areas. The observed low (1.9%) seroprevalence in veterinarians in Finland is consistent with our previous results for staff members and medical students born in Finland (3.1%) (*12*). The seroprevalence of bufavirus in the United States was similar to that in Finland, although the major genotype was different. In contrast to the diverse epidemiology of antibodies against bufavirus, antibodies against cutavirus appeared globally and were much more evenly distributed and showed a low prevalence. These results provide new insights on the global distribution and identify areas to which protoparvoviruses are endemic.

Because the difference in seroprevalence between persons born in Finland and staff born in Asia in our previous study could also be caused by more frequent animal contacts for 5 persons from Asia (*12*), we included veterinarians in this current study. However, no specific animal contacts for veterinarians from Finland were associated with bufavirus IgG or cutavirus IgG seropositivity. Although species jumps have occurred within protoparvoviruses (*28*), this result is consistent with the general rule of host-order specificity of parvoviruses (*1*). No animal contact information was available for persons from the Middle East, Kenya, or the United States.

Table 3. Seroprevalence of bufavirus IgG in adult cohorts, by age, in study of global distribution of human protoparvoviruses*

Cohort	Age group, y	No. persons	No. (%) bufavirus IgG+ (95% CI)	p value	Mean age, y (range)
Finland	<40	165	1 (0.6) (0.0–3.7)	0.096	31.6 (19–39)
	≥40	149	5 (3.4) (1.2–7.8)		49.7 (40–79)
United States	<40	38	1 (2.6) (0.0–14.7)	0.731	26.4 (18–39)
	≥40	46	2 (4.3) (0.4–15.3)		53.5 (40–72)
Iraq	<40	45	40 (88.9) (76.1–95.6)	0.325	30.8 (18–39)
	≥40	54	44 (81.5) (69.0–89.8)		47.2 (40–60)
Iran	<40	54	21 (38.9) (27.0–52.2)	0.0003	30.1 (18–39)
	≥40	53	39 (73.6) (60.3–83.7)		55.0 (40–77)
Kenya	<40	52	37 (71.2) (57.7–81.8)	0.890	29.5 (18–39)
	≥40	65	47 (72.3) (60.4–81.8)		54.3 (40–88)

*The exact age of 2 adults from Kenya and 10 veterinarians from Finland were not known and they were excluded from the analysis. However, the 2 Kenyans were defined as adults in the overall prevalence calculations on the basis of education and marital status in the questionnaire. All veterinarians were defined as adults on the basis of work-related sample collection site. +, positive.

The age group results from Kenya, which showed continuously increasing seroprevalences of all 3 bufavirus genotypes, also showed that bufaviruses infect persons of all ages. The lower seroprevalence in children <5 years of age than in older children and adults indicates that the age of acquisition of bufavirus greatly differs from that of human bocavirus 1 (*27*) but resembles that for human parvovirus B19. Also for persons 5–17 years of age, an age-dependent increase was evident, but statistical power was insufficient to further divide these children into narrower age groups. In adults, the age-dependent increase in seroprevalence was detectable in Iran, but not in Kenya or Iraq. This difference could be caused by decreased bufavirus circulation in Iran during the past 30–40 years or to socioeconomic or cultural changes over time. In Finland and the United States, age group prevalences were similar, albeit at low levels.

HIV-positive adults in Kenya did not have a higher seroprevalence of bufavirus IgG than HIV-negative adults. Bufavirus infection route(s) could therefore be hypothesized to differ from those for HIV infection.

The predominant bufavirus genotype (1 or 3) varied between countries studied. Bufavirus 2 was the second most common genotype in the 3 high-prevalence countries (Iraq, Iran, and Kenya). In sharp contrast, bufavirus 2 DNA has hitherto been found in the fecal sample of only 1 child in Burkina Faso, whereas all other bufavirus DNA–positive samples had genotypes 1 or 3 (*2*,*5*,*6*,*8*–*11*,*14*). Our serologic data indicate that bufavirus 2 infections exist and are common in certain areas. Further studies of patients with primary infection should elucidate whether sample type(s) most suitable for detection of bufavirus 2, and also for genotypes 1 and 3, is stool or another type of sample.

In humans, IgG is induced against all 3 bufavirus genotypes and cutavirus, and immune reactions appear to be strong. In our previous report on bufavirus IgG, the 3 bufavirus genotypes were shown to have no mutual cross-reactivity (*12*). In this study, some cross-reactivity was observed between the 3 bufavirus genotypes, particularly among high OD samples in high-seroprevalence cohorts. In addition, cutavirus and bufavirus 2 cross-reactivity was common, which is consistent with the fact that amino acid identities are high within the VP2 gene (82% identity for the amino acid sequence). However, both genotype and species cross-reactivities could be distinguished from specific reactivity in the competition assay, similarly to what is shown for the 4 human bocaviruses (*28*).

Despite some VLP cross-reactivity in the EIA, the 3 bufavirus genotypes do not appear to be cross-protective. Several persons had antibodies against 2 or even 3 protoparvoviruses. Whether the previously formed antibodies against the first virus protects the human host against possible symptoms of the second related virus infection, or worsen the symptoms through antibody-dependent enhancement, is not known. However, it appears that immunity toward different protoparvoviruses does not hamper a sequential infection by heterologous virus (bufavirus genotype or cutavirus) or formation of specific antibodies toward this virus, which is in contrast to the phenomenon of original antigenic sin seen among human bocaviruses (*27*,*29*). Longitudinal studies are needed to assess antibody and protection patterns, both during acute primary infections and during subsequent infections by the other human protoparvoviruses. It will be useful to determine the clinical pictures in these contexts, whether these viruses cause similar primary symptoms and illnesses, and whether they have the same or different tissue tropisms.

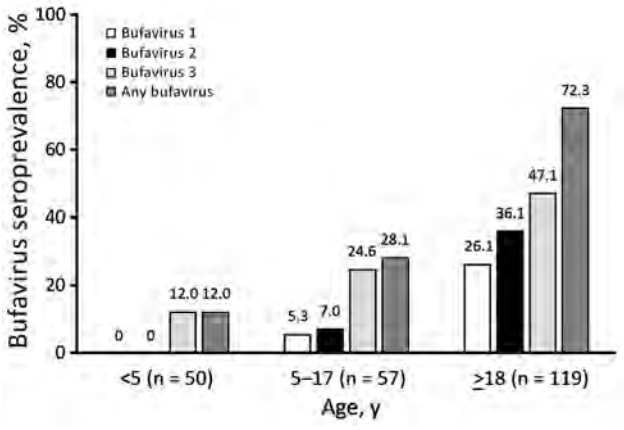

Figure 2. Seroprevalence of bufavirus in Kenya, by age. Several persons (mostly adults) had IgG against ≥1 bufavirus genotypes; such persons are counted as 1 person in the bufavirus column. Differences in overall bufavirus seroprevalences were statistically significant between younger children vs. older children (p = 0.04345), younger children vs. adults (p<0.000001), and older children vs. adults (p<0.000001).

We did not detect tusavirus IgG in any cohort. This finding is consistent with results of 2 previous human studies, which reported no or infrequent evidence of tusavirus (*3*,*12*). In addition, a study has reported sequences with some resemblance to tusavirus: a metagenomic analysis of fur seals in Brazil described partial sequences with 39%–82% amino acid similarity to tusavirus (*23*). Further studies on tusavirus DNA or antibodies are needed to determine whether tusavirus is a human or an animal parvovirus whose original detection in human feces was caused by consumption of meat or other products of a tusavirus-infected animal.

In conclusion, we observed major differences in seroprevalence of bufavirus when we compared Finland and the United States with the Middle East and Kenya. The high seroprevalence of bufavirus in the Middle East and Africa provides new opportunities for detecting bufavirus primary

infections because these infections seem to be endemic to these regions. The predominant bufavirus genotype varied: bufavirus 1 was the most prevalent type in Finland and in the Middle East, and bufavirus 3 was the most prevalent type in the United States and in Kenya. Although IgG cross-reactivity was commonly observed, virus-specific antibodies could be distinguished from cross-reactivity by the competition assay. In contrast to bufavirus infections, cutavirus infections were distributed evenly and at found at low prevalences in all countries studied; for blood donors in the United States no virus IgG was detected. Tusavirus IgG was not detected in any cohorts studied.

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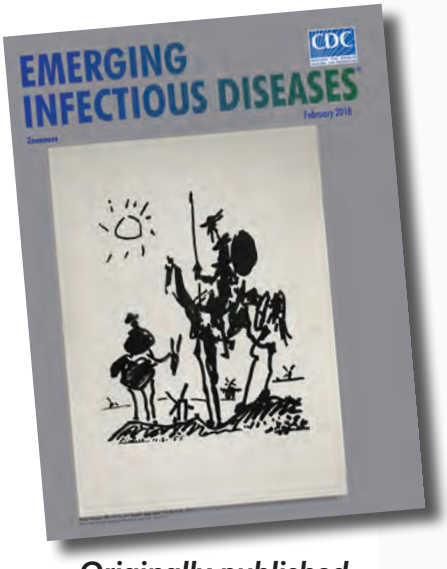
etymologia revisited

Parvovirus [pahr′ vo-vi″res]

Viruses of the family *Parvoviridae* (Latin *parvum* [meaning small or tiny]) are among the smallest viruses described, 18–28 nm in diameter. There are 2 subfamilies of the family *Parvoviridae*: *Parvovirinae* and *Densovirinae* (Latin denso [thick or compact]). *Parvovirinae* may infect humans, but *Densovirinae* infect only arthropods. Structurally, these viruses are nonenveloped, icosahedral viruses that contain a single-stranded linear DNA genome.

The small size of these viruses might account for their late discovery. In 1974, the first pathogenic human parvovirus was discovered and named B19 from the coding of a serum sample, number 19 in panel B, that gave anomalous results during testing for hepatitis B. Although human B19 infections are more often asymptomatic or lead to mild rash illnesses and arthralgias, they can also cause severe anemia in fetuses and in persons with underlying hemoglobinopathies.



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Mapping of the US Domestic Influenza Virologic Surveillance Landscape

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Influenza virologic surveillance is critical each season for tracking influenza circulation, following trends in antiviral drug resistance, detecting novel influenza infections in humans, and selecting viruses for use in annual seasonal vaccine production. We developed a framework and process map for characterizing the landscape of US influenza virologic surveillance into 5 tiers of influenza testing: outpatient settings (tier 1), inpatient settings and commercial laboratories (tier 2), state public health laboratories (tier 3), National Influenza Reference Center laboratories (tier 4), and Centers for Disease Control and Prevention laboratories (tier 5). During the 2015–16 season, the numbers of influenza tests directly contributing to virologic surveillance were 804,000 in tiers 1 and 2; 78,000 in tier 3; 2,800 in tier 4; and 3,400 in tier 5. With the release of the 2017 US Pandemic Influenza Plan, the proposed framework will support public health officials in modeling, surveillance, and pandemic planning and response.

Influenza viruses cause a substantial burden of illness each year in the United States, estimated at 9.2–35.6 million cases of infection, 4.3–16.7 million clinic visits, 140,000–710,000 hospitalizations, and 12,000–56,000 deaths (*1*). To monitor these constantly changing viruses, the Centers for Disease Control and Prevention (CDC), in collaboration with public health partners, collects and analyzes data from multiple surveillance systems (*2*). These efforts track currently circulating influenza viruses, identify novel influenza viruses of public health importance, monitor antiviral drug susceptibility, and characterize circulating seasonal viruses for guiding influenza vaccine virus selection.

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The data and specimens used for influenza virologic surveillance originate from ambulatory patient care facilities, academic and community hospital laboratories, public health laboratories, and commercial laboratories. Recently, CDC has initiated efforts to improve the efficiency of national virologic surveillance and to introduce next-generation, whole-genome sequencing into routine activities. As a first step in this process, we explored existing influenza testing practices and constructed a comprehensive overview of the US virologic surveillance landscape. We evaluated key elements of the system and present a framework for analyzing system strengths and limitations. These findings can be used for informing future modeling efforts, ongoing rightsizing of surveillance, and preparing for a surge in testing during a pandemic response (*3*). In addition, the recently revised 2017 Pandemic Influenza Plan from the US Department of Health and Human Services has initiated a cascade of pandemic influenza plan revisions among other federal, state, and local government partners (*4*). Our proposed framework will support these efforts, providing a common diagnostic operating picture for all levels of influenza testing.

Methods

To characterize the specimen and data flow used to inform influenza virologic surveillance, we conducted open-ended interviews with clinicians, state public health laboratory (PHL) directors, epidemiologists, and laboratorians from CDC and the Association of Public Health Laboratories (APHL) staff, asking them to share their understanding of all aspects of the data and specimen flow with which they were familiar. We mapped major flows of respiratory specimens and test data contributing to virologic surveillance into a process map, tracing sources, routes, and destinations. We identified 5 virologic surveillance tiers in which specimens were collected or tested: outpatient care settings (tier 1), inpatient care settings and commercial laboratories (tier 2), state and local public health laboratories and health departments (tier 3), laboratories at CDC-sponsored National

Influenza Reference Centers (NIRCs) (tier 4), and laboratories within the CDC National Center for Immunization and Respiratory Diseases Influenza Division (tier 5). We defined testing activities within each tier (Table) and incorporated them into a framework to characterize domestic influenza virologic surveillance. We used stakeholders' reviews and comments for revisions to finalize the framework.

To observe trends in the type and amount of influenza testing performed in both outpatient and inpatient healthcare settings, we used MarketScan Research Databases (Truven Health Analytics, Atlanta, GA, USA) and Medicare and commercial carrier reimbursement claims, which provided test counts from 95,176,178 covered lives during 2010–2015 (*5*). We examined Current Procedural Terminology (CPT) codes for virus isolation (87252, 87253, and 87254), immunofluorescence (87275 and 87276), PCR (87501, 87502, 87503, 87631, 87632, 87633, and 87798), and rapid influenza diagnostic test (RIDT) (87400, 87449, 87804). We also used inpatient testing trends from published literature (*6*). To capture the volume and type of tests performed at clinical laboratories contributing to influenza virologic surveillance and at PHLs, we analyzed reports submitted to CDC from clinical providers, and state and local public health authorities participating in influenza surveillance. The CDC Influenza Division provided counts of surveillance tests performed in 3 CDC-supported NIRC laboratories and CDC laboratories.

Results

We categorized the multiple elements of the US domestic influenza virologic surveillance into the 5 tiers and captured the interrelationships of decisions and specimen and data submission in a process map (Figure 1). The tiers reflect the sequential flow of viruses, information, and location of activities contributing to virologic surveillance.

Tiers 1 and 2

Tier 1 consists of outpatient care facilities, predominantly physician offices and urgent care centers. Specimens collected in this tier are used primarily for diagnosis and treatment decisions. Only a subset of care-seeking patients with influenza-like illness (ILI) will have respiratory specimens tested for influenza (*7*); of these respiratory specimens, only a subset is sent to the PHLs represented in tier 3. These specimens may be collected by ILINet providers and tier 1 providers designated by their state as influenza surveillance partners. ILINet is a network of >2,800 outpatient healthcare providers located in all 50 states, Puerto Rico, the District of Columbia, and the US Virgin Islands. Each week during influenza season, ≈2,000 ILINet provider-participants report total and ILI visits to CDC (*2*).

Clinicians may use many criteria when deciding whether and how to test for influenza. Physicians often test for influenza when there is a suspected outbreak in a facility or closed setting; when epidemiologic factors indicate the potential for severe disease; or when travel history, animal exposure, or both indicate possible infection with a potential pandemic virus. An analysis of claims data indicates that RIDTs were clinicians' predominant testing choice during 2010–2015 (Figure 2) (*8*). Most RIDTs can be performed by healthcare providers in settings such as physicians' offices or small clinics. Although RIDTs may exhibit high specificity, the suboptimal sensitivity of some RIDTs can produce false-negative results (*9*).

Tier 2 comprises laboratories with higher-complexity testing capabilities, such as hospital and commercial

Table. Characteristics of influenza test types used for US domestic influenza virologic surveillance*

Characteristic	RIDTs†	Virus isolation	Direct fluorescent antibody tests	Molecular tests‡	Antiviral resistance functional tests	Antigenic tests§	Genetic sequencing
Result type	Influenza positive or negative AND type A or B (for most tests)	Virus growth	Influenza positive (type A or B), negative, or inconclusive	Influenza type and/or subtype positive, negative, or inconclusive	Resistant or not to adamantanes and neuraminidase inhibitors	Antigenic relatedness of viruses to vaccine or reference viruses	Genetic structure and relationship to previously circulating influenza viruses
Time to results	<30 min; most differentiate positive influenza A and B	Traditional: 3–10 d Rapid: 1–3 d	1–4 h	15 min–6 h	≈1 d	5–8 h	3–5 d (excluding isolation)
CLIA¶ category	Varies: CLIA-waived to moderate complexity	High complexity	Varies: moderate to high complexity	Varies: CLIA-waived to high complexity	High complexity	High complexity	High complexity

*CLIA, Clinical Laboratory Improvement Amendment; RIDT, rapid influenza diagnostic tests.
†http://www.cdc.gov/flu/professionals/diagnosis/rapidlab.htm#table2.
‡http://www.cdc.gov/flu/pdf/professionals/diagnosis/molecular-assay-table-1.pdf.
§Hemagglutination inhibition, microneutralization, and focus-reduction assays (https://www.cdc.gov/flu/professionals/laboratory/antigenic.htm).
¶ CLIA categories for laboratory complexity (https://wwwn.cdc.gov/clia/Resources/TestComplexities.aspx).

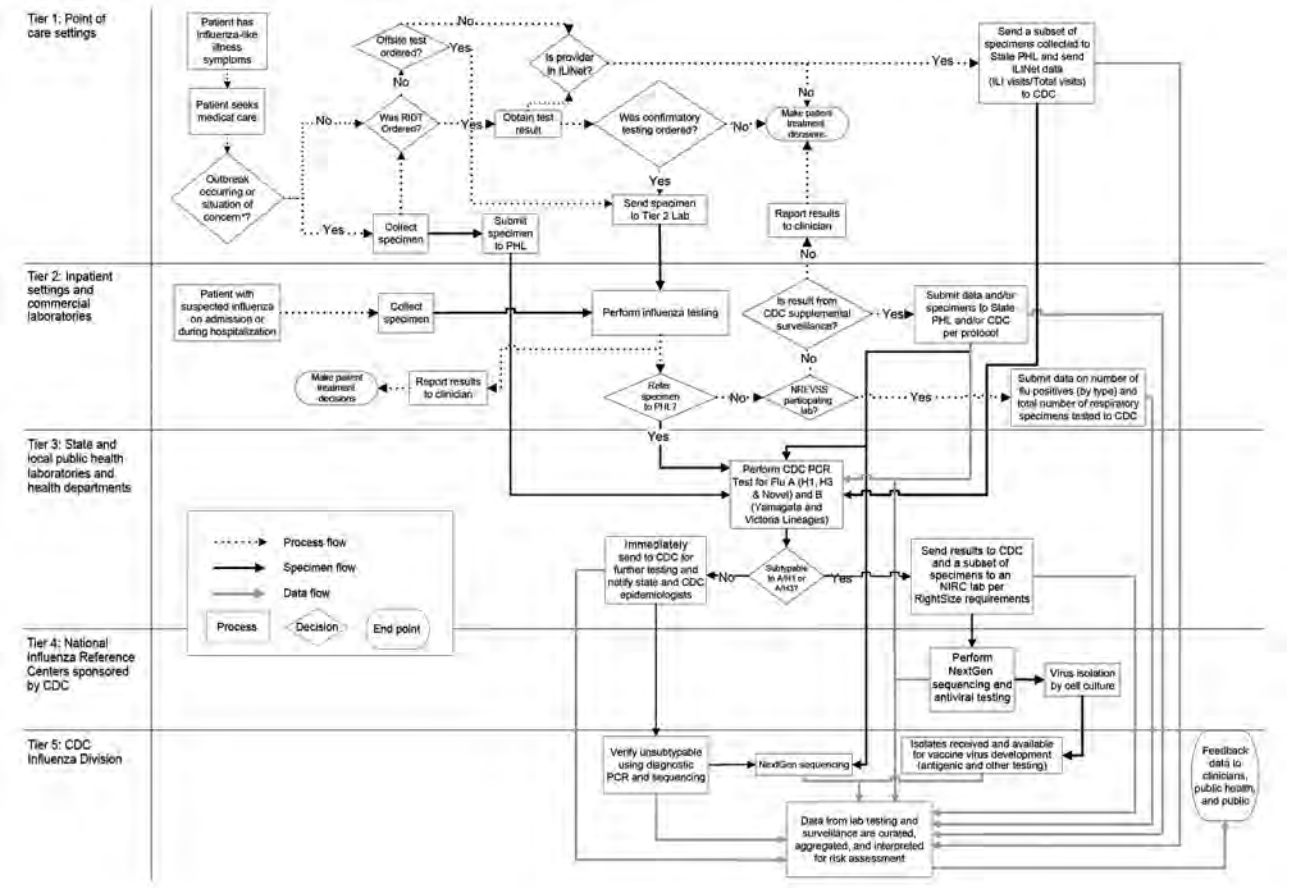

Figure 1. Influenza Virologic Surveillance Landscape illustrating the processes and the flow of specimens and test data through 5 tiers of testing activity. CDC, Centers for Disease Control and Prevention; ILI, influenza-like illness; ILINet, Influenza-Like Illness Surveillance Program; NGS, next-generation sequencing; NIRC, National Influenza Reference Center; NREVSS, National Respiratory and Enteric Virus Surveillance System; PHL, public health laboratory; RIDT, rapid influenza diagnostic test. *Situation of concern: epidemiologic factors indicating outbreak, potential for severe disease, resistant infection, or possible novel virus infection.

laboratories. Providers in tier 1 may send specimens to tier 2 laboratories to obtain more sensitive initial testing or as a follow-up to confirm RIDT results. These laboratories report test results to clinicians who can use them to validate or modify treatment decisions. However, tier 1 laboratories that participate in public health surveillance networks may also submit specimens directly to tier 3 for validation of results.

Tier 2 laboratories may forward a subset of specimens and data to PHLs in their jurisdictions as part of state-requested surveillance, or for further testing of unusual clinical cases, suspect novel events, or potential antiviral drug resistance. If the tier 2 laboratory is 1 of the ≈300 clinical laboratories that report test results through the National Respiratory and Enteric Virus Surveillance System (*2*), it will submit weekly counts of positive and total influenza tests to CDC.

Tiers 1 and 2 represent most influenza testing in the United States. The total number of influenza tests performed each year at tier 1 and 2 facilities is not known. Figure 2 shows the relative use and trend during 2010–2015 of 4 different influenza test types used in tiers 1 and 2. RIDT claims were ≈4 times more common than all other test claims combined, and the total number of test claims per 10,000 enrollees rose 2.5-fold during 2010–2015. In general, RIDTs are most frequently used in tier 1 facilities. Tests used in tier 2 facilities, such as virus isolation, direct immunofluorescence, and PCR, are generally more complex. PCR test claims increased >3-fold during 2010–2015. The estimated number of tests in the 2015–16 season from tier 2 that directly contributed to US influenza virologic surveillance was 804,000.

Tier 3

Tier 3 comprises ≈100 state and local PHLs, in all 50 US states, that collaborate with CDC for influenza surveillance. These laboratories use standard CDC-supplied reverse transcription PCR (CDC RT-PCR) test reagents to detect

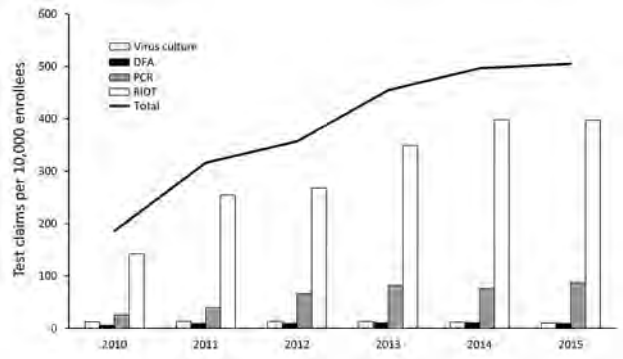

Figure 2. Number of influenza test claims per 10,000 enrollees in Truven Health Analytics' Database 2010–2015, demonstrating that the total number of influenza tests has consistently increased, with RIDTs comprising the largest percentage of tests. DFA, direct fluorescent antibody test; RIDT, rapid influenza diagnostic test.

influenza viruses A(H1N1)pdm09, A(H3N2), B/Yamagata lineage, B/Victoria lineage, A(H5N1), A(H7N9), and other novel influenza viruses. These laboratories test influenza specimens primarily for surveillance or outbreak investigations. Results are reported to state health departments and to CDC. A subset of specimens is also sent to CDC or a CDC-designated NIRC on the basis of guidelines established for each influenza season. Any specimen producing inconclusive results (i.e., influenza A with no subtype identified by CDC RT-PCR) may indicate infection with a novel influenza virus with epidemic or pandemic potential. These specimens are to be sent directly, as soon as possible, to CDC for rapid diagnostic confirmation and comprehensive characterization with notification to state and CDC epidemiologists. Some PHLs participate in 1 of 3 supplemental surveillance systems that ask participants to send additional influenza-positive specimens, related data, or both to CDC. Supplemental surveillance systems include CDC's Influenza Hospital Surveillance Network (FluSurv-Net), the Influenza Incidence Surveillance Program, and the US Influenza Vaccine Effectiveness Network. FluSurv-Net monitors hospitalizations related to laboratory-confirmed influenza in 13 states (>70 counties) (*1*). The Influenza Incidence Surveillance Program consists of a convenience sample of primary outpatient practices in 6 states, recruited by their state health departments, that collect respiratory specimens from all ILI patients (*10*). The US Influenza Vaccine Effectiveness Network includes ambulatory care facilities affiliated with 5 major medical centers in Washington, Wisconsin, Michigan, Pennsylvania, and Texas. These facilities provide data and specimens to CDC from patients seeking care for acute respiratory infections (*11*).

Tier 3 laboratories tested ≈78,000 respiratory specimens during the 2015–16 influenza season. During 2009–2016, the number of tests reported to CDC by public health laboratories varied by season; most reported tests were PCR, using the CDC RT-PCR assay (Figure 3). Virus culture was performed less frequently. Tier 3 laboratories are key for novel influenza A virus detection. In addition to testing for all currently circulating human influenza viruses, the CDC RT-PCR allows tier 3 laboratories to presumptively identify human infection with swine variant influenza viruses and avian influenza A(H5N1) and A(H7N9) viruses. CDC training of the tier 3 laboratories and the use of common platforms and test methods permit rapid deployment of new assays in the event of a public health emergency.

Tier 4

Tier 4 consists of 3 state PHLs designated by CDC as NIRC laboratories. These laboratories receive specimens from tier 3 PHLs, isolate viruses in cell culture to sufficient volumes and titers, and assess susceptibility of viruses to antiinfluenza medications. The number and influenza type/subtype of specimens sent from tier 3 PHLs is determined using an online calculator tool for determining each jurisdiction's sample size for submission. This effort, termed rightsizing, began implementation in 2013 and was used for the 2015–16 season in all submitting jurisdictions in all 50 US states (*12*).

Since 2015, NIRC laboratories have also begun using next-generation whole-genome sequencing (NGS) directly from clinical specimens to characterize viruses. Sequence data from NIRC laboratories are immediately available to CDC during sequence runs through a cloud-based sequence analysis platform. All remaining original clinical specimens and virus isolates are sent to CDC for further characterization. Since 2010, from 2,100 to 3,000 specimens from domestic surveillance have been tested in tier 4 each season (Figure 4). For the 2015–16 season, 2,800 specimens were tested in tier 4.

Tier 5

Tier 5 represents laboratories at CDC. These laboratories receive specimens and isolates from the NIRCs and specimens collected by PHLs during case or outbreak investigations from clinicians concerned about novel or drug-resistant influenza virus infections in humans. Laboratories at CDC also receive specimens and isolates from international laboratories for virologic surveillance. Viruses that have not undergone NGS at a NIRC undergo NGS in CDC laboratories. CDC scientists analyze all genetic sequencing data to identify viruses of epidemiologic and clinical importance. Final gene sequences and related information are submitted to the GISAID database (*13*) and GenBank (*14*). GenBank produces an annotated collection of all publicly available DNA sequences. The GISAID initiative focuses exclusively on influenza viruses and provides open access to sequences, clinical and epidemiologic data, and geographic data.

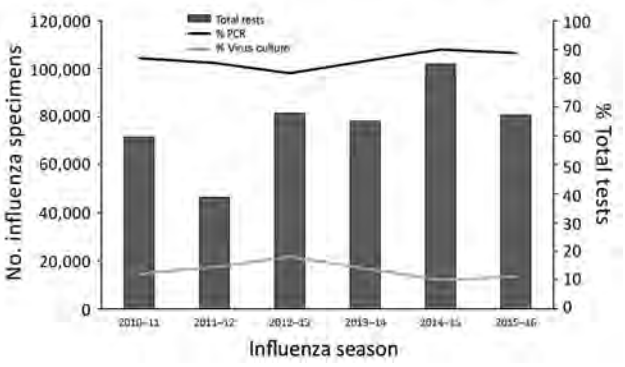

Figure 3. Number of influenza tests reported by public health laboratories to CDC since 2010. The number of specimens tested varies with the severity of the season. Since 2010, an average of 77,000 specimens has been tested annually. Multiple tests may be performed on a single specimen. Most tests have been PCR.

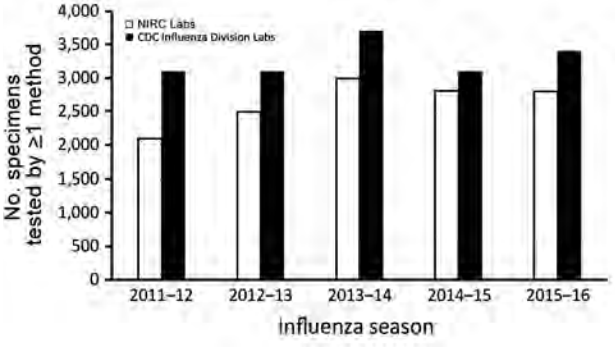

Figure 4. Number of influenza specimens tested for domestic surveillance in tier 4 (NIRCs) and tier 5 (CDC, Atlanta) laboratories. NIRCs receive specimens from tier 3 laboratories and are a major source of specimens for tier 5 laboratories. CDC, Centers for Disease Control and Prevention; NIRC, National Influenza Reference Center.

Influenza viruses from the NIRCs and those sent directly to CDC are characterized by hemagglutination inhibition, microneutralization, or focus-reduction assays to determine antigenic relatedness to vaccine viruses. Specimens sent directly to CDC are also tested for susceptibility to antiviral drugs. Supplemental PCR testing is done on specimens for which subtyping performed elsewhere is inconclusive or requires confirmation. CDC scientists analyze all results to identify viruses of epidemiologic and clinical importance and to prepare information documents for the WHO twice-yearly consultation meetings on composition of influenza vaccines.

CDC epidemiologists and laboratorians aggregate and analyze data from PHLs, NIRCs, and other designated surveillance laboratories; outpatient illness data; influenza-associated hospitalization and mortality data; and state epidemiologist reports of the geographic spread of influenza to identify currently circulating viruses and their clinical impact. These data are used to produce FluView (*15*), a weekly surveillance report, as well as other communication products that share surveillance data with clinicians, public health officials, and the public. The data are also used for periodic risk assessments of newly emerging novel influenza viruses (*16*).

Multiple influenza tests are conducted in tier 5. During the 2015–16 season, CDC's influenza laboratories tested ≈3,400 influenza viruses from the domestic surveillance system; most of these viruses were received from tier 4 NIRCs for additional analysis (Figure 4). Specimens collected during case and outbreak investigations were also submitted to CDC, as were specimens submitted by tier 3 laboratories when results generated using the CDC RT-PCR required confirmation or advanced laboratory testing. Nearly 70% of viruses received were antigenically characterized by hemagglutination inhibition. A subset, including those unable to be tested with hemagglutination inhibition, was subsequently tested in supplementary microneutralization assays. Almost all specimens received directly underwent NGS. The percentage of specimens with a record of sequencing activity increased from 27% in 2011 to 100% in 2016. Sequencing before 2015 included some traditional Sanger sequencing of the hemagglutinin (HA) and neuraminidase (NA) (and sometimes matrix [M]) gene segments.

Combining information from all tiers during the 2015–16 influenza season, CDC reported ≈804,000 influenza test results from tier 2 National Respiratory and Enteric Virus Surveillance System laboratories, 78,000 from tier 3 PHLs, 2,800 from tier 4 NIRCs, and 3,400 from CDC laboratories.

Discussion

The US influenza virologic surveillance landscape is a system that has developed over >40 years. In 1973, US virologic surveillance consisted of 60 cooperating laboratories mailing weekly reports to CDC of specimens submitted and specimens positive for influenza isolation (*17*). The current system includes a much wider compilation of participants and laboratory practices, including NGS and automated electronic laboratory reporting. Using several data sources, we developed a framework and process diagram of 5 testing tiers to assist efforts of diagnostic modelers, public health officials improving the efficiency of surveillance, and agencies revising pandemic plans.

The US influenza virologic surveillance landscape is complex. A diversity of system participants, each functioning with its distinct testing purposes, sampling approaches, testing algorithms, and test methods, contributes to complexity both within and between the tiers (Figure 1). The purpose of influenza testing influences the amount of testing performed and the test methods. Ultimately, all

influenza virologic surveillance relies on specimens collected from symptomatic patients during medical encounters in tiers 1 and 2, where the purpose of testing is primarily patient diagnosis rather than surveillance. Influenza tests are most useful for individual patients when likely to give results helpful for diagnosis and treatment decisions. The decision to test ILI outpatients for influenza is based on the individual physician's knowledge, background, experience, and interest, as well as current influenza activity, resulting in diverse testing practices. Specimens are collected and subsequently available for public health surveillance from only a subset of patients seeking medical care, which in turn is a subset of those experiencing symptoms. Data obtained from test results in tier 1, therefore, may be subject to bias. As tests improve in sensitivity and specificity, samples from more patients may be tested and found positive, potentially leading to better treatment and illness outcomes, as well as improvements in the quality of influenza surveillance.

The types of tests in the diagnostic landscape are changing. Because RIDTs are the least expensive influenza tests, do not require complex testing capabilities, and can be performed in most physicians' offices and outpatient clinics, they continue to comprise the greatest percentage of tier 1 influenza tests. However, the use of molecular (e.g., PCR) tests has increased. According to a survey of 931 clinical laboratories, the adoption of molecular test methods, some of which subtype influenza viruses, detect multiple respiratory pathogens, or both, more than doubled during the 2009 H1N1 pandemic (*18*). Increases in the use of PCR in tier 2 were also observed in FluSurv-NET, rising from <10% during 2003–2008 to ≈70% during 2009–2013 (*6*). Our data show that overall influenza testing increased 2.5-fold and PCR testing increased >3-fold during 2010–2015 (Figure 2). These increases may be the result of an increase in awareness of influenza following the 2009 pandemic and may be attributable to physician demand for more sensitive and specific influenza diagnostics.

One notable change in testing is evident in tiers 4 and 5, where NGS is now routine. In 2015, CDC began the Sequence First Initiative to introduce NGS for all specimens sent to CDC for virologic surveillance. The project continued in 2016, as NGS began to be implemented at CDC-supported NIRCs. As of August 2017, all specimens tested at CDC or NIRCs undergo NGS using bioinformatics and computational science, both at CDC and through cloud services, for rapid data sharing and analysis. NGS reveals the genetic variation among different virus particles in a single specimen and allows public health laboratorians to confirm the genetic identity of circulating viruses (*2*). These sequence data are also now a critical component of the twice-yearly WHO influenza vaccine virus selection process and are used in molecular modeling and forecasting. As the cost of NGS drops and the availability of more rapid sequencing platforms increases, NGS may begin to serve as a routine approach for influenza virologic surveillance in tier 3 laboratories as well (*19*).

Specimen collection and testing practices were found to vary across tier 3 state and local PHLs; however, new efforts have introduced a more standard approach for surveillance at the tier 4 and 5 levels. Since 2013, PHLs have been able to access right-size calculators to calculate an optimal number of specimens required for effective surveillance. These calculators, developed through a collaborative effort between CDC and APHL, use statistical tools to determine the amount of testing required for desired confidence levels of surveillance (*12*). These calculators allow state and local PHLs to evaluate their virologic surveillance systems and to improve the efficiency, representativeness, and timing of specimen submissions to CDC (*20*). Through rightsizing the submission of specimens, CDC now has a more systematic approach to identifying early drift in seasonal influenza viruses, detecting unsubtypable and potentially pandemic viruses, and selecting more representative and timely viruses for use in annual influenza vaccines.

Finally, we have provided an operating framework of specimen testing and surveillance that can support pandemic planning and response efforts. In 2017, the US Department of Health and Human Services released an update to the Pandemic Influenza Plan originally released in 2005, prompting the need for revised operational plans at the federal, state, and local levels (*4*). A critical component of those plans will require outlining how early detection and reporting of influenza viruses will be executed to respond rapidly to an emerging pandemic. The virologic surveillance landscape provided here delineates the various public health agency roles and responsibilities for virologic surveillance for seasonal, as well as pandemic, influenza. In addition, the landscape framework, along with the described rightsize calculators, provides estimates for specimens tested at each tier and ways to determine how many specimens are expected and needed during surge. CDC will also use the framework to estimate the needed number of PCR reagent kits it distributes from the International Reagent Resource (*21*) to the nearly 100 PHLs in the United States that participate in tier 3 virologic surveillance. Finally, resource, reimbursement, and logistics modelers can use the framework and estimates for developing or revising tools for use by planners and response agencies.

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Pediatric Complicated Pneumonia Caused by *Streptococcus pneumoniae* Serotype 3 in 13-Valent Pneumococcal Conjugate Vaccinees, Portugal, 2010–2015

Catarina Silva-Costa, Maria João Brito, Marcos D. Pinho, Ana Friães, Sandra I. Aguiar,[1] M. Ramirez, Jose Melo-Cristino, on behalf of the Portuguese Group for the Study of Streptococcal Infections[2] and the Portuguese Study Group of Invasive Pneumococcal Disease of the Pediatric Infectious Disease Society[2]

Despite use of 7-valent pneumococcal conjugate vaccine, incidence of pleural effusion and empyema (pediatric complicated pneumococcal pneumonia [PCPP]) is reportedly increasing globally. We cultured and performed PCR on 152 pleural fluid samples recovered from pediatric patients in Portugal during 2010–2015 to identify and serotype *Streptococcus pneumoniae*. We identified only 17 cases by culture, but molecular methods identified *S. pneumoniae* in 68% (92/135) of culture-negative samples. The most frequent serotypes were 3, 1, and 19A, together accounting for 62% (68/109) of cases. Nineteen cases attributable to 13-valent pneumococcal conjugate vaccine (PCV13) serotypes (mostly serotype 3) were detected among 22 children age-appropriately vaccinated with PCV13. The dominance of the additional serotypes included in PCV13 among PCPP cases in Portugal continues, even with PCV13 available on the private market (without reimbursement) since 2010 and with average annual coverage of 61% among age-eligible children. Our data suggest reduced effectiveness of PCV13 against serotype 3 PCPP.

Streptococcus pneumoniae (pneumococcus) is the leading cause of bacterial pneumonia in children and is the most common pathogen isolated in pleural effusions and empyemas (*1*–*3*). In this article, we will refer to pediatric pneumococcal pneumonias occurring with either parapneumonic effusion or empyema as pediatric complicated pneumococcal pneumonias (PCPPs). Several studies reported an increasing incidence of PCPP in the 1990s and early 2000s (*1*,*2*,*4*), a trend that was observed to persist or even accelerate after the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7), which covered serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F (*1*,*2*,*4*,*5*). Several factors could be responsible for an apparent increase in PCPP, including greater awareness and improved diagnostics (*1*). However, temporal trends or vaccine-induced changes in serotype prevalence and the particular propensity of certain serotypes to cause PCPP have also been implicated (*1*). Serotypes 1, 3, 7F, 14, and 19A are the dominant serotypes in PCPP (*1*), and some studies associated the increase in PCPP after the introduction of PCV7 with increasing incidence of PCPP by serotypes 1, 3, and 19A, which are not targeted by PCV7 (*1*,*2*,*6*).

Culture of pleural fluid or blood of PCPP case-patients is frequently negative; the yield of cultures is as low as 8% for pleural fluid and only slightly higher for blood (*7*,*8*). Although one of the reasons behind this low yield might be previous antimicrobial drug treatment (*9*,*10*), a recent study showed that serotype 3 is infrequently cultured from children's samples but is readily detected by PCR-based assays, even without prior exposure to antibiotics (*10*). Although PCR-based assays for the direct detection of pneumococci from clinical samples have limitations (*11*), they also offer several advantages, such as speed, high sensitivity, and being independent of bacterial viability (*9*,*10*). Given these advantages, PCR-based techniques are increasingly being used as important tools in the diagnosis of pneumococcal invasive infections and the epidemiologic surveillance of the characteristics of unculturable pneumococci (*10*).

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PCV7 was introduced in Portugal in 2001. PCV10, which added serotypes 1, 5, and 7F to the PCV7 serotypes, became available in mid-2009. PCV13, which added serotypes 3, 6A, and 19A to the PCV10 serotypes , became available in early 2010. All PCVs were offered on the private market without any reimbursement. Even without reimbursement, 75% of children born in 2008 were vaccinated with PCV7, although soon afterward a decline in vaccination coverage occurred; coverage reached 63% in 2012 (*12*) and remained stable at 61% until 2014 (unpublished data from IQVIA, Pfizer, and Instituto Nacional de Estatística, assuming a 4-dose schedule). In July 2015, PCV13 was introduced in the national immunization plan (NIP) for children born after January 2015. We conducted a prospective study to determine the serotypes causing PCPP in children in Portugal during 2010–2015, a period when PCV13 was being administered outside of the NIP.

Materials and Methods

Patient Samples

We asked 61 hospital laboratories and pediatric departments located throughout Portugal to report all cases of possible PCPP (in patients <18 years of age) for which pleural fluid was available for analysis and to submit these samples for characterization. We included in our study only samples recovered during January 2010–December 2015, but we performed no audit that would ensure reporting compliance. Because our network includes all secondary and tertiary care hospitals in which PCPP is likely to be treated and a pleural fluid sample obtained, we assume our catchment population is the entire population of Portugal <18 years of age. During 2010–2015, this population steadily decreased, from 1,929,331 in 2010 to 1,802,196 in 2015 (average 1,865,288) (https://www.ine.pt).

Whenever possible, the vaccination status of the patient was also reported. We used guidelines from the Vaccines Committee of the Pediatric Infectious Diseases Society and Portuguese Pediatric Society from 2014 (*13*) and 2009 (*14*) to define age-appropriately vaccinated children with PCV7 and PCV13 (Table 1). The study was approved by the Institutional Review Board of the Centro Académico de Medicina de Lisboa.

Culture-Positive Samples

We identified all bacteria as *S. pneumoniae* by colony morphology, hemolysis on blood agar plates, optochin susceptibility, and bile solubility. We performed serotyping with the standard capsular reaction test by using the chessboard system and specific serum samples (Statens Serum Institute, Copenhagen, Denmark). For this study, we classified serotypes as vaccine serotypes (i.e., those in PCV7; the additional 3 serotypes included in PCV10 [addPCV10]; the additional 3 serotypes included in PCV13 [addPCV13]) and nonvaccine serotypes.

Culture-Negative Samples

When identification of disease etiology by conventional microbiologic methods failed, we sent pleural fluid to the central laboratory, where total DNA was extracted from 200 μL of the patient sample by using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) or DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturers' instructions. We used a conventional PCR amplification of 2 human genes (encoding human β-actin and RNaseP) as control for the quality of the purified DNA. We initially tested the presence of *S. pneumoniae* by using conventional PCR to target the *lytA* and *wzg* genes (2010–2011) and later (2012 and later) by using real-time PCR (rPCR). We serotyped positive samples by conventional PCR, rPCR, or a combination of both. We confirmed negative results by conventional PCR by using rPCR on samples stored at –80°C.

Conventional PCR

We used a multiplex PCR for the amplification of the genes encoding human β-actin and RNaseP (*15*,*16*) and *S. pneumoniae* genes (*lytA* and *wzg*) (*17*,*18*). We serotyped PCR-positive samples by using multiplex PCR, with primers targeting serotypes 1, 3, 4, 5, 6A, 6B, 6C, 7F,

Table 1. PCV immunization schemes according to guidelines from the Vaccines Committee of the Pediatric Infectious Diseases Society and Portuguese Pediatric Society*

Vaccine	Start of vaccination scheme	Primary immunization course	Booster vaccination
PCV7	2–6 mo	3 doses, at 2, 4, and 6 mo	12–15 mo
	7–11 mo	2 doses	Booster dose at 12 mo†
	12–23 mo	2 doses‡	NA
	24–59 mo	Risk group only: 2 doses	NA
PCV13	2–6 mo	2 or 3 doses§	11–15 mo¶
	7–11 mo	2 doses§	11–15 mo¶
	12–23 mo	2 doses‡	NA
	≥24 mo	1 dose	NA

*NA, not applicable; PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV; PCV13, 13-valent PCV.
†8 weeks after the second dose.
‡≥8 weeks apart.
§Minimum interval between doses 4 weeks.
¶Minimum interval from last dose 8 weeks.

8, 9V, 14, 15A, 15B/C, 16, 18C, 19A, and 23F (*17*,*19*). We performed PCR reactions in a final volume of 25 μL containing 4 μL of DNA, 1X polymerase buffer (Biotools, Madrid, Spain), 3U of polymerase GoTaq (Biotools, Madrid, Spain), 10 mmol/L dNTPs, and 10 pmol of each primer (with the exception of human β-actin primers, for which 7 pmol was used). The PCR program consisted of 4 min denaturation at 95°C, 30 cycles of 95°C for 40 s, 58°C for 40 s, and 65°C for 2 min, followed by a final extension at 65°C for 10 min.

rPCR

We performed rPCR on the Rotor-Gene 6000 (Corbett Research, Cambridge, United Kingdom) by using the Platinum quantitative PCR SuperMix-UDG (Thermo Fisher Scientific, Waltham, Massachusetts, USA). PCR reactions contained 5 μL of DNA, 5 pmol/L of each primer (targeting *lytA* and *wzg* genes) (*18*,*20*), 2.5 pmol/L of each probe, 12.5 μL of PCR SuperMix-UDG, 1.5 μL of $MgCl_2$ (50 nmol/L), and water, for a final volume of 25 μL. Cycling conditions were as follows: 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. We performed detection of *lytA* and *wzg* in singleplex PCR. We defined negative results as those with cycle threshold (C_t) >40 and positive results as those with $C_t \leq 35$ (*21*). If a C_t value >35 and ≤40 was obtained, we considered the result inconclusive and varied the amount of DNA in the reaction by using twice as much DNA and diluting over a 50-fold range. If still no reaction yielded a $C_t \leq 35$, we considered the sample negative.

For serotyping by rPCR (*22*), we performed 7 multiplex reactions targeting 3 serotypes or serogroups each: 3, 7F/7A, and 19A; 1, 15B/15C, and 23F; 14, 18C, and 19F; 4, 6, and 9V/9A; 5, 11A/11D, and 16F; 8, 12F/12A/12B, and 22F/22A; and 15A, 23A, and 33F/33A/37. We also used rPCR to serotype positive samples that tested negative for all serotypes by conventional PCR. The rPCR scheme for serotyping did not enable discrimination between some serotypes within a few serogroups, as indicated in the description of each multiplex reaction.

Statistical Methods

We used the Fisher exact test to evaluate differences in the prevalence of the most frequent addPCV10 and addPCV13 serotypes, as well as the number of PCPPs among vaccinated and nonvaccinated children. We consider a p value <0.05 statistically significant. We report patient age as median years and interquartile range (IQR).

Results

Patient Samples

During January 2010–December 2015, we analyzed 152 pleural fluid samples; 17 cases were identified by culture to have pneumococcal etiology by the participating laboratories. We submitted 135 culture-negative samples to the central laboratory for molecular testing. In 43 samples, we did not detect *S. pneumoniae* in the pleural fluid by molecular methods, but we confirmed the remaining 92 cases (68%) to be PCPP by molecular methods. The total numbers of requests for molecular testing and samples positive for *S. pneumoniae* were approximately constant over the years (Table 2).

PCPP Case-Patients

Among the 109 PCPP case-patients (17 identified by culture and 92 by molecular methods), 56 were male and 52 female; the sex of 1 patient was not available. Patient age ranged from 4 months to 17 years (median age 4 [IQR 2.3–6] years); 27 cases were in children ≤2 years of age. A total of 34 (31%) PCPP cases occurred in nonvaccinated children (median age 2.6 [IQR 1.4–8.5] years), and the vaccination status was unknown for 28 (26%) patients. The remaining 47 (43%) PCPP cases occurred in children who received ≥1 vaccine dose (median age 3.2 [IQR 3–5] years). All vaccinated children received PCV7, PCV13, or both; PCV10 had not been administered to any child in the study.

Serotypes of PCPP Case-Patients

Except for 18 (16%) samples, we were able identify the serotypes of the pneumococci responsible for illness (Table 3). Overall, the most frequent serotype was serotype 3, responsible for 36% of the PCPP cases (n = 40; median patient age 3 [IQR 2–5] years), followed by serotype 1 (n = 21 [19% of cases]; median patient age 5.5 [IQR 4–13.5] years). Other serotypes found were 19A (n = 7); 7F/7A (n = 4); 14 (n = 3); 5, 16F, and 7F (n = 2 each); and 6B, 6, 8, 9V, 9V/A, 10A, 15A, 19F, 23F, and 33F/33A/37 (n = 1 each); median patient age for all of these cases was 3 (IQR 1–6) years. Although serotype 1 cases occurred more frequently among older children than serotype 3 cases (p = 0.005 by

Table 2. Number of requests for laboratory testing and confirmed positive cases of *Streptococcus pneumoniae* infection in children, Portugal, 2010–2015*

	Year						
Cases and requests	2010	2011	2012	2013	2014	2015	Total
Positive for *S. pneumoniae*	29	18	19	18	10	15	109
Negative for *S. pneumoniae*	2	3	8	10	6	14	43
Requests	31	21	27	28	16	29	152

*Among the cases are 17 for which pneumococci were cultured from pleural fluid (2010, n = 5; 2011, n = 3; 2012, n = 2; 2013, n = 3; 2014, n = 0; and 2015, n = 4).

Table 3. Serotype distribution among the 152 pediatric case-patients with *Streptococcus pneumoniae* infection included in this study, by PCV vaccination status, Portugal, 2010–2015*

	Vaccination status						
Serotype	AP_PCV13	AP_PCV7	NA_PCV13	NA_PCV7	Not vaccinated	Unknown	Total
14	1				2		3
19F						1	1
23F						1	1
PCV7	1		1		2	2	6
1	1	3		2	7	8	21
5		1				1	2
PCV10	2	4	1	4	9	11	31
3	17	6		1	11	5	40
19A		1			5	1	7
PCV13	19	11	1	5	26	17	79
16F						2	2
8				1			1
15A					1		1
10A			1				1
7F/7A		1		3†		2	6
6					2‡		2
9V/9A			1§		1		2
33F/33A/37			1				1
Nonvaccine type		1	2	2	3	4	12
Not identified	3	1	1	1	5	7	18
Total	22	13	4	8	34	28	109

*AP_PCV13, age-appropriately vaccinated with PCV13; AP_PCV7, age-appropriately vaccinated with PCV7; NA_PCV13, not age-appropriately vaccinated with PCV13; NA_PCV7, not age-appropriately vaccinated with PCV7; PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV; PCV13, 13-valent PCV.
†Serotype 7F (n = 2).
‡Serotype 6B (n = 1).
§Serotype 9V (n = 1).

Fisher exact test), the age distribution of the case-patients infected with each of these serotypes was not significantly different from that of PCPP case-patients infected with all other serotypes ($p \geq 0.198$ by Fisher exact test). Whenever possible, conventional PCR serotyping enabled the identification of serotypes 7F, 6B, and 9V, whereas rPCR detected only the groups 9V/9A, 7F/7A, and 6 without further discrimination. On the 17 culture-positive samples, only 5 serotypes were found: 1 and 3 (n = 7 each) and 14, 10A, and 19A (n = 1 each). Apart from serotype 10A, which was detected exclusively in 1 isolate, all other serotypes were also detected by PCR from culture-negative samples. Most of the PCPP case-patients for whom a serotype was unambiguously identified were infected with the addPCV13 serotypes (n = 47 [43%]), followed by the addPCV10 serotypes (n = 25 [23%]), whereas PCV7 serotypes were responsible for a small fraction of PCPP cases (n = 7 [6%]). Among the remaining cases, 6 were caused by nonvaccine serotypes, and in another 6 cases, we were unable to classify capsular types as vaccine or nonvaccine type. These cases included samples for which rPCR tested positive for serogroup 6 (n = 1), 7F/7A (n = 4), or 9V/9A (n = 1).

Vaccination Status of PCPP Case-Patients

Among the 47 vaccinated children, 35 were age-appropriately vaccinated (Table 3). Of these 35 children (median age 4 [IQR 5.5–6] years), 13 had received 4 doses of PCV13 and 13 (median age 5 [IQR 3–5] years) had received 4 doses of PCV7; the remaining children had received fewer doses (n = 6) or a combination of both PCVs (n = 3). Among the 22 children age-appropriately vaccinated with PCV13, 19 had infections caused by serotypes included in PCV13, representing vaccine failures. These cases occurred throughout the study period (1 case in 2010, 2 in 2012, 9 in 2013, and 7 in 2015). Among this group were 12 children (median age 3 [IQR 3–3.5] years) who had completed a 4-dose scheme of PCV13; 11 had serotype 3 PCPP and 1 serotype 1 PCPP. We also identified serotype 3 in 4 of 6 PCPPs in children who were age-appropriately vaccinated and had received 3 doses of PVC13 (1 case in a 1.5-year-old child, 2 in 2-year-old children, and 1 in a 3-year-old child). Among children who received a combination of both conjugate vaccines, a series of 3 or 4 doses of PCV7 followed by 1 dose of PCV13 serotype 3 was detected in 2 case-patients (both 6-year-olds) and serotype 14 in 1 case-patient (a 3.5-year-old). Among the 13 children age-appropriately vaccinated with PCV7 (median age 5 [IQR 3–5] years), all had received 4 PCV7 doses, and we detected no cases caused by PCV7 serotypes. Most cases were caused by addPCV13 serotypes; serotype 3 was most prevalent (n = 6), followed by serotype 1 (n = 3), and serotypes 5 and 19A (n = 1 each).

We compared the number of addPCV10 and addPCV13 PCPP cases (i.e., those caused by serotypes 1, 3, 19A, and 7F) among children age-appropriately vaccinated with PCV13 and among the other case-patients for

whom vaccination status was known and serotype could be unambiguously determined. Serotype 3 was overrepresented among PCPP cases in children age-appropriately vaccinated with PCV13 ($p<0.001$ by Fisher exact test). We detected a similar number of positive samples for *S. pneumoniae* among children age-appropriately vaccinated with PCV13 (n = 22/27) and among the other case-patients for whom vaccination status was known (n = 59/71) (p = 1 by Fisher exact test).

Discussion

The detection of *S. pneumoniae* in pleural fluid samples was greatly improved by the use of molecular methods, as previously reported (*3*); only 17 culture-positive cases of 109 were confirmed PCPPs (16%). Serotype 3 and the other major serotypes found (1, 19A, 7F/7A, and 14) have already been associated with complicated pneumonias and pneumococcal empyemas worldwide (*3*,*9*,*23*–*26*). Serotypes 1, 19A, and 14 were important causes of pediatric invasive pneumococcal disease (IPD) in Portugal during 2008–2012 (*12*) and were also among the most prevalent in PCPPs; however, serotype 3, which accounted for a small fraction of pediatric IPD cases during 2008–2012 (n = 9 [2%]) was the most frequent serotype in PCPPs. Although several factors might explain this difference, serotype 3 isolates might be more prone to specifically invade the pleural space and cause complicated pneumonia, with or without empyema. Supporting this hypothesis, a previous study found that pneumonia caused by serotype 3 was associated with an increased risk for necrotizing pneumonia, associated parapneumonic empyema, and increased severity of illness (*25*).

In the group of children vaccinated with PCV13, the most frequent serotype was 3, despite the potential protection conferred by vaccination. In fact, serotype 3 cases were more prevalent among children age-appropriately vaccinated with PCV13 than among the other case-patients for which vaccination status was known. One possible explanation for this could be a more limited protection of PCV13 against serotype 3 PCPP and a more effective protection against other serotypes, namely the other most prevalent serotypes (1, 7F and 19A), which would increase the likelihood that any PCPP cases in this group would be caused by serotype 3.

The effectiveness of PCV13 against serotype 3 has been questioned in several studies. In a large surveillance study performed in the United States, no reduction in IPD caused by serotype 3 was observed despite reductions in IPD incidence and evident decreases in IPD caused by PCV13 serotypes 19A and 7F (*27*). A postlicensure indirect cohort study in England, Wales, and Northern Ireland to assess vaccine effectiveness against IPD indicated that, for serotype 3, the calculated correlate of protection was 2.83 μg/mL, which is much higher than the 0.35 μg/mL aggregate correlate of protection used during licensing, suggesting a potential explanation for the reduced effectiveness of PCV13 against this serotype (*28*). The cases of serotype 3 PCPP in our study among children age-appropriately vaccinated with PCV13 occurred mostly in children ≥3 years of age (n = 12/17), but the distribution was similar to that of serotype 3 case-patients not vaccinated with PCV13 (p = 0.353 by Fisher exact test), so it does not seem likely that this was attributable to faster waning of the immune response to this serotype. In fact, cases of serotype 3 PCPP occurred in younger children than did cases caused by serotype 1, a serotype also included in PCV13 and for which only 1 vaccine failure was detected. Because the synthesis of the serotype 3 capsular polysaccharide proceeds through a synthase mechanism, the polysaccharide is not covalently linked to the peptidoglycan and can be released during growth, thereby potentially reducing opsonophagocytosis (*29*,*30*). Free capsular polysaccharide in pleural effusions in vitro can also neutralize type-specific anticapsular antibody, further reducing the efficacy of antibody-mediated clearance (*26*), and the considerable amounts of capsule produced could further enhance these effects in serotype 3 strains. Taken together, these data suggest that PCV13 might offer more limited individual protection against serotype 3, particularly in the context of complicated pneumonia.

The proportion of children asymptomatically colonized with serotype 3 increased in the period preceding PCV13 introduction in Portugal (*31*), but no data are available for more recent years. However, serotype 3 is currently the most important serotype among pneumococcal infections in adults (*32*,*33*), suggesting substantial circulation of these strains. Even so, if PCV13 in the NIP reduces colonization by serotype 3 isolates, its overall effectiveness could be much higher than the individual protection afforded because a reduction of the circulation of serotype 3 would also mean less opportunities to cause infection. Such an effect could be behind the decrease in PCPP observed with the introduction of PCV13 in several countries (*2*,*5*).

In agreement with our observations, other reports document serotype 3 vaccine failures. A study in Greece found 5 cases of complicated pneumonia caused by serotype 3 pneumococci among vaccinated children, although most vaccine failures occurred in children who received a single dose of PCV13, which could offer only limited protection (*34*). A more recent report, also from Greece, found 4 cases of empyema caused by serotype 3 pneumococci among children vaccinated with either a 3-plus-1 schedule (n = 3) or a booster dose at the age of 21 months (n = 1) (*35*), which can be considered vaccine failures. A study from Catalonia, Spain, also found 9 vaccine

failures among 86 cases of IPD, mostly caused by serotype 3 (n = 6), including 2 cases in children who had received 4 vaccine doses (*36*).

Although our study was prospective and involved both pediatric and microbiology departments, it was not designed to estimate the incidence of PCPP because it did not identify cases in which there were clinical or radiographic criteria for complicated pneumonia and for which pneumococci were identified in either blood or respiratory samples. Although this certainly resulted in an underestimation of PCPP, the use of conventional PCR and rPCR techniques greatly enhanced the ascertainment of PCPP cases by improving the detection of *S. pneumoniae* in pleural fluid samples and emphasizes the potential role of molecular techniques when evaluating disease incidence. Another limitation of our study is lack of detailed information about the immune status or other underlying conditions in age-appropriately vaccinated children with PCPP by vaccine serotype. However, given the high prevalence of serotype 3 in this group, it is unlikely that all cases could be explained by host characteristics, indicating that specific properties of serotype 3 must be responsible for this behavior.

In summary, we describe data that are compatible with a lower individual effectiveness of PCV13 against PCPP, a presentation of IPD for which PCV13 is specifically recommended. The public health consequences of such possible lower protection might be mitigated by a reduction in circulating serotype 3 by vaccination with very high coverage, such as those achieved through inclusion in NIPs. Carriage studies and continued surveillance are necessary to determine the group effect of the introduction of PCV13 in the NIP to clarify the effectiveness of PCV13 in the prevention of infections caused by serotype 3.

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Virus RNA Load in Patients with Tick-Borne Encephalitis, Slovenia

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We determined levels of tick-borne encephalitis (TBE) virus (TBEV) RNA in serum samples obtained from 80 patients during the initial phase of TBE in Slovenia. For most samples, levels were within the range of 3–6 $\log_{10}$ copies RNA/mL. Levels were higher in female patients than in male patients, but we found no association between virus load and several laboratory and clinical parameters, including severity of TBE. However, a weak humoral immune response was associated with a more severe disease course, suggesting that inefficient clearance of virus results in a more serious illness. To determine whether a certain genetic lineage of TBEV had a higher virulence potential, we obtained 56 partial envelope protein gene sequences by directly sequencing reverse transcription PCR products from clinical samples of patients. This method provided a large set of patient-derived TBEV sequences. We observed no association between phylogenetic clades and virus load or disease severity.

Tick-borne encephalitis (TBE) is one of the major virus infections of the human central nervous system (CNS) in Europe and Asia. This disease is caused by TBE virus (TBEV) (family *Flaviviridae*, genus *Flavivirus*). Three main subtypes of this virus have been recognized (European, Siberian, and Far Eastern), and their geographic distribution closely resembles the distribution of their tick vectors, namely *Ixodes ricinus* ticks for the European subtype and *I. persulcatus* ticks for the Siberian and Far Eastern subtypes (*1*,*2*).

Approximately 10,000–15,000 TBE cases are reported annually; 3,000 of them are in Europe. However, because reporting of TBE is not established in all disease-endemic countries, the real numbers are most likely higher (*2*,*3*). Humans acquire TBEV infection mainly through tick bites and only rarely (≈1%) by consuming unpasteurized milk or milk products from infected livestock, particularly goats (*4*–*6*). Thus, most TBE cases occur in the warm months of the year (April–November), which corresponds with the main period of tick activity (*7*,*8*).

Most (70%–98%) TBEV infections are believed to be asymptomatic (*9*,*10*). In ≈75% of patients with TBE caused by the European subtype, the disease has a typical biphasic course. The first phase, which follows an incubation period with a median of 8 days (range 2–28 days) after a tick bite, and which correlates with viremia, is characterized by nonspecific symptoms, such as fever, fatigue, general malaise, headache, and body pain, which are often associated with leukopenia or thrombocytopenia. The initial phase lasts for 2–7 days and is followed by an improvement or even an asymptomatic interval of ≈1 week (range 1–21 days). The second phase manifests as meningitis (≈50% of adult patients), meningoencephalitis (≈40%), or meningoencephalomyelitis (≈10%) (*11*–*13*). The severity of TBE increases with the age of patients (*14*,*15*). Unfavorable outcomes, including long-term sequelae, are more often seen in patients with severe acute illness (*16*,*17*) and other clinical and laboratory findings (*12*,*16*,*18*). However, the exact mechanisms leading to more severe disease and unfavorable outcome in an individual patient are not known.

After a tick bite, TBEV replication occurs locally in dendritic skin cells. From there, the virus reaches other organs, especially the spleen, liver, and bone marrow. It is believed that production of high levels of virus in the affected organs, resulting in viremia, is a prerequisite for the virus to cross the blood–brain barrier because the capillary endothelium is not easily infected. However, the exact mechanism by which TBEV accesses the brain is not known (*13*,*19*,*20*). Furthermore, some authors reported a correlation between a low concentration of neutralizing antibodies and more severe disease, suggesting that a delayed formation of neutralizing antibodies could be associated with high viremia (*16*,*21*). However, no information is available on the level of viremia in patients with TBE and its effect on disease severity. The main purpose of this study was to determine levels of TBEV RNA in clinical samples of patients with TBE and correlate these levels with several laboratory and clinical parameters, including severity of the disease.

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Patients and Methods

Patients and Samples

Patients eligible for study were those given a diagnosis of TBE at the Department of Infectious Diseases, University Medical Center Ljubljana (Ljubljana, Slovenia), during 2003–2013 who were seen at the initial and second (meningoencephalitic) phases of TBE and in whom TBEV was identified by PCR in serum specimens obtained during the initial phase of the disease. Initial-phase serum samples were obtained either during a prospective study on the etiology of febrile illness after a tick bite or represented remnants of the samples collected as a part of routine diagnostic testing of a patient with a febrile illness in whom TBE later developed. In addition, available cerebrospinal fluid (CSF) samples obtained during the meningoencephalitic phase of the illness from the same patients were also included in the study. The specimens were stored at –80°C until further processing.

Definitions

The initial phase of TBE was classified as a febrile illness that, after a clinical improvement lasting ≤21 days, was followed by neurologic involvement. TBE was defined as clinical signs or symptoms of meningitis or meningoencephalitis, increased CSF leukocyte counts (>5 × 10^6 cells/L), and serum TBEV IgM and IgG or TBEV IgG seroconversion in paired serum samples. TBE was categorized as mild (only signs or symptoms of meningeal involvement), moderate (monofocal neurologic signs or mild-to-moderate signs/symptoms of CNS dysfunction), or severe (multifocal neurologic signs or signs/symptoms of severe dysfunction of the CNS) (*11*).

In addition to this simple clinical classification, a quantitative evaluation of the severity of the disease was performed by using a standardized questionnaire as reported (*22*). Points (1–9) were assigned for the presence, intensity, and duration of headache, fever, vomiting, and meningeal signs; the presence of tremor, pareses, urine retention, and cognitive function disturbances; the presence and intensity of conscious disturbances; and the need for and duration of treatment for increased intracranial pressure. The absence of a particular symptom/sign scored 0. A score <9 corresponded to clinically mild disease, 9–22 to moderate disease, and >22 to severe disease (*22*).

Ethics Considerations

The study was conducted according to the principles of the Declaration of Helsinki, the Oviedo Convention on Human Rights and Biomedicine, and the Slovene Code of Medical Deontology. The study was approved by the National Medical Ethics Committee of Slovenia (no. 152/06/13, no. 178/02/13, and no. 37/12/13). Patients whose specimens were obtained in the study on the etiology of febrile illness after a tick bite signed an informed consent form that included the use of collected specimens for further studies. The Ethics Committee waived the need for written informed consent for patients for whom remnants of routinely collected serum specimens were used.

TBEV Antibody Levels

We determined the presence and concentration of TBEV antibodies in serum samples by using the Enzygnost Anti-TBE/FSME Virus (IgM, IgG) test (Siemens AG, Munich, Germany) according to the manufacturer's instructions. Specificities of the test were 99.5% for IgG and 99.9% for IgM, and sensitivities were 96.8% for IgG and 98.8% for IgM.

TBEV RNA Load

We extracted total RNA from serum and CSF samples by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. For a quantitative reverse transcription PCR (RT-PCR), we used the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Carlsbad, CA, USA). This RT-PCR was performed as reported (*23*). For analysis purposes, we converted virus loads to $\log_{10}$ values.

Sequencing and Phylogenetic Analysis

We obtained sequences by direct sequencing of RT-PCR products from serum samples of patients with TBE. A partial TBEV envelope (E) protein gene was amplified and sequenced by using primer pair TBE ENV 3F (5′-TGA GGG GAA GCC TTC AAT-3′) and TBE ENV 3R (5′-TCA TGT TCA GGC CCA ACC A-3′), and sequence analysis was performed as reported (*24*).

Statistical Analysis

Numerical data were summarized as means and SDs or medians and interquartile ranges (IQRs) and categorical variables as frequencies and percentages. We calculated 95% CIs for means or percentages of some variables. We assessed the association between variables and TBEV RNA by using univariate linear regression; $\log_{10}$-transformed TBEV RNA counts were used as the outcome variable. We used a similar approach to assess the association between phylogenetic clades and severity of disease.

We displayed observed associations with outcome variables graphically by using box and whisker plots for categorical variables and scatter plots for numerical variables. We added a loess regression (locally weighted scatterplot smoothing) line (*25*) with 95% CIs fitted by using the geom_smooth function in the ggplot2 R software (*26*). We conducted analyses by using R statistical language (*27*).

Results

Patients and Samples

We obtained basic demographic data, clinical characteristics, and laboratory findings for 80 patients with established TBEV RNA in serum during the initial phase of TBE (Table). Second-phase CSF samples were available for 48 of these 80 patients.

TBEV RNA Load

TBEV RNA was detected in serum of 80 patients with febrile illness in whom neurologic involvement later developed and who fulfilled criteria for TBE. CSF samples obtained at the meningoencephalitic phase of illness from 48 of these 80 patients all showed negative results for TBEV RNA. On the day of virus load detection, only 3 patients were positive for TBE IgM in the serum, and all other patients were negative for TBEV IgM and IgG.

The mean (SD) of the logarithmic transformation of TBEV RNA levels in serum was 4.65 (1.13) $\log_{10}$ copies RNA/mL. For most (95%) patients, this level showed a range of 3–6 $\log_{10}$ copies RNA/mL (Figure 1, panel A). RNA-positive serum results were obtained as early as the first day and as late as the tenth day (median fifth day) of the initial phase of TBE. For 1 patient, we detected virus RNA in the serum sample on day 14 of disease, which corresponded clinically to the seventh day of the asymptomatic interval. We did not find any substantial differences in numbers of RNA copies within this time frame (Figure 1, panel B).

TBEV RNA levels were higher in female patients than in male patients (mean [SD] 4.86 [1.25] vs. 4.4 [0.93] $\log_{10}$ copies RNA/mL; p = 0.064). The log-transformed number of detected RNA copies did not appear to be associated with ages of patients, leukocyte and platelet counts determined on the same day as RNA load (Figure 2, panels A–D), duration of the initial phase of TBE, duration of the asymptomatic interval between the initial phase and second phase of TBE, CSF cell count determined in the meningoencephalitic phase of illness, severity of TBE according to quantitative assessment, and simple clinical classification (mean [SD] virus load values in patients with clinically mild, moderate, and severe disease were 4.54 [0.75], 4.68 [1.27], and 4.74 [1.71] $\log_{10}$ copies RNA/mL, respectively; p = 0.856) (Figure 3, panels A–D). Associations between these variables and log-transformed TBEV RNA in the serum were not statistically significant, and they did not appear to have any potential clinical role.

We observed no differences in distribution of detected virus RNA levels when compared with concentrations of specific TBE IgG in initial follow-up serum samples of

Table. Characteristics of 80 patients with TBEV RNA in serum obtained during initial phase of TBE, Slovenia*

Characteristic	Value	95% CI†
Sex		
F	43 (53.8)	42.2–64.9
M	37 (46.2)	35.0–57.8
Median age, y (IQR)	48.5 (31–60.8)	42.0–50.5
F	52 (30–63)	41.1–53.2
M	47 (33–59)	39.1– 51.4
History of tick bite	68 (85.0)	75.3–92.0
Initial (first) phase of TBE		
Leukopenia‡	71 (88.8)	79.7–94.7
Thrombocytopenia§	52 (65.0)	53.5–75.3
Duration of first phase (days), median (IQR)	6 (5–8)	5.9–6.7
Asymptomatic interval		
Duration, d, median (IQR)	10 (7–13)	9.5–11.7
Second (meningoencephalitic) phase of TBE		
CSF findings		
Cell count × 10^6/L, median (IQR)	57 (25–104.8)	64.6–122.6
Protein concentration, mg/L, median (IQR)	0.54 (0.40–0.74)	0.54–0.68
Serum antibodies to TBEV¶		
IgM	80 (100.0)	95.5–100.0
IgG	71 (88.7)	79.7–94.7
Severity of acute illness		
Quantitative assessment		
Mild	28 (35.0)	24.7–46.5
Moderate	45 (56.3)	44.7–67.3
Severe	7 (8.7)	3.6–17.2
Clinical classification		
Mild	29 (36.2)	25.8–47.8
Moderate	45 (56.3)	44.7–67.3
Severe	6 (7.5)	2.8–15.6

*Values are no. (%) patients unless otherwise noted. CSF, cerebrospinal fluid; IQR, interquartile range; TBE, tick-borne encephalitis; TBEV, TBE virus.
†For the population percentage or the population mean.
‡Blood leukocyte count <4 × 10^9/L.
§Blood platelet count <140 × 10^9/L.

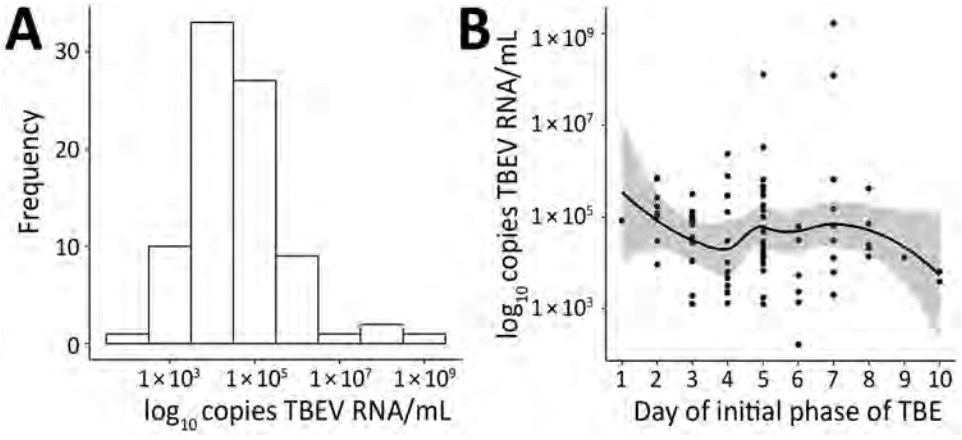

Figure 1. Distribution of virus RNA load in patients with TBE, Slovenia (A), and by day of initial phase of TBE (B). Solid line indicates a loess regression line, and shaded area indicates 95% CIs. TBE, tick-borne encephalitis; TBEV, TBE virus.

patients obtained during the second phase of the disease (Figure 4, panel A). However, we observed a significant association between TBEV antibody titers and disease severity ($p = 0.005$); on average, lower IgG levels were observed for patients with more severe illness (Figure 4, panel B).

Phylogenetic Analysis

We obtained 56 partial E protein gene sequences by direct sequencing of RT-PCR products from serum samples of patients with TBE and used a 1,272-bp segment of the E protein gene for phylogenetic analysis. Analysis showed that in Slovenia, sequences grouped into 6 clades (S1–S6). Nucleotide sequence identity was 95.8%–100% (divergence 0%–4.2%) and amino acid identity was 98.1%–100% (divergence 0%–1.9%). To assess a potential association of sequence divergence with geographic locations, we plotted permanent residencies of patients on a map of Slovenia according to their respective phylogenetic clustering on the basis of E protein gene sequence analysis. Results showed a high level of regional clustering (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/7/18-0059-Techapp1.pdf).

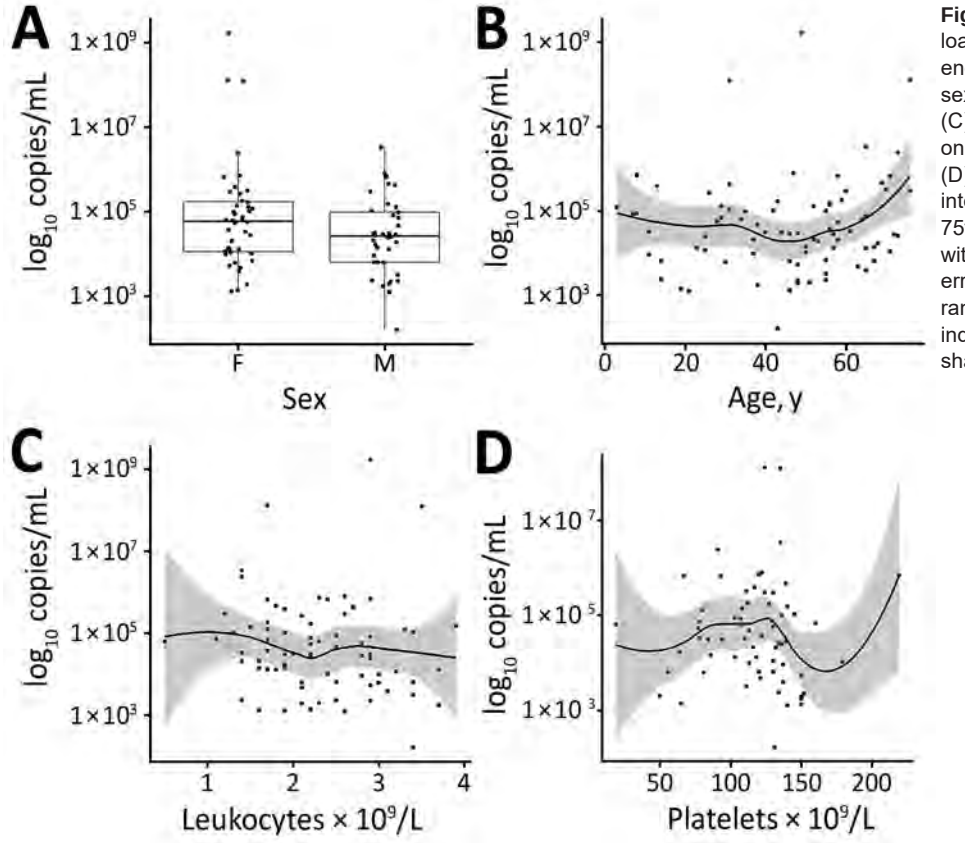

Figure 2. Distribution of virus RNA load in patients with tick-borne encephalitis, Slovenia, by patient sex (A), age (B), leukocyte count (C), and platelet count determined on the same day as RNA load (D). Boxes in panel A indicate interquartile ranges and 25th and 75th percentiles, horizontal lines within boxes indicate medians, and errors bars indicate 1.5× interquartile ranges. Solid lines in panels B–D indicate loess regression lines, and shaded areas indicate 95% CIs.

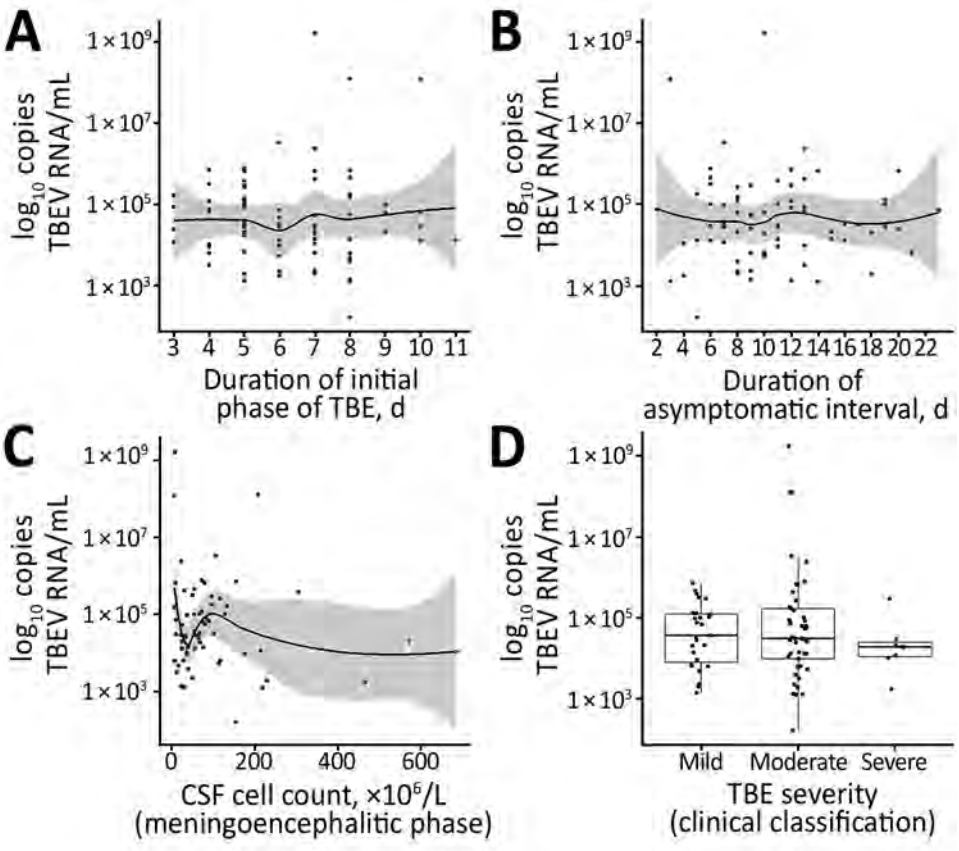

Figure 3. Distribution of virus RNA load in patients with TBE, Slovenia, by duration of initial phase of TBE (A), duration of asymptomatic interval (B), CSF cell count determined in the meningoencephalitic phase (C), and severity of TBE according to clinical classification (D). Solid lines in panels A–C indicate loess regression lines, and shaded areas indicate 95% CIs. Boxes in panel D indicate interquartile ranges and 25th and 75th percentiles, horizontal lines within boxes indicate medians, and error bars indicate 1.5× interquartile ranges. CSF, cerebrospinal fluid; TBE, tick-borne encephalitis; TBEV, TBE virus.

To determine whether a certain genetic lineage of TBEV had a higher virulence potential, we compared phylogenetic clades with levels of TBEV RNA and disease severity. Because there were too many groups to perform a meaningful statistical analysis, we combined some clades on the basis of their sequence identities (Figure 5). Although levels of TBEV RNA were somewhat lower in patients infected with viruses belonging to S4 and S5 phylogenetic clades, differences were not significant ($p = 0.116$). Also, we observed no association between phylogenetic clades and disease severity (Figure 5).

Discussion

Although TBE is one of the major and serious neuroinfections in Europe and Asia, some crucial steps in the development of the disease remain poorly understood. It has been postulated that high virus replication in the primarily affected organs, which maintains viremia in the first phase of the disease, is a prerequisite for the virus to cross the blood–brain barrier because viruses with a low capacity to generate viremia in peripheral tissue can be classified as having low neuroinvasiveness, regardless of their intrinsic neurovirulence potential (*13*,*19*,*20*). However, levels of viremia have so far been reported only in a few (individual) TBEV-infected patients (*23*,*28*).

In our study, we determined TBEV RNA loads in clinical samples and their association with laboratory and clinical parameters in a large group of patients with TBE. TBEV RNA levels were measured and detected in 80 first-phase serum samples obtained from 80 patients in whom neurologic involvement later developed and who were hospitalized for TBE during 2003–2013. TBEV RNA was detected in the time frame of 1–14 days from the beginning of the initial phase of illness. At the time of TBE RNA measurement, 79 patients were febrile (i.e., they were in the initial phase of TBE clinically). For 1 patient, TBEV RNA was detected 7 days after defervescence and 8 days before recurrence of fever; thus, this patient was clinically interpreted to be in the asymptomatic interval. For 95% of patients, levels of TBEV RNA were 3–6 $\log_{10}$ copies/mL and showed a mean (SD) value of 4.65 (1.13) $\log_{10}$ copies RNA/mL. In comparison, levels of viremia in blood or plasma samples of persons infected with West Nile virus (WNV) were somewhat lower (mean values 3–4 $\log_{10}$ copies RNA/mL), but higher virus loads have been reported in urine samples (*29*–*32*). However, studies on WNV virus load included patients with symptomatic (West Nile fever and West Nile neurologic disease), as well as patients with asymptomatic infections; all of our patients were symptomatic and had neurologic manifestations. Data are not available for

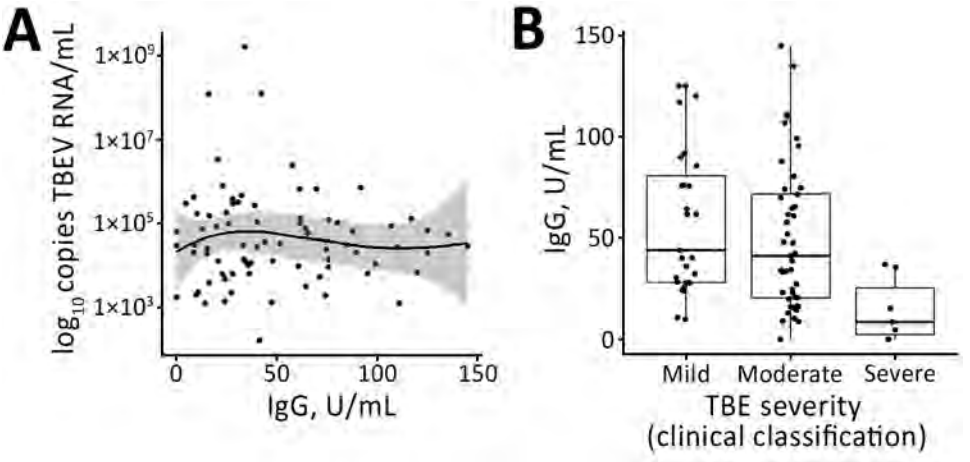

Figure 4. A) Distribution of virus RNA load in patients with TBE, Slovenia, by levels of TBEV IgG. B) Levels of TBEV IgG according to disease severity (clinical classification). Solid line in panel A indicates loess regression line, and shaded area indicates 95% CIs. Boxes in panel B indicate interquartile ranges and 25th and 75th percentiles, horizontal lines within boxes indicate medians, and errors bars indicate 1.5× interquartile ranges. TBE, tick-borne encephalitis virus; TBEV, TBE virus.

viremia levels in patients infected with Japanese encephalitis virus, another major neurotropic flavivirus.

Although we determined that TBEV RNA was readily detected in first-phase TBE serum samples, TBEV RNA could not be detected in any of the second-phase CSF samples. This finding confirms previous findings that PCR examination of serum is a valuable approach for a diagnosis of TBEV infection in the first phase of the disease, whereas corresponding testing of CSF obtained in the meningoencephalitic phase of TBE is not a valuable approach (*33*).

We detected higher levels of TBEV RNA in female patients than in male patients. However, we found no association with age of patients. We do not have an obvious explanation for higher viremia in female patients, but female patients did not differ from male patients by age, day of illness when virus load was measured, severity of disease, and several other parameters.

To evaluate the role of TBEV RNA levels in the pathogenesis of disease, we assessed the association between measured TBEV RNA load and several clinical parameters, including duration of the first phase of the disease, duration of the asymptomatic interval, TBE severity, and clinical presentation. However, univariate linear regression showed no association between virus load and any of the observed variables (Figure 3). This finding is consistent with findings from studies of laboratory mice, in which no association was detected between levels of TBEV, as well as WNV viremia and survival rates of animals (*34*,*35*). Studies of humans infected with WNV showed that higher virus loads in blood, plasma, or urine were present in patients with symptomatic infections than in those with asymptomatic infections, but no association was found between WNV burden and disease course (*29*,*31*,*32*,*36*). Because our study included only patients with symptomatic infection (i.e., those with biphasic TBE), we were unable to compare virus loads of the 2 viruses in asymptomatic infections.

Furthermore, our study found no association between virus RNA levels and laboratory parameters measured during the course of the disease, including leukocyte and platelet counts determined on the same day as RNA load and CSF cell counts and TBEV IgG levels determined at the beginning of encephalitic phase of TBE. However, a strong association was observed when TBEV IgG levels were compared with disease severity; the highest concentrations of antibodies were detected in patients with a mild form of the disease and the lowest concentrations were detected in patients with a severe form of the disease. This finding might suggest that although higher levels of TBEV

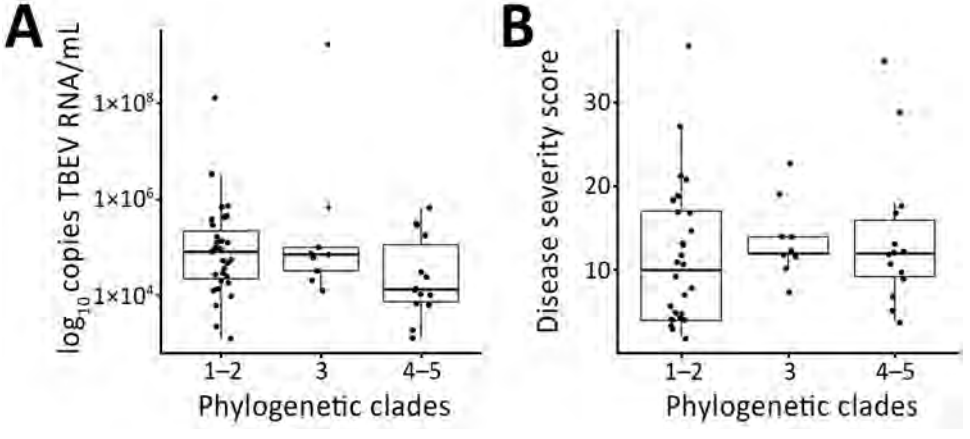

Figure 5. Distribution of virus RNA load in patients with tick-borne encephalitis, Slovenia, by A) virus phylogenetic clades and B) disease severity scores according to Bogovic et al. (*22*). Boxes indicate interquartile ranges and 25th and 75th percentiles, horizontal lines within boxes indicate medians, and errors bars indicate 1.5x interquartile ranges. TBEV, tick-borne encephalitis virus.

RNA are not directly associated with a more severe disease course, a limited or delayed humoral response results in a more severe illness caused by failure of the host to clear the virus. Thus, such prolonged viremia could result in a more pronounced infection of neuronal cells and subsequently in a more severe clinical presentation. This hypothesis is supported by previous studies of TBE patients (*16*) and experimentally infected laboratory animals (*21*,*35*,*37*), in which low concentrations of TBEV-specific antibodies and TBEV neutralizing antibodies in serum coincided with subsequent appearance of TBE and more severe disease.

Severity of TBE has been reported to vary in different geographic regions because severity is related to the subtype of the virus causing the infection. Thus, disease caused by the European TBEV subtype is considered to be milder than TBE caused by Siberian and Far Eastern subtypes, for which higher case-fatality rates and severe neurologic sequelae rates have been reported (*1*,*38*). However, differences in clinical presentation of TBE have also been reported in areas where only 1 virus subtype was present (*11*,*17*,*39*).

In our study, we obtained 56 partial E protein gene sequences by directly sequencing RT-PCR products from clinical samples of patients to avoid occurrence of mutations that could arise in the process of virus culturing or cloning. Relatively high genetic variability of TBEV from Slovenia was observed, which corroborates the results of Fajs et al. for a smaller number of patients in Slovenia (*24*). Previous studies of genetic diversity of TBEV have also shown that multiple sequence variants are present in relatively small geographic areas (*40*–*43*). In our study, we identified 6 phylogenetic clades by analyses of patient-derived E protein gene sequence analyses. Although the phylogeographic analysis of human samples included locations of residence, which do not necessarily correlate with site of infection, we observed an association between geographic and phylogenetic clustering, which suggested that most patients become infected near their homes, as reported previously (*44*). However, we found no association between phylogenetic clades and levels of TBEV RNA. Somewhat lower levels were detected in patients infected with viruses belonging to S4 and S5 phylogenetic clades, but we observed no major differences.

We also observed no association between phylogenetic clades and disease severity. These results do not demonstrate that differences in clinical presentation of TBE observed in a small geographic area are attributable to different genetic variants of the virus circulating in the area. Although in studies conducted by Belikov et al. (*45*) and Leonova et al. (*46*) in which full-genome sequences of Far Eastern TBEV strains isolated from patients with variable disease severity were analyzed, these authors found that the position of the strain on the phylogenetic tree and presence of specific mutations showed a strong correlation with pathogenicity of TBEV strains and disease severity; mutations found in the E protein gene sequence did not correlate with the degree of pathogenicity of TBEV strains. Also, other studies reported that mutations in genome regions other than E protein gene could be responsible for changes in neuroinvasivenes and neurovirulence (*47*–*49*).Therefore, phylogenetic analysis of more genome sequences is needed for better understanding of potential differences in pathogenicity of virus strains circulating in Slovenia.

In conclusion, for most patients with TBE, levels of TBEV RNA in serum in the first stage of illness are 3–6 $\log_{10}$ copies RNA/mL. The findings of our study do not indicate that levels of TBEV RNA in the first stage of TBE are directly associated with clinical presentation or severity of disease, or that levels can be used as a prognosis factor. Nevertheless, a weak humoral immune response seems to be associated with more severe acute illness, which suggests that inefficient clearance of virus results in a more serious infection of the CNS. The pathogenesis of TBE is most likely a complex and multifactorial process driven by properties of TBEV, as well as by the immune responses of the host. Further studies are needed to substantiate the association between the efficiency of virus clearance, prolonged viremia, and clinical presentation, and to better elucidate the immunopathogenesis of infections with TBEV.

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Strengthening Global Public Health Surveillance through Data and Benefit Sharing

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Equitable sharing of public health surveillance data can help prevent or mitigate the effect of infectious diseases. Equitable data sharing includes working toward more equitable sharing of the public health benefits that data sharing brings and requires the engagement of those providing the data, those interpreting and using the data generated by others, those facilitating the data-sharing process, and those deriving and contributing to the benefit. An expert consultation conducted by Chatham House outlined 7 principles to encourage the process of equitable data sharing: 1) building trust, 2) articulating the value, 3) planning for data sharing, 4) achieving quality data, 5) understanding the legal context, 6) creating data-sharing agreements, and 7) monitoring and evaluation. Sharing of public health surveillance data is best done taking into account these principles, which will help to ensure data are shared optimally and ethically, while fulfilling stakeholder expectations and facilitating equitable distribution of benefits.

Global outbreaks, including those of severe acute respiratory syndrome (SARS), Middle East respiratory syndrome, and Ebola virus disease, remind us that a public health event in a single location can rapidly become a global crisis. Control of infectious diseases can therefore be considered a global public good, and public health surveillance is a tool that helps achieve it. Timely sharing of public health surveillance data enables better preparedness and response, locally and globally.

Public Health Surveillance

Public health surveillance has been defined as "the ongoing, systematic collection, analysis and interpretation of health-related data with the a priori purpose of preventing or controlling disease or injury and identifying unusual events of public health importance, followed by the dissemination and use of such information for public health action" (*1*). Public health surveillance data are often collected without requiring individual patient consent. This practice is ethically and legally justified as a part of a government's responsibility to protect the public's health (*2*) and as a basic interest of persons in a pluralistic society (*3*). These justifications are tempered by the state's responsibility to use data for public health purposes only, engage stakeholders, and ensure protection of personal information.

Healthcare professionals are traditionally mandated to notify public health authorities about cases of specified diseases within a certain timeframe. The authorities then analyze the data and take appropriate action. Surveillance systems therefore tend to be the responsibility of the government. Most countries provide routine surveillance data to multilateral agencies (*4*), which analyze and disseminate information on disease trends at the regional or global level. These agencies also receive data from countries when the impact of a public health event crosses national borders, a standard of practice codified by the 2005 International Health Regulations (IHR 2005) (*5*), the international legal instrument aimed at assisting the global community to prevent and respond to public health threats that have the potential to affect populations worldwide.

Sources of Data

Public health authorities increasingly complement notifications with laboratory data (*6*), although in practice, this practice is often limited to high-income countries because it requires considerable laboratory capacity and advanced information technology infrastructure. Syndromic surveillance, in which health-related data such as the number of consultations for a specific diagnosis are reported, is used in high- and low-income settings. In some low-income settings, nongovernment actors, such as nongovernmental organizations (NGOs), academic institutions, private companies, or foreign medical teams, sometimes fill surveillance gaps (*7*), in particular during public health emergencies in which temporary, early warning surveillance systems based on syndromic surveillance are deployed in response to an increased outbreak risk (*8*).

Increasingly, online data not necessarily collected with an a priori health objective are used for public health purposes. Online technologies that provide data for disease

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or event detection are known as digital disease detection (DDD) systems. Data sources include search engine and social media queries (*9*,*10*), crowd-sourced event-based information queries (*11*), machine learning (*12*), natural language processing, and geolocalization (*13*). In 2014, DDD identified the resurgence of poliomyelitis and the Ebola outbreak in West Africa before the World Health Organization (WHO) officially reported them (*14*), 2 events WHO later declared as Public Health Events of International Concern. As DDD data quality and accuracy improve, DDD will likely assume a more prominent role, particularly in settings where the infrastructure to support public health surveillance systems is lacking (*10*). However, DDD needs more systematic integration into the formal, government-owned surveillance landscape as well as ties to response mechanisms to maximize its potential (*10*,*15*). The systematic use of nongovernment, informal surveillance systems is beginning to gain traction with surveillance systems such as WHO's new Epidemic Intelligence from Open Sources, an event-based system receiving alerts from a range of informal sources, planned for launch in the near future (*16*). DDD data raise new ethical and legal challenges that need to be addressed as they become integrated into conventional surveillance systems (*17*). These data also create competition for resources or the generation of data that are not consistent with data from conventional surveillance systems; these factors can give rise to trust and acceptability issues. Nevertheless, evidence for the added value of these data is building, and they are increasingly being incorporated into conventional surveillance systems.

Why Share Public Health Surveillance Data?

Public health surveillance data require timely sharing to ensure more coordinated and effective risk management for public health response (*18*). Sharing public health surveillance data between countries improves capacity for disease detection and response (*19*) and can help identify an outbreak source when national-level data cannot (*20*). This sharing can also reduce the potential or actual impact of a global health crisis. For example, the Global Influenza Surveillance and Response System, a laboratory network that shares information to detect the emergence of novel influenza viruses with pandemic potential (*21*), helped prevent SARS from becoming endemic after the 2003 outbreak (*22*). This network also improved the timeliness of the response to the 2009 influenza A(H1N1) pandemic (*21*). Besides being useful for outbreak management, sharing of routine public health surveillance data enables national and international collaboration, capacity strengthening, insight into public health system performance, and ultimately better control of infectious diseases (*18*).

Real or perceived risks, in particular those risks linked to travel and trade restrictions, can lead to a reluctance by governments to share data, leading to adverse public health and economic consequences. The 2003 SARS outbreak cost an estimated US $40–$80 billion to the global economy, with travel and tourism industries badly affected (*23*). China's delay in sharing information about the 2003 SARS outbreak contributed to the disease's spread and the delayed global response (*24*) as well as economic and reputation damage to China (*25*). With Middle East respiratory syndrome, the incomplete assessment of the disease origin and source has largely been attributed to a reluctance to share public health surveillance data in a timely fashion (*26*). Sharing of data helps achieve appropriate public health action while limiting risks to travel and trade. IHR 2005 is designed to ensure maximum public health benefit while keeping restrictions to a minimum (*5*). Nevertheless, public health surveillance data are not always freely shared because of perceived or real technical, political, economic, motivational, ethical, and legal barriers (*27*). Sharing public health surveillance data must become the norm.

Which Stakeholders Are Concerned?

Government actors implement most conventional public health surveillance systems and generate most data and can be complemented by nongovernment actors. In addition to having value at the national level, a country's routine public health surveillance data enable multilateral organizations to generate intelligence on specific diseases at the regional and global level. These organizations provide standards and advice on data sharing to facilitate the process by individual countries and conduct their own surveillance activities (*28*); examples include the WHO's global measles surveillance system (*4*) and the European Union's surveillance system (TESSy), which has standardized surveillance across the European Union (*29*). Such supranational systems come with their own challenges, such as the additional burden placed on individual countries to report data already analyzed nationally and the difficulties associated with comparing different types of data resulting from surveillance systems with different national legal bases. Institutions that do not generate data themselves but seek to reuse data for academic or public health purposes are also part of this data-sharing landscape (Figure).

Stakeholders can be divided into 3 groups that need to be engaged for optimal data sharing: 1) data providers, who generate public health surveillance data either from the community, the healthcare system, or nonhealth sources; 2) data recipients, who interpret and use data generated by others; and 3) data sharing facilitators, those who make sharing between data providers and recipients possible.

Individual stakeholders can commonly belong to ≥1 group at a time and can assume a different role in different situations. For example, a country might provide surveillance data to a multilateral agency and receive data from a

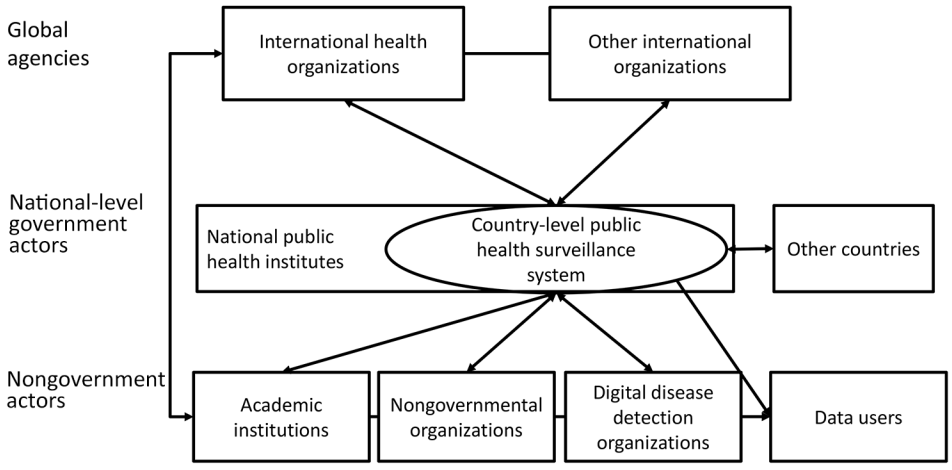

Figure. The global public health surveillance landscape, 2018.

neighboring country simultaneously; alternatively, a multilateral agency might provide data sharing guidelines to countries, acting as facilitator, while also receiving and analyzing event-based data.

Disease outbreaks caused by Ebola virus (2014–2016) and Zika virus (2016), among others, have increased awareness of the importance of data sharing among global health stakeholders. In addition to several other recent calls to share research data during emergencies (*30*,*31*), in 2016, a group of ≈40 international public health leaders published a statement calling on stakeholders to share "all public health surveillance data, as necessary to improve and protect public health" (*18*; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/7/15-1830-Techapp1.pdf). This statement further outlined that public health surveillance data sharing should be the norm, rather than the exception, with public health surveillance data made accessible in a timely manner while taking appropriate steps to safeguard the privacy of individuals and other legitimate public interests. Accordingly, the statement asked stakeholders to commit to 1) sharing public health surveillance data by default when a public health need is identified; 2) using public health surveillance data responsibly, with the intention of protecting and improving the health of the population; 3) making the benefits explicit; 4) ensuring that public health surveillance data are shared with as few restrictions as possible and in an ethical way.

Principles for Sharing Public Health Surveillance Data

Formulating principles to promote and facilitate data sharing in public health is not a new concept. An example is the Pandemic Influenza Preparedness Framework (*32*). This guidance document is restricted to 1 disease and resulted only after extensive negotiations (*32*). In addition to such international efforts, individual funders often have specific data-sharing policies (*33*).

The principles we outline here are the result of a consultation process with ≈100 experts, including those from the fields of public health, law, ethics, politics, and data sharing, including experts from WHO, the World Organisation for Animal Health (OIE), and the Food and Agriculture Organization of the United Nations. The consultation was convened by the Chatham House Centre on Global Health Security (*34*). The principles form the basis of a guide to sharing public health surveillance data and benefits. This guide, available in hard copy and as an online interactive tool (https://datasharing.chathamhouse.org), addresses perceived and real barriers and is intended to facilitate equitable sharing of public health surveillance data and benefits. Equitable in this context is taken to mean that data and benefits are shared among stakeholders according to individual, organizational, and public health needs. The guide enables sharing without the need for prolonged negotiation by creating an environment conducive to sharing data and achieving good practice.

The Principles

This approach is governed by 7 principles. Each incorporates the ethical concepts most relevant to data sharing: social beneficence, respect, justice, and transparency. Those principles also strive to ensure that sharing data when a need is identified leads to equitable sharing of public health benefits and capacity-building where necessary and appropriate. This component is particularly important when the parties sharing the data have different capacities to benefit because of unequal resources. The principles encourage parties who are better resourced to ensure that others benefit from the process according to need.

1. Building Trust

Trust facilitates successful data sharing, which in turn further reinforces trust. Two principal dimensions to trust are brought to bear when public health surveillance data are

shared. First, organizations sharing public health surveillance data should do so in a transparent manner and should be able to demonstrate to communities from which the data originate how their data are collected, analyzed, used, and protected. Second, trust-building measures between organizations or individuals sharing data, whether at the personal or organizational level, help create an environment where public health surveillance data can be shared. Transparency with regard to what data are shared with whom, and for what purpose, is a prerequisite. Trust includes ensuring that the shared data are used responsibly and not made available to other parties or publicly without consulting the data providers. When the purpose of sharing data is clear and explicit, and those persons involved in sharing know each other, understand each other's expectations, and carry out commitments as agreed, a trust relationship can emerge. Established trust increases the likelihood of collaboration and shared benefits and promotes core surveillance capacity through the creation of surveillance networks (*19*). Building trust for routine data sharing can provide strong foundations for emergency surveillance and response. Building trust can be hard, but losing trust is all too easy.

In practice, trust often translates into developing appropriate professional relationships with counterparts in other countries or regions (*35*). Trust-building measures sometimes take the form of face-to-face meetings, regional workshops, desktop exercises, or joint outbreak investigations (*19*). For data recipients, providers must be trustworthy in providing high-quality data. Therefore, improving data quality through capacity-building (for example, by sharing technical expertise) is in their interest.

2. Articulating the Value

The benefits of sharing should be explicitly articulated when public health surveillance data are shared. However, loss of rights over the data and the potential for misuse can increase the risk of data providers being reluctant to share because of real or perceived reputational damage and loss of benefits, either in terms of public health or, for example, publication opportunities (*35*). Because of these potential negative outcomes, some public health authorities have used legal or operating standards to restrict data sharing (*36*). When initiating data sharing, the purpose must be explicit, and all stakeholders should be able to understand the value of sharing the data, who will have access to the data, and how the data will be used. Stakeholders must also be assured that they will benefit from the sharing process in an equitable manner in terms of collaboration opportunities and public health benefits. In situations where no direct benefits to the data providers exist, the sharing process should ensure that, at a minimum, those providing data do not suffer adverse public health consequences or lose opportunities for publication, collaboration, or otherwise.

Such assurances maximize the utility of the data while allowing data providers to retain control over the data, thereby encouraging data sharing. Any data use viewed as data harvesting (i.e., when data recipients use data while no benefit is enjoyed by the data provider) is unjust and unfair. Such a practice increases reluctance to share and jeopardizes sharing globally. Conversely, organizations claiming ownership of, and restricting access to, public health surveillance data when such actions would decrease potential health benefits derived from those data is unacceptable.

3. Planning for Data Sharing

Public health surveillance data should be collected with potential sharing in mind. Sharing is most successful when expectations of all stakeholders are met and it addresses a need, whether real or perceived (*35*), which should be identified in advance to help ensure timeliness of sharing.

An a posteriori approach to sharing might not maximize benefits, particularly when timeliness is a key element of success, such as in emergencies. Planning for data sharing extends to all steps of the data-management lifecycle (i.e., data collection, processing, analysis, preservation, access, reuse, and disposal) (*37*). This effort requires technical capacity, information technology infrastructure, and a workforce with data-management skills.

Planning also requires a professional ethic for responsibility to protect identifiable data, which are often collected without individual consent. Preserving confidentiality of individual-level data is critical because societies can sometimes respond to persons with infectious diseases in stigmatizing and discriminatory ways.

Data-sharing and data-management standards, in particular with regard to metadata, help maximize quality, utility, and reuse potential. Data recipients benefit from high standards, which ensure that they will be able to reuse data according to their agreed purpose. The time and skills required to collect and manage data in adherence with relevant standards should be taken into account when hiring and training staff. Having a data provider with the human resource and technical capacity to provide the data to required standards is in the data recipient's interest. As such, data sharing can be an opportunity for IHR 2005–mandated capacity-building.

4. Achieving Quality Data

High-quality data enable the generation of high-quality evidence and therefore lead to better public health outcomes. Surveillance data can be evaluated for relevance, accuracy, timeliness, accessibility, interpretability, and coherence, among other characteristics (*38*). Generally, trade-offs exist between these characteristics, and the attributes to prioritize should be considered when sharing the data. Overall, data accessibility and sharing subject the data to feedback and

therefore improves quality. Technical and human resource implications of data quality exist; for example, standardization and automation can make sharing less expensive, more effective, and easier (*35*). Standardization also improves the validity and public health benefit of comparative analyses, which are particularly challenging to interpret if data from nonstandardized surveillance systems are aggregated. High-quality data production requires a skilled workforce to develop, manage, and evaluate surveillance systems (*35*). However, when a public health situation warrants the rapid sharing of data, concerns about quality should not be a reason not to share, providing sufficient confidence in the data to inform public health action exists. Quality should be balanced with timeliness.

5. Understanding the Legal Context

The legal implications of data sharing and the most suitable type of agreement depend on geographic location, type of institution involved, type of data, level of public health threat, and other contextual factors. Parties should understand the legal implications and tools available. Sharing public health surveillance data across borders has legal implications when the type of data shared is protected by national or international law. This concern applies mainly to disaggregated data containing confidential or personal information. In current practice, guidance on the legal implications of cross-border public health data sharing is not readily available. Where this guidance does exist, the balance between making data accessible, safeguarding privacy, and protecting intellectual property is not well regulated or standardized, which can result in protective policies (*27*). Governments are often more likely to focus on safeguarding their institutions against liability when creating agreements, whereas nongovernment institutions sometimes focus more on intellectual property concerns.

Data-sharing agreements can help resolve differences or ambiguities in law and are most successful when the context is defined as precisely as possible, supported by local knowledge, and when relevant laws and regulations are taken into account. In some instances, an agreement that is not legally binding may be more suitable than using legal means.

6. Creating Data-Sharing Agreements

Formal data-sharing agreements are unnecessary if informal arrangements are sufficient to accomplish the goal of sharing. The rights and interests of stakeholders should be properly taken into account whatever arrangements are made. When more formal agreements are required, they can take different shapes, from short memoranda of understanding to detailed, legally binding data-sharing agreements. Depending on the context, the agreement can take place at the local, national, regional, or global level. Whatever form they take, successful and sustainable data-sharing agreements require consideration of the needs and expectations of all parties. Agreements drafted before the needs and expectations of all parties are understood can lead to inequities in the sharing of benefits (*35*). This imbalance can also result in missed opportunities for knowledge and skills capacity-building. Parties should collaborate and ensure that the terms of reference are acceptable to all, data providers have the opportunity to take part in any data analysis if they wish so, benefits are shared equitably, and potential harms to individuals and communities are minimized. Tools and resources to help parties initiate or revise data sharing agreements are available online (https://datasharing.chathamhouse.org)

7. Monitoring and Evaluation

Sharing data only leads to public health benefit if a need is addressed and the data are visible and usable. Therefore, it is important to ensure that the data are shared according to the plan, used for the intended purposes once they have been shared, and achieve the desired effect. If these outcomes are not achieved, the reasons should be analyzed. As new sources of surveillance data emerge and as data are successfully shared, recording and disseminating success stories that demonstrate the added value of data sharing also are important. These stories can help trigger a "norm cascade" that creates a critical mass of stakeholders who adopt data sharing as a normative expectation (*39*). In addition, in situations where sharing did not have the expected result or a lack of data sharing contributed to negative public health outcomes, the process should be documented and analyzed to help understand and make improvements in the future.

Conclusions

Sharing surveillance data improves public health. We propose an approach to data sharing that creates an environment conducive to sharing, encourages good practice, and ensures that the benefits derived from the sharing process are equitably distributed.

The public health surveillance landscape is complex, with a range of government and nongovernment stakeholders who can provide and receive data as well as facilitate sharing. Optimal sharing requires an understanding of the roles and responsibilities of these stakeholders. The 7 principles for public health surveillance data sharing we propose address good practice for the sharing of public health surveillance data. Those principles serve as the basis for comprehensive guidance with actionable recommendations for all stakeholders. The complete guidance is available online (https://datasharing.chathamhouse.org).

Sharing of public health surveillance data is best done with an agreement that takes into account those principles,

which will help to ensure that data are shared optimally and ethically, while fulfilling the expectations of stakeholders and facilitating equitable distribution of benefits. We encourage stakeholders, and in particular multilateral organizations, to consider these principles when strengthening frameworks and capacity for data sharing.

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Detection of Respiratory Viruses in Deceased Persons, Spain, 2017

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During the 2016–17 influenza season in Spain, we tested specimens from 57 elderly deceased persons for respiratory viruses. Influenza viruses were detected in 18% of the specimens and any respiratory virus in 47%. Only 7% of participants had received a diagnosis of infection with the detected virus before death.

Increases in all-cause deaths usually occur during annual influenza and respiratory syncytial virus (RSV) epidemics (*1*,*2*). This excess is higher in seasons dominated by influenza virus A(H3N2) (*3*,*4*). Ecologic design approaches have been used to estimate deaths caused by influenza and other respiratory viruses on the basis of weekly virus surveillance data (*1*–*5*). Because a small proportion of persons are tested for influenza virus before death (*6*), the actual contribution of influenza to all-cause mortality is not well known. Other respiratory viruses are responsible for some influenza-like illnesses and related deaths (*7*) and have been related to deaths of unknown cause (*8*). We conducted a pilot study to evaluate the feasibility of detecting influenza and other respiratory viruses in recently deceased persons and of estimating the prevalence of infections in persons who died within an influenza epidemic period.

The Study

We performed this study in Navarre, Spain, during January 23–February 19, 2017, during the seasonal influenza epidemic (*9*). Recruitment was conducted in 2 morgues by trained professionals. Persons ≥65 years of age who had died of natural causes regardless of the reported cause of death were included, after we obtained written informed consent from their closest relatives. We obtained nasopharyngeal swab specimens before the bodies were prepared for burial; we tested the swabs for influenza and RSV by reverse transcription PCR (RT-PCR). We tested negative samples for other respiratory viruses using multiple PCR (Allplex Respiratory Panel; Seegene, Seoul, South Korea).

We obtained demographic information and previous diagnoses from the epidemiologic surveillance system. We retrieved hospitalization and laboratory confirmation for respiratory viruses within the 30 days before the death from electronic healthcare databases. We obtained the underlying causes of death from the regional mortality register and grouped them into 5 categories according to the International Classification of Diseases, 10th Revision: neoplasms (codes C00–D49), nervous system diseases (codes G00–G99), circulatory system diseases (codes I00–I99), respiratory system diseases (codes J00–J99), and all other causes. We used the 2-tailed Fisher exact test to compare proportions.

The study period included the last 4 weeks of the 2016–17 influenza epidemic in Navarre, starting 2 weeks after the peak. This period was characterized by a high but descending number of hospitalizations of patients with laboratory-confirmed influenza and 27% excess in all-cause mortality (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/7/18-0162-Techapp1.pdf).

During the study period, 460 deceased persons ≥65 years of age were registered, 106 were attended in the participating morgues, and 57 (54%) were enrolled in the study. Nonparticipation resulted mainly from logistic problems and lack of signed consent.

Of the 57 participants in the study, 29 (51%) were women, 23 (40%) were <85 years of age, 50 (88%) had major chronic conditions, 5 (9%) had been resident in nursing homes, and only 12 (21%) had been hospitalized before death. Nonparticipants did not differ in these characteristics (online Technical Appendix Table).

Respiratory viruses were detected in the postmortem study in 27 (47%) participants, but only 4 (7%) had received this diagnosis before death (Figure 1). Ten (18%) participants tested positive for influenza virus A(H3N2), 7 (12%) for RSV (4 subgroup A and 3 subgroup B), 7 (12%) for coronavirus (6 type 229E and 1 type OC43), and 4 (7%) for rhinovirus. Although postmortem detection of any

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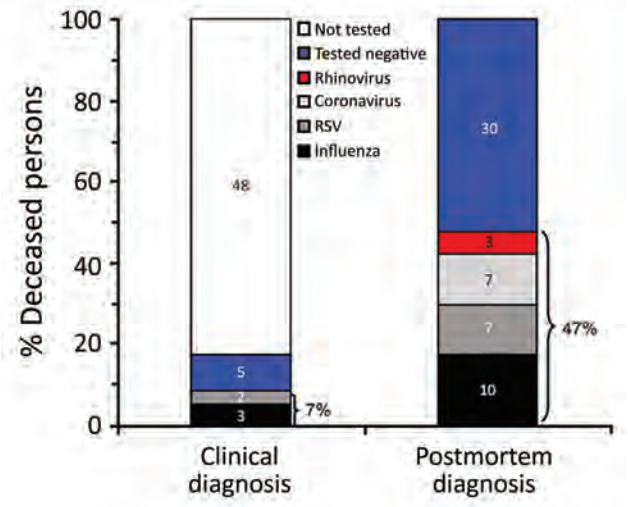

Figure 1. Clinical and postmortem detections of respiratory viruses among 57 deceased persons ≥65 years of age, Spain, 2017. As indicated, 47% of deceased patients tested positive for respiratory virus infection postmortem, but only 7% had received the same diagnosis before death. RSV, repiratory syncytial virus.

respiratory virus was more likely among previously hospitalized persons, it was also frequent among those not hospitalized (75% vs. 40%; p = 0.050) (Table).

The postmortem detection of influenza or other respiratory viruses was not statistically associated with the analyzed covariates, with 2 exceptions: respiratory viruses other than influenza were detected more frequently in deceased men (46%; 13/28) than in women (14%; 4/29; p = 0.010), and respiratory viruses were more frequently detected among deceased persons who were reported with respiratory system diseases as the underlying cause of death than in those reported with other causes (86% vs. 42%; p = 0.045) (Table). Nevertheless, the percentage of deceased persons whose specimens tested positive for any respiratory virus was notable in all groups of nonrespiratory causes of death (range 38%–50%) (Table; Figure 2). Only 1 person (10%) whose specimen was detected as having influenza virus in the postmortem test had influenza registered as the cause of death; 5 (50%) were registered as having a cardiorespiratory cause of death.

Conclusions

This study demonstrates the feasibility of the detection of respiratory viruses in samples from deceased persons. Respiratory viruses were found in nearly half of the persons who died of natural causes in an influenza epidemic period, and 18% were confirmed for influenza virus A(H3N2), which was the same influenza virus subtype that dominated in patients during the 2016–17 season (*10,11*). The 2016–17 influenza season was characterized in Europe by an increase in deaths (*2*). Other respiratory viruses were detected during the influenza circulation period and may have contributed substantially to hospitalizations and deaths (*1,5,12*). RT-PCR seems to have high sensitivity for the detection of respiratory viruses in deceased persons, as previously shown in studies based on coronial autopsies (*8,13*).

Respiratory virus infections are characterized by sudden onset; death may occur suddenly, even before the symptoms are evident. Respiratory viruses can trigger secondary bacterial infections or exacerbate existing chronic conditions, and these concurrent conditions usually prevail as the underlying cause of death. Half of

Table. Factors associated with postmortem detection of influenza and other respiratory viruses among deceased persons, Spain, 2017

Patient characteristics	Total no. patients	No. (%) patients					p value†
		Influenza virus	Respiratory syncytial virus	Coronavirus	Rhinovirus	Any respiratory virus*	
Total	57	10 (18)	7 (12)	7 (12)	4 (7)	27 (47)	
Sex							0.189
M	28	3 (11)	4 (14)	6 (21)	4 (14)	16 (57)	
F	29	7 (24)	3 (10)	1 (3)	0	11 (38)	
Age, y							0.889
65–74	7	2 (29)	0	0	1 (14)	3 (43)	
75–84	16	3 (19)	3 (19)	1 (6)	0	7 (44)	
≥85	34	5 (15)	4 (12)	6 (18)	3 (9)	17 (50)	
Major chronic conditions‡	50	8 (16)	7 (14)	6 (12)	4 (8)	24 (48)	1.000
Nursing home residence	5	1 (20)	0	1 (20)	1 (20)	2 (40)	1.000
Hospitalization before death	12	3 (25)	2 (17)	2 (17)	3 (25)	9 (75)	0.050
Premortem diagnosis	4	3 (75)	1 (25)	0	0	4 (100)	0.044
Cause of death							
Neoplasms	16	1 (6)	1 (6)	4 (25)	0	6 (38)	0.391
Nervous system condition	8	2 (25)	0	1 (13)	0	3 (38)	0.709
Circulatory system condition	14	3 (21)	2 (14)	0	1 (7)	6 (43)	0.765
Respiratory system condition	7	2 (29)	3 (43)	0	1 (14)	6 (86)	0.045
Other causes	12	2 (17)	1 (8)	2 (17)	2 (7)	6 (50)	1.000

*One person's specimen tested positive for both coronavirus and rhinovirus.
†The 2-tailed Fisher exact test was used to compare the proportions of patients with any respiratory virus infection for the listed variables.
‡Major chronic conditions included heart disease, respiratory disease, renal disease, cancer, diabetes mellitus, cirrhosis, dementia, stroke, immunodeficiency, rheumatic disease, and morbid obesity.

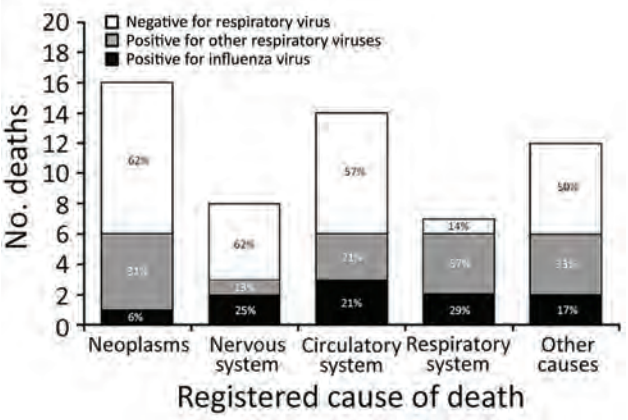

Figure 2. Postmortem detection of influenza and other respiratory virus infection by underlying cause of death among 57 deceased persons ≥65 years of age, Spain, 2017.

the deaths with influenza virus detection in the postmortem test were registered as having a noncardiorespiratory cause of death, which is consistent with a previous hospital study (*6*). This finding demonstrates the difficulty in estimating the deaths related to respiratory viruses by using the mortality registers.

These results raise relevant implications. Only a small proportion of deceased persons whose respiratory virus was detected in the postmortem test had been hospitalized and received this diagnosis before dying; therefore, the contribution of viral infections to death may be underestimated. Deaths related to respiratory viruses could be distributed among all causes of death. Although the burden of death has been estimated by indirect approaches (*1*–*5*), this study offers a complementary novel approach to assess the impact in terms of the proportion of all-cause deaths with respiratory virus detection (*14*).

The surveillance of influenza based on laboratory-confirmed cases is implemented in primary healthcare and in hospitalized patients (*9*,*11*). Our results open the possibility and show the potential interest of adding a sentinel virological surveillance based on persons who die during the influenza season.

Caution should be paid in the interpretation of these results, however. Virus detection does not necessarily imply a causal relationship between virus infection and death because respiratory viral shedding has been described in asymptomatic persons (*15*). Our study included 12% of deaths in the region during the last 4 weeks of the influenza epidemic, but the peak was not included; therefore, the representativeness is limited. Similar characteristics of participants and nonparticipants rule out selection bias. We cannot rule out false-negative results, however, because samples were obtained postmortem and the time from symptom onset to swabbing was unknown. Only negative samples for influenza and RSV were tested for other respiratory viruses, which might underestimate the frequency of the other respiratory codetections.

In summary, we demonstrate the feasibility of detecting respiratory viruses in recently deceased persons. We frequently detected respiratory viruses postmortem in winter deaths, although most of these infections were not clinically diagnosed. Respiratory virus surveillance systems could be complemented by testing persons who die during the influenza circulation period for respiratory virus infections.

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The study protocol was approved by the Navarre Ethical Committee for Medical Research (Pyto2016/129).

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Microevolution and Patterns of Transmission of *Shigella sonnei* within Cyclic Outbreaks of Shigellosis, Israel

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Whole-genome sequencing unveiled host and environment-related insights to *Shigella sonnei* transmission within cyclic epidemics during 2000–2012 in Israel. The Israeli reservoir contains isolates belonging to *S. sonnei* lineage III but of different origin, shows loss of tetracycline resistance genes, and little genetic variation within the O antigen: highly relevant for *Shigella* vaccine development.

Shigellosis is common all over the world and is hyperendemic to developing countries where children with the disease have an increased risk for persistent diarrhea, arrested growth, and death (*1*–*3*). The annual incident cases of shigellosis are estimated at ≈190 million in developing countries, where *Shigella flexneri* is the most common cause of shigellosis, and ≈1 million in industrialized countries, where *S. sonnei* predominates (*4*–*7*).

The Study

Despite the improved socioeconomic conditions, Israel has remained an area where shigellosis is highly endemic, reporting an annual incidence rate of culture-proven shigellosis of ≈97 cases per 100,000 population. Cyclic outbreaks during 2000–2012 occur every 2 years; *S. sonnei* is the pathogen for >85% of the cases. It has been shown that the ultraorthodox Jewish communities, which are overcrowded and have a high number of children <5 years of age, were the epicenter of these epidemics during the past 15 years (*5*). We used whole-genome sequencing (WGS) to provide a high-resolution view to better understand the local microevolution and patterns of *S. sonnei* transmission within the cyclic outbreaks in Israel.

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A total of 281 *S. sonnei* isolates were subject to WGS (Figure 1; online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/7/17-1313-Techapp1.pdf). We collected data from isolates during the epidemic years 2000, 2002, 2004, 2006, 2008, and 2012, and the nonepidemic years 2001 and 2003. All isolates were from children of various sanitary, socioeconomic, cultural, and ethnic backgrounds: ultraorthodox Jews, secular Jews, and Israeli Arabs. The ultraorthodox Jews represent ≈11% of the total population of Israel. This population group resides in towns or neighborhoods separated from the secular Jewish population (*8*) and also in mixed ones. The Israeli Arabs, who are estimated to account for 20% of the total population, reside mostly in rural areas and in towns or neighborhoods separated from the Jewish population; but they also live in towns inhabited by both Jews and Arabs (*8*). Of the 281 isolates, 263 (93.5%) were collected from Jewish children (mainly from ultraorthodox communities) and 18 (6.4%) isolates were from Israeli Arab children (mainly Bedouins living in southern Israel).

The WGS analysis showed that the clones within the Israeli reservoir formed 2 distinct subclades: a major subclade (subclade I) containing ≈94% of the Israeli collection, which is more prevalent among Jewish children (92% originated from Jewish children); and a minor subclade (subclade II) containing ≈5.7% of the Israeli collection, which is more prevalent among Israeli Arab children (82%). Only 1 isolate (≈0.3%) did not cluster with any of the Israeli isolates (Figure 1).

A comparison to global analyses (*9*) suggests that even though both subclades belong to *S. sonnei* lineage III, they are of different origins. Subclade II clones were more closely related to isolates that originated in Egypt and Iran than to the Israeli subclade I clones that seem to be endemic and have a distinctive recombination site, as previously described for 1 sequenced isolate from a patient in Israel in 2003 (*9*; online Technical Appendix Table 2). They were also found to distinguish *S. sonnei* among Jewish Orthodox communities of various countries (*10*). Nine of 13 Israeli Arab strains in clade II were isolated from Bedouins living in the vicinity of the Egyptian border. The frequent migration over the Israel–Egypt border of Bedouins often belonging to the same tribe

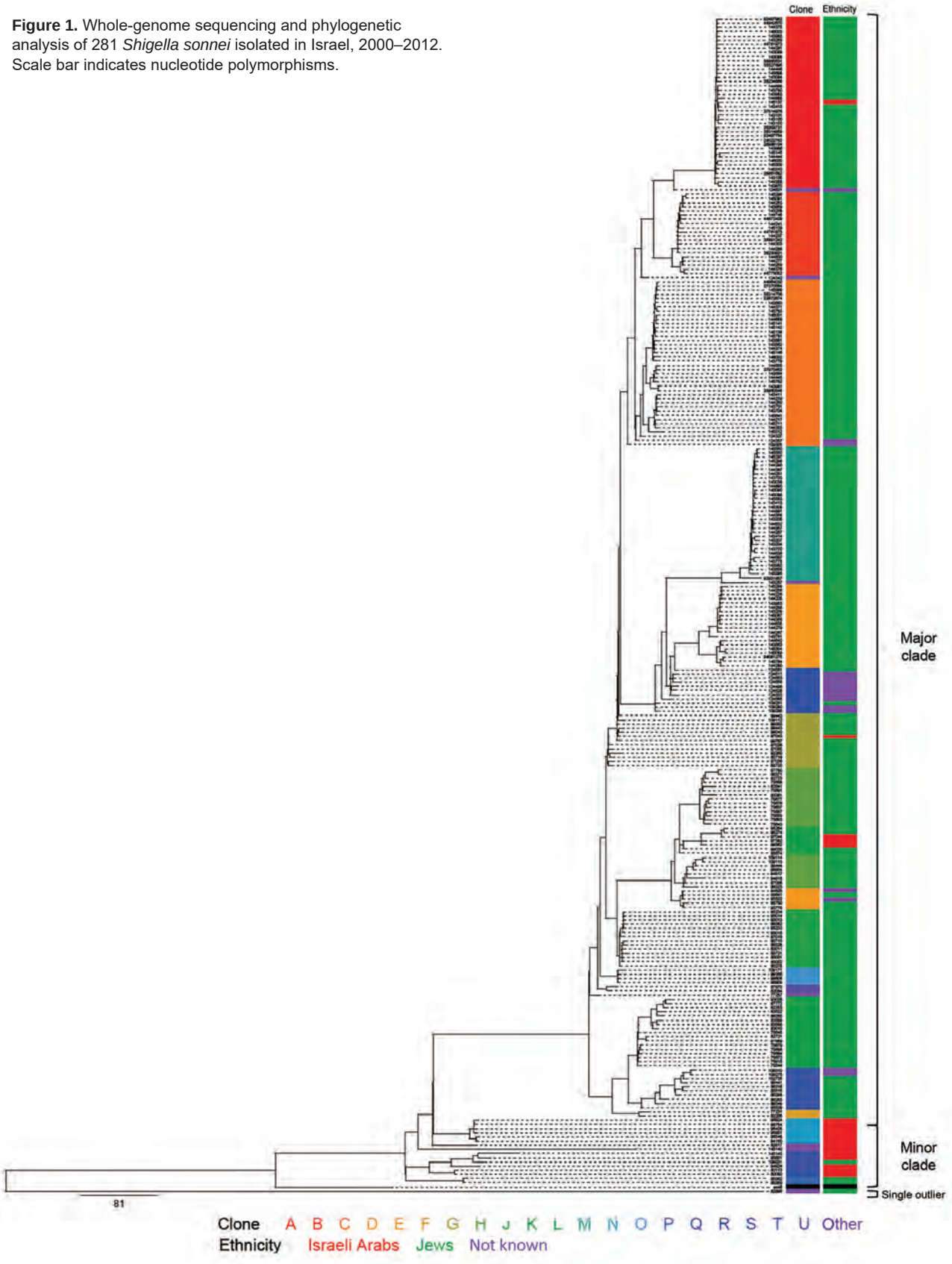

Figure 1. Whole-genome sequencing and phylogenetic analysis of 281 *Shigella sonnei* isolated in Israel, 2000–2012. Scale bar indicates nucleotide polymorphisms.

could explain the possible importation of subclade II *S. sonnei* from Egypt and/or through Egypt, similar to the recent transborder silent spread of poliovirus type 1, another fecal–orally transmitted enteropathogen in southern Israel (*11*). Our results also indicate that in general, isolates from Israeli Arab children who reside in mixed settlements and in close proximity to Jewish children commonly have positive test results for *Shigella* strains in clade 1. Only 5 (1.9%) isolates in clade I originated from Israeli Arabs (Figure 1). Of note, 4 of the 5 isolates were obtained from samples from Arab children residing in Beer Sheva (3 isolates) and Mevaseret Zion (1 isolate), cities inhabited by both Jews and Arabs. Consequently, it appears that a combination of both biogeography and ethnicity forming microhabitats for *S. sonnei* clone circulation shapes the differences observed between Jewish and Israeli Arab children.

Each subclade could be further subdivided into clonal groups consisting of clusters of isolates with ≤30 chromosomal single-nucleotide polymorphism (SNP) differences from the nearest neighboring cluster. We defined a total of 20 unique and distinct *S. sonnei* endemic clones

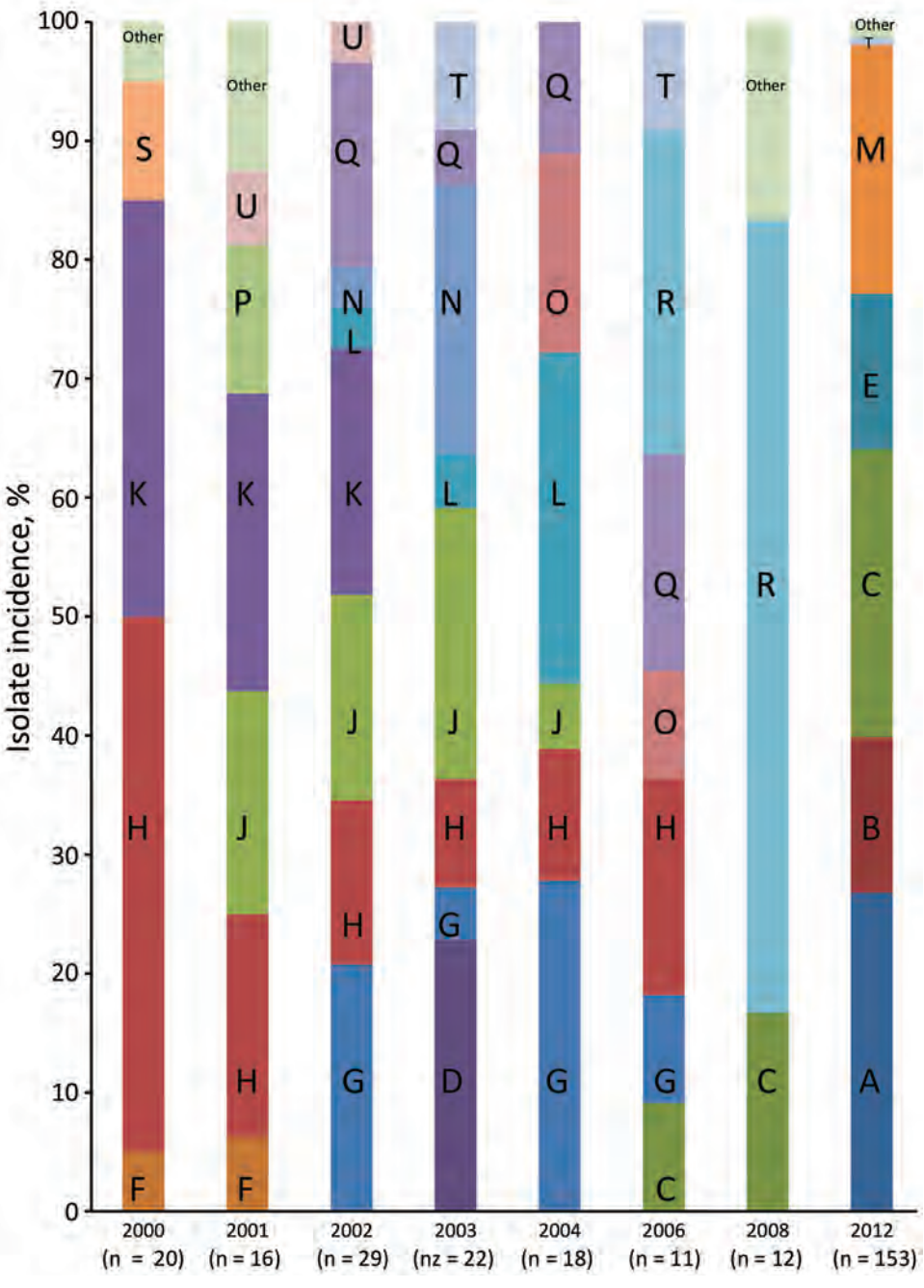

Figure 2. The relative distribution of the various whole-genome sequencing defined *Shigella sonnei* clones per year of isolation, 2000–2012.

circulating in the Israeli population (Figure 2, panels A–U). The majority of the clones (≈69%; Figure 2) can be found throughout the years regardless of the shigellosis outbreaks that occurred in Israel every 2 years during 2000–2012, suggesting some mechanism of persistence (*5*). Contrary to our hypothesis, neither the establishment and dynamics of persistent or dominant clones could explain the Israeli cyclic outbreaks. Moreover, we found no specific genetic attributes that could distinguish them from other clones. Therefore, we postulate that the cyclic peaks of morbidity rates associated with *S. sonnei* are the result of changes in the level of natural immunity, as was shown by several observational studies (*5*,*12*,*13*). An outbreak of shigellosis occurring among children 0–4 years of age will lead to an increase in the level of natural immunity to the homologous *Shigella* organism (*S. sonnei*), which will also provide the level of herd immunity sufficient to prevent the onset of a new epidemic. After 1 or 2 years, declining levels of antibodies, together with the intake of a new cohort of naive newborns, will lead to a decrease in the level of herd immunity below a critical level. High and continuous exposure to a variety of circulating *S. sonnei* clones in children 0–4 years of age who live in crowded conditions will lead to the renewal of the epidemic transmission of these clones (*5*).

Conclusions

Although we excluded all *S. sonnei* plasmids from the phylogenetic analysis, plasmid reads were mapped and the assembled sequences compared with the reference plasmid sequences. Our data suggest that plasmid spA is undergoing degradation as a result of the loss of tetracycline resistance genes over time. This finding is consistent with the results of Holt et al. for the Middle East (III) clade (*9*) and with laboratory examination showing that the *S. sonnei* Israeli reservoir is becoming less resistant to tetracycline (*5*) (p-value for linear trend <0.01) (online Technical Appendix Table 3).

Although notoriously unstable when *S. sonnei* is grown on laboratory media, invasive plasmid pINVB was present in ≈58% of our isolate sequences. Our results demonstrate that *S. sonnei* O antigen encoded on this plasmid is well-conserved within the *S. sonnei* Israeli reservoir. No SNPs were detected in genes that belong to the O antigen gene cluster in ≈97% of the plasmids, and pINVB seems to be under very little immune selection as has been also shown in other studies (*9*,*14*). We identified a single SNP leading to a nonsynonymous substitution, in gene *wbgW* within the O antigen gene cluster that was shared by only 4 (2.4%) isolate plasmids. We also identified in 1 (≈0.6%) isolate 1 SNP, a nonsynonymous change in gene *wbgY*. To date, *Shigella* vaccine development has mainly focused on serotype-targeted vaccines that are based on *shigella* O antigen (*15*). Thus, our findings may have implications for public health as the need for a safe and effective *Shigella* vaccine becomes more pressing (*15*).

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Diphtheria Outbreak in Amerindian Communities, Wonken, Venezuela, 2016–2017

Adriana Lodeiro-Colatosti, Udo Reischl, Thomas Holzmann, Carlos E. Hernández-Pereira, Alejandro Rísquez, Alberto E. Paniz-Mondolfi

In February 2017, a diphtheria outbreak occurred among Amerindians of the Pemón ethnic group in Wonken, Venezuela. A field investigation revealed ≈10 cases; clinical presentation did not include cutaneous or neurologic signs or symptoms. To prevent future outbreaks in Venezuela, Amerindian communities need better access to vaccination and healthcare.

Diphtheria is a contagious acute bacterial infection caused by toxin-producing, gram-positive *Corynebacterium diphtheriae* and other *Corynebacteria* ssp., such as *Corynebacterium ulcerans* (*1*,*2*). Humans are a known reservoir, but bacteria can also be isolated from horses and cats. Transmission occurs primarily through contact with airborne respiratory secretions or exudation from infected skin lesions (*3*–*5*). The incidence of diphtheria in the Western Hemisphere has decreased dramatically over the past few decades, although the disease has remained endemic in some developing countries around the globe. Diphtheria was eradicated in Venezuela 25 years ago; the last reported case occurred in 1992 (*6*).

However, in November 2016, the International Health Regulations National Focal Point of Venezuela updated the Pan American Health Organization and World Health Organization about diphtheria in the country, reporting that 16 of 24 federal agencies had reported 183 suspected cases of the disease during September–November 2016 (*6*). During weeks 1–49 of 2017, suspected and confirmed diphtheria cases were reported in 4 countries in the Americas: Brazil (4 cases), the Dominican Republic (3 cases), Haiti (152 probable cases), and Venezuela (227 cases) (*7*).

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The Study

In February 2017, a cluster of ≈10 cases of an illness characterized by swollen neck occurring in 7 children and 3 adults (including 2 deaths) was reported in 3 Amerindian communities (Urimpatá [5.128429°N, –61.380956°E]; Damasko [5.127997°N, –61.504152°E]; Atanao [5.128429°N, –61.380956°E]) of the Great Savannah in Bolivar, Venezuela (Table). These settlements, which are part of the greater Weiyekupotá community, are home to the seminomadic populations of the Pemón aboriginals, who migrate for long periods to perform agricultural, hunting, fishing, and mining activities, with regular return visits to their home villages. Reaching these isolated communities can only be achieved by river navigation or small aircraft. Access to healthcare for this population is limited (≈2-day walk to closest hospital); according to reports from the Ministry of Health, the estimated diphtheria vaccination coverage rates during the first half of 2016 were <24%. This cluster of diphtheria cases prompted an epidemiologic investigation in the affected communities.

In Urimpatá, a 31-year-old Amerindian man (case-patient 1) who had recently returned home from a gold mining camp in Apoipó (4.744573°N, –61.477692°E) and 2 members of his household, his 4-year-old daughter (case-patient 2) and 9-year-old niece (case-patient 3), sought treatment for symptoms they had been experiencing for over a week. All 3 exhibited classic signs of diphtheria (Figure 1; Table) and did not have cutaneous lesions or neurologic signs or symptoms.

Pharyngeal samples from the index case-patient were collected on swabs and applied to glass slides, which were submitted for real-time PCR testing, as previously described (*8*). Compared with collecting the sample by scraping the dried sample from the glass slide, collecting the sample by rubbing the slide with a moist swab (wetted with phosphate-buffered saline) led to ≈100-fold higher yields of DNA in subsequent PCR assays. Samples were positive for *C. diphtheriae* toxin gene (*tox*) by real-time reverse transcription PCR; we observed cycle thresholds of ≈30

Table. Demographics and clinical characteristics of 10 Amerindians with suspected diphtheria cases, Wonken, Venezuela, 2017*

Case-patient no.	Age, y/sex	Location†	Signs and symptoms	Duration	Treatment	Outcome
1	31/M, returning miner	Urimpatá	Hyperthermia; dysphagia; odynophagia; dysphonia; gray adherent membranes; massive cervical lymphadenopathy	9 d	Azithromycin (500 mg, 2×/d for 10 d), 7-d cycle ampicillin/sulbactam, penicillin G benzathine (1.2 million units, IM, 1 dose), adult Td to contacts	Survived
2	4/F, household contact of case-patient 1	Urimpatá	Dysphagia; odynophagia; hemoptysis; fever; gray adherent membrane formation; cervical lymphadenopathy	7 d	Azithromycin (10 mg/kg,1×/d for 7 d), cefotaxime/clarithromycin at admission, Tdap vaccination	Survived, admitted to reference hospital
3	9/F, household contact of case-patient 1	Urimpatá	Abrupt onset of odynophagia; barking cough; dysphonia; stridor and gray adherent pseudomembranes covering tonsils, uvula, and pharynx	7 d	Azithromycin (10 mg/kg, 1×/d, 7 d), penicillin G benzathine (0.6 million units, IM, 1 dose), Tdap vaccination	Survived
4	14/F	Atanao	Fever; dysphonia; dysphagia; odynophagia	≈1 wk	No data	Died
5	4/M	Atanao	Dysphagia; odynophagia; dysphonia; hyporexia	≈1 wk	No data	Died
6	9/F	Urimpatá	Odynophagia; barking cough; dysphonia; stridor and gray pseudomembrane covering tonsils, uvula, and pharynx	≈1 wk	Azithromycin (10 mg/kg, 1×/d, 7 d), penicillin G benzathine (0.6 million units, IM, 1 dose), Tdap vaccination	Survived
7	9/F	Damasko	Dysphagia; odynophagia; dysphonia; fever; gray pseudomembrane covering tonsils, uvula, and pharynx	≈1 wk	Azithromycin (10 mg/kg, 1×/d for 7 d), penicillin G benzathine (0.6 million units, IM, 1 dose), Tdap vaccination	Survived
8	13/F	Damasko	Odynophagia; fever; small grayish membranes admixed with vesicles covering pharynx	≈1 wk	Azithromycin (10 mg/kg, 1×/d for 7 d), penicillin G benzathine (0.6 million units, IM, 1 dose), Tdap vaccination	Survived
9	Unknown	Atanao, in transit to Vista Alegre community	Reported as signs and symptoms suggestive of diphtheria	Unknown	No data	Unknown
10	Unknown	Atanao, in transit to Vista Alegre community	Reported as signs and symptoms suggestive of diphtheria	Unknown	No data	Unknown

*None of the case-patients were previously immunized or received diphtheria antitoxin as treatment. No case-patients had cutaneous or neurologic signs or symptoms. IM, intramuscular; Td, tetanus-diphtheria; Tdap, tetanus-diphtheria-acellular pertussis.
†Location coordinates: Urimpatá (5.128429°N, −61.380956°E); Atanao (5.128429°N, −61.380956°E); and Damasko (5.127997°N, −61.504152°E).

and the characteristic melting temperature by LightCycler hybridization probe (Sigma-Aldrich, St. Louis, MO, USA) melting curve analysis.

Persons with suspected diphtheria were given penicillin G benzathine and azithromycin (Table). Because erythromycin and penicillin G procaine were not available and to broaden antimicrobial coverage, we additionally gave case-patient 1 a 7-day course of ampicillin/sulbactam and case-patient 2 cefotaxime/clarithromycin. Case-patient 2 was transferred to the nearest hospital for further assistance. None of the case-patients identified in this outbreak were given diphtheria antitoxin because of supply shortages nationwide. A few days before case-patients 1–3 sought treatment, 2 deaths were reported in Atanao in persons exhibiting the same symptoms: a 14-year-old girl (died in the community) and 4-year-old boy (transferred to Boa Vista, Brazil, and died later) (Table). Our team could not reach the rest of the case-patients with suspected diphtheria in distant mines and villages, but local personnel registered cases in adult miners in Atanao. None of these case-patients had been previously immunized. All 41 Amerindians examined by the investigation team and their contacts from 3 different villages received toxoid immunization.

Conclusions

Although diphtheria is declining or has been eliminated from many countries because of high and widespread immunization coverage, the disease remains endemic to some developing countries, especially in regions under extreme poverty and low vaccine coverage (*3*). Over the past 4 years, Venezuela has faced a sharp reduction in oil revenue and undergone economic and political developments

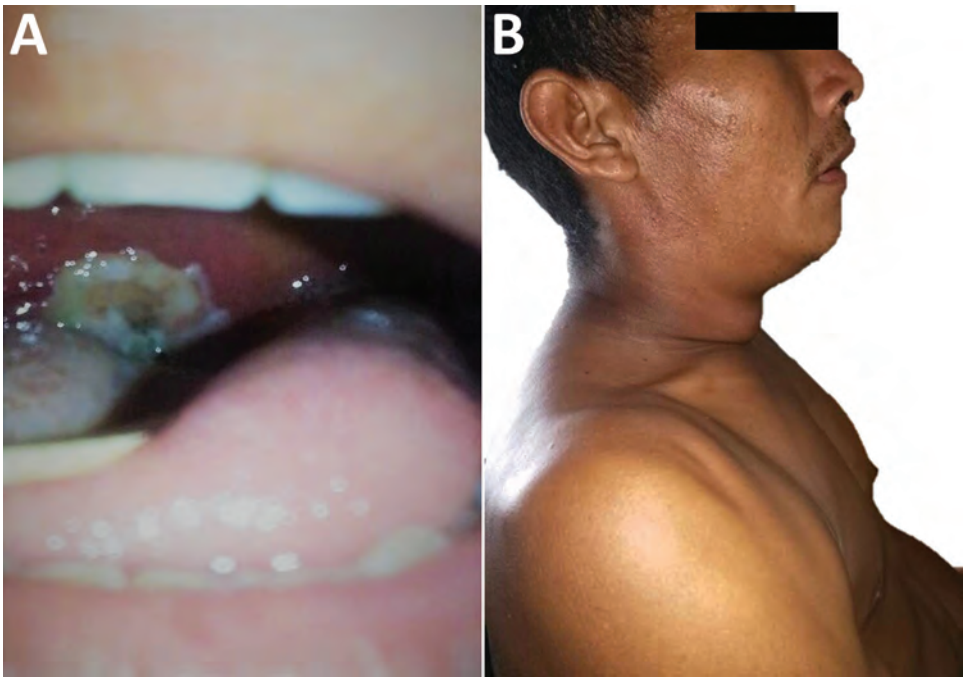

Figure 1. Physical characteristics of 31-year-old Amerindian male index case-patient with diphtheria, Wonken, Venezuela, 2017. A) Firmly adherent gray-white pseudomembrane in pharynx. B) Typical bull-like neck swelling with massive cervical adenopathies.

that have led to high inflation, impoverishment, and scarcity of basic resources largely affecting the public health infrastructure, resulting in long-term shortages of essential medicines and medical supplies, including vaccines for universal immunization programs and the immunization of specific risk groups against specific diseases (*9*). In addition, job shortages have pushed many locals into the practice of informal economy, food speculation, and, particularly, illegal gold mining.

The state of Bolivar is the largest federal entity in the country and the richest in mineral deposits. Legal and illegal mining activity is ongoing and rapidly growing, especially since the government announced the uncontrolled opening of the mining arch of the Orinoco River in 2011. This situation has led to an unprecedented increase in vectorborne disease transmission in these areas (*8*). From week 1 in 2016 through week 48 in 2017, a total of 609 suspected cases were reported in Venezuela, 227 of which were laboratory confirmed, with a case-fatality rate (CFR) of 15.5% (*7*). As of week 24 in 2017, a total of 282 (63%) cases were reported from Bolivar (Figure 2, panels A, B), with most occurring in the highly populated municipalities of Heres and Sifontes (*10*). However, to the best of our knowledge, diphtheria cases among the isolated Amerindian communities of the Savannah Plateau we examined has not been reported elsewhere.

Diphtheria is primarily controlled by vaccination and ensuring optimal herd immunity through high immunization coverage (*3*). The occurrence of diphtheria outbreaks reflects inadequate vaccination coverage. This outbreak was probably the consequence of the reintroduction of previously eradicated diseases by infected migrants traveling through mining districts and low vaccination rates.

Although calculated as 15.5%, the CFR of this epidemic cannot be accurately estimated because of the geographic isolation and elusive nature of most Amerindian communities. However, the CFR is expected to be higher because of the low vaccination rates and complete absence of effective diphtheria treatments in most of the region.

This outbreak highlights 2 issues: the unknown epidemiologic effect of diphtheria on isolated, immunologically naive Amerindian tribes in Venezuela and the difficulty of diagnosing diphtheria when clinicians are unfamiliar with the disease, tribe members have limited access to healthcare, and doctors lack treatment and laboratory facilities. Of note, the diagnosis of 1 diphtheria case was made by using pharyngeal samples applied to glass slides that were later processed by molecular methods; the enhanced DNA detection seen by using wet swabs is a valuable observation, potentially making diagnosis more accessible for resource-poor communities.

Reports of diphtheria affecting other aboriginal communities in Venezuela, such as the Kariña population (Gran Kashaama, Guanipa Plateau, Anzoategui), indicate that further investigation is necessary to elucidate the true extent of diphtheria. The public health challenge of improving the provision of preventive services and access to medical care for the isolated and underserved communities in Bolivar is needed to prevent future diphtheria outbreaks.

This study was supported in part by the Scottish Funding Council Global Challenges Research Fund (SFC/AN/12/2017).

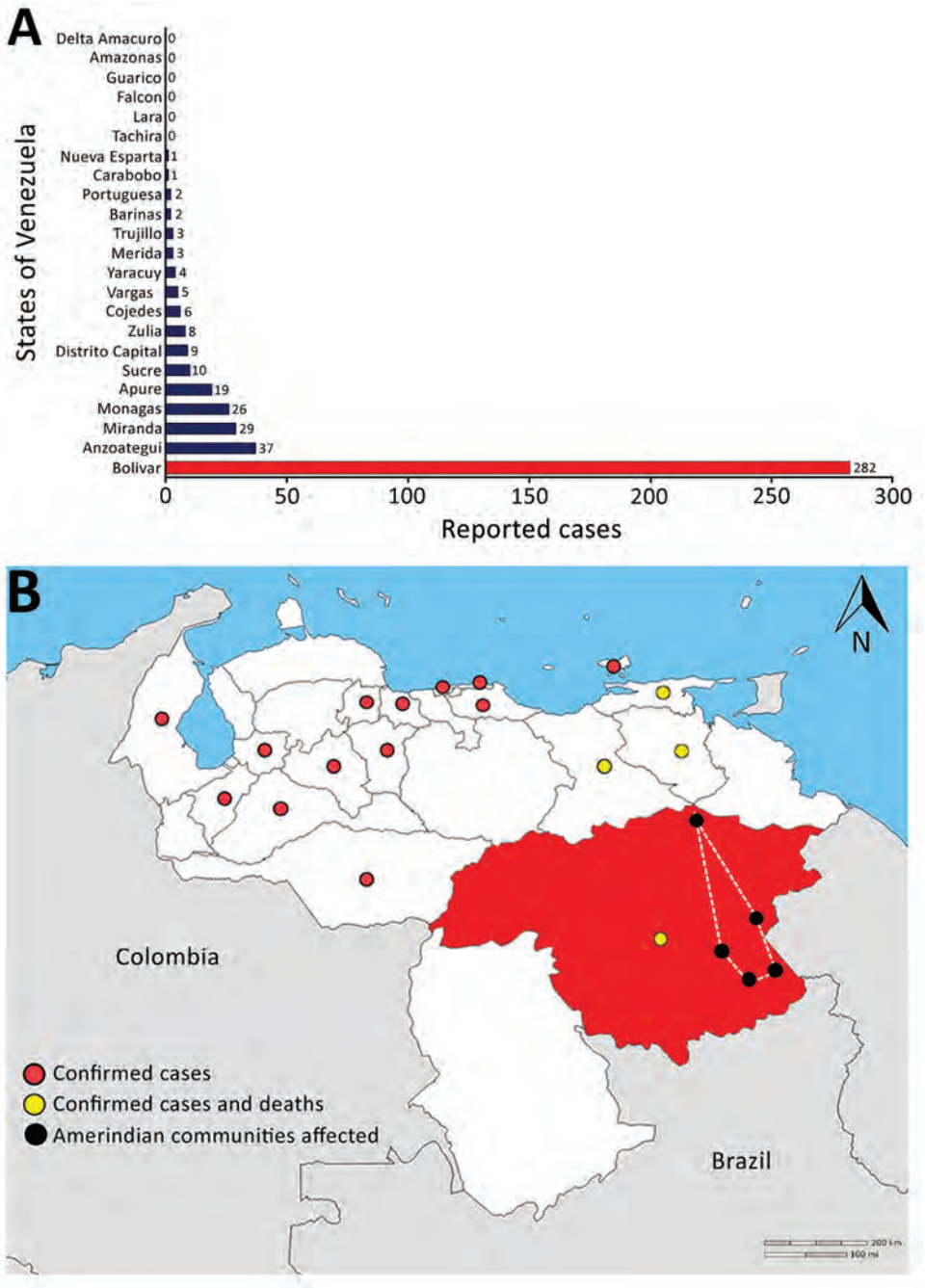

Figure 2. Suspected and confirmed diphtheria cases and deaths, by state, Venezuela, 2016–2017. The highest number of cases occurred in the state where Amerindians reside (Bolivar, red). A) Number of suspected cases of diphtheria reported from week 28 of 2016 through week 24 of 2017, by state. B) Location of confirmed cases and deaths, Venezuela, 2017. The affected Amerindian communities reside in the area within the dotted line. Map obtained from d-maps (http://d-maps.com/carte.php?num_car=4080&lang=es).

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Legionnaires' Disease Outbreak on a Merchant Vessel, Indian Ocean, Australia, 2015

Timothy J.J. Inglis, Chantal Spittle, Hilary Carmichael, Jaala Downes, Marilina Chiari, Adrian McQueen-Mason, Adam J. Merritt, Meredith Hodge, Ronan J. Murray, Gary K. Dowse

Two cases of Legionnaires' disease and 1 of Pontiac fever occurred among the crew of a merchant ship operating off the shores of Australia. PCR assays identified potential sources in the ship's cabins. Modification of maritime regulations for Legionnaires' disease prevention in commercial vessels is needed for nonpassenger merchant ships.

The risk for Legionnaires' disease (LD) is known on cruise liners (*1*–*3*) and is matched by recommendations for preventive measures (*4*,*5*). Environmental sources of *Legionella pneumophila* in ships are prone to transmit LD over several years through resistance to decontamination (*6*,*7*). As opposed to cruise liners, there are few reports of LD on working vessels, where occupational health risks differ (*8*). *Legionella* was detectable in potable water systems on 58% of 350 merchant vessels in a recent survey (*9*). There was no established precedent for environmental risk assessment or control when 2 LD cases occurred on a merchant ship off the northwestern Australian Indian Ocean coast in 2015. We therefore conducted an extended field investigation.

The Study

The first LD case-patient on the merchant ship sought treatment at the nearest hospital emergency department, and provided no alternative exposure source. After laboratory confirmation of this case, the crew disembarked and the vessel was required to lie at anchor offshore. After using emergency control measures by a private contractor, we obtained information on the ship's plumbing, including potable, fresh, and hot water systems; water storage; air conditioning; food preparation areas; and sleeping quarters.

We then boarded the ship for environmental investigation on August 27, 2015, to collect samples from potential fomites around the vessel at 33 locations, including cabins and potable water outlets. We collected PCR swab samples in duplicate from inside showerheads and sink faucets (also known as mixer taps) aerators in sleeping quarters and food preparation areas, including those used by LD case-patients and their neighbors. The contractor disinfected the water system by using super chlorination the next day, and collected a second environmental sample series on September 4. Additional targeted control measures included replacement of showerheads and removal of faucet aerators from cabins.

We collected a series of PCR swab samples from original test locations on October 12 to assess the residual health threat, and tested 24 of these samples on the ship (*10*). Duplicate samples were then tested in the reference laboratory (*10*). We analyzed showerheads removed from cabins (Figure 1). We tested samples of the inside surface of each showerhead and its O-ring gaskets by using PCR assays. We collected swab samples from potential reservoirs and tested for *Legionella* species: the O-rings; rinse samples from showerhead parts in sterile 0.08% NaCl solution for *Legionella* species; peptone water washings, showerhead contents, debris from a thermal mixing valve, fresh and pre–UV-treated water, showerheads, air conditioners, and faucets from cabins (*11*). We identified presumptive *Legionella* cultures on MWY and BMPA agars by using *Legionella* Latex Agglutination antisera (Oxoid; ThermoFisher Scientific Australia Pty Ltd, Scoresby, Melbourne, Victoria, Australia), and cultured for amoeba on showerhead rinse specimens. Detailed methods are provided in Technical Appendix Part 1 (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/24/7/17-1978-Techapp1.pdf).

In August 2015, the Western Australia Department of Health was notified of Legionnaires' disease confirmed by *L. pneumophila* serogroup 1 urinary antigen test in a member of the vessel's crew (case-patient 1), and was informed that other crew members had mild febrile

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Figure 1. Dismantled showerhead from nonpassenger merchant vessel showing multiple inner parts, including 7 O-rings, all of which were in contact with water passing through shower, Australia, 2015.

respiratory illness (Table 1). Later that day, another crew member, who had symptoms of severe bilateral pneumonia and pneumothorax, arrived at the regional hospital and required aeromedical evacuation for intensive care (case-patient 2). LD was confirmed by urinary antigen testing and PCR assay on bronchial washings. Other crew members who had nonpneumonic respiratory and other symptoms were investigated for legionellosis by using urinary antigen tests and serologic tests which proved negative, except in case-patient 3, who had *L. pneumophila* seroconversion and Pontiac fever that did not require hospital admission. The 3 cases all satisfied Australian LD case definitions (*12*). Case-patients 1 and 2 occupied adjacent cabins and case-patient 3 was 2 cabins away from case-patient 2 (Figure 2).

L. pneumophila was not isolated from any environmental samples. Legionella PCR result was positive in 7/10 cabins tested (13/27 samples) (Table 2). A PCR result was positive for showerheads or residual water from sink faucets in the cabins of 2 LD cases. In 5 other cabins, only faucets were positive (Figure 2). Detection of sludge or biofilm in the showerheads and faucets prompted replacement with better-designed showerheads and removal of faucet aerators. Only 2/79 samples collected on the second visit on September 4 were Legionella PCR positive; a significant reduction (χ^2, Yates' correction; 15.98, $p<0.001$). Only 1 of the 58 samples from the third series of samples was clearly PCR positive, from a faucet in a cabin unconnected to LD cases. The in-field PCR results were identical to the confirmatory reference laboratory replicate results. All 10 types of showerhead were rust-stained inside and smelled of chlorine. The most common showerhead types had either 7 silicone rubber O-rings or 1 complex silicone rubber gasket. Showerhead swabs and agar O-ring impressions grew profuse mixed bacteria, commonly *Pseudomonas aeruginosa*. Nonpneumophila *Legionella* sp. was isolated from 1 showerhead. Legionella PCR assays produced unambiguous positives in 13/16 showerheads (19/32 samples). Almost all O-rings from the common showerhead types were *Legionella* positive (Technical Appendix Part 2).

A recent study of nonpassenger merchant vessels (NPMVs) highlighted the risk for *Legionella* contamination of potable water systems (*9*), but did not establish a link with confirmed infections. Our investigation of *L. pneumophila* serogroup 1 infection in a merchant vessel's crew highlights the need to control *Legionella* in NPMV water systems, and the challenge of using PCR assays, which do not detect viable bacteria. Culture-dependent methods did not contribute to determination of the environmental source or route of dissemination. Preliminary control measures by external contractors may have prevented *Legionella* isolation from our environmental samples, but have doubtful long-term preventive value without sustained control measures because environmental persistence occurs in ships despite biocide treatment (*6*).

The survey vessel had a gross tonnage of 2,620, was 64 m long, 16 m wide, a draft of 4.7 m, and a crew of 27. It had 2 water storage tanks with 60,000 L capacity, an ultraviolet water sterilization unit, and 2 hot water geysers. These tanks were refilled from bunkers while in port, and replenished at sea by reverse osmosis. Showers were highlighted in a previous study of NPMV potable water systems (*9*), and aerator devices have been implicated as bacterial amplification sites in tropical and nosocomial outbreaks (*13*,*14*).

Multiple positive PCR results from water outlets in the cabins implicated the showers and faucets as means of

Table 1. Summary of confirmed legionellosis cases and results of environmental PCR testing in the case-patients' merchant vessel cabins, August 2015*

Case-patient	Age, y	Onset	Infection	Hospital	UAT	Serology	PCR†	Cabin no.	Cabin samples (Aug 27)		
									Shower water	Showerhead swab	Bathroom sink faucet
1	54	Aug 12	Lower respiratory	Regional	+	–	+	22	+	–	+
2	55	Aug 19	Lower respiratory	Tertiary	+	–	+	18	+	+	+
3	48	Aug 10	Mild respiratory	Not required	–	Conversion (0–2,048)	–	29	NA‡	NA‡	NA‡

* NA, not available; UAT, urinary antigen test; +, positive; –, negative.
†PCR-positive *Legionella pneumophila.*
‡Cabin in use on August 27, 2015. Water from hand basin faucet collected on August 20 by private agency was culture negative.

Figure 2. Accommodation deck plan, Australia, 2015. Cabins (n = 10) and other rooms (ACU, air conditioning unit) from which environmental samples were collected on August 27, 2015, are indicated in dark gray. PCR-positive locations are indicated by semicircles; upper, shower water or swab; lower, mixer tap water or swab. The 3 case-patients occupied cabins 18, 22, and 29.

infection. All showerheads on the vessel had interior moving parts to control spray settings and were the leading PCR-positive location. A rust-colored biofilm inside most showerheads indicated possible deterioration of iron pipes in the ship's distribution system, and persistence of *Legionella* in biofilms (*15*). The silicone rubber O-rings from the showerheads supported profuse growth of aquatic bacteria and were PCR positive for *L. pneumophila*. The O-rings formed a permanently wet niche for bacterial growth, and their movement will shear bacteria from biofilms. Faucet aerators also promote turbulent flow by mixing water and air under pressure. These results highlight the potential for *Legionella* aerosol generation. We recommended replacing the showerheads with a simpler plastic design, more suited to periodic removal, decontamination, and cleaning, and gravity drainage after daily use.

Conclusions

A cluster of *L. pneumophila* serogroup 1 infections in a vessel working in waters near Australia led to an environmental health assessment in which molecular methods enabled the field investigation team to implicate water outlets in crew quarters and tailor environmental controls. Deployment of quantitative PCR assays extended our investigative reach offshore, enabling faster return of the vessel to active service. The leadership and crew of nonpassenger merchant vessels operating in tropical waters need heightened *Legionella* awareness and require control measures more stringent than those applied in passenger vessels.

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T.J.J.I. and R.J.M. managed the patients and the corresponding clinical laboratory investigations. T.J.J.I. initiated the public health laboratory investigation in collaboration with G.K.D., who coordinated the public health response to the outbreak, liaised with the shipping company, maritime authorities, and regional public health unit. C.S. assisted with environmental

Table 2. *Legionella pneumophila* PCR results from environmental samples collected on merchant vessel, Australia, 2015*

	Samples collected on vessel, by date								Dismantled showerheads	
	August 27		September 4		October 12					
	Reference laboratory testing		Reference laboratory testing		In-field testing		Reference laboratory testing		Reference laboratory testing	
Sample type	Total	PCR+	Total	PCR+	Total	PCR+	Total	PCR+	Total	PCR+
Cabin shower heads	6	3	36	0	12	0	29	0	32	19
Cabin faucets	14	9	33	1	12	0	29	1	NA	NA
Air conditioning	4	0	NA	NA	NA	NA	NA	NA	NA	NA
Water supply	2	0	NA	NA	NA	NA	NA	NA	NA	NA
Others	1	1	10	1	NA	NA	NA	NA	NA	NA
Total results	27	13	79	2	24	0	58	1	32	19
PCR controls										
Positive, *Legionella* DNA extract	2	2	2	2	2	2	2	2	2	2
Negative, ultrapure water	6	0	16	0	5	0	12	0	6	0

*NA, not applicable; +, positive.

specimen collection from the start of the investigation and collected subsequent PCR sample series with the support of H.C. The first boarding party comprised T.J.J.I., C.S., and J.D., who together collected, documented, secured, and forwarded environmental samples and their contextual data. M.C. and A.M.-M. conducted the environmental bacteriology, in consultation with and under the guidance of M.H. T.J.J.I. conducted the *Legionella* PCR assays in the field. A.J.M. verified these in the reference laboratory and conducted the additional PCR analyses. T.J.J.I. collated input from the other authors and wrote the first draft, which was then edited by A.J.M. and G.K.D. before circulation to the other authors. All authors have contributed to this report, and have reviewed and checked its content for accuracy.

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Guiana Dolphin Unusual Mortality Event and Link to Cetacean Morbillivirus, Brazil

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During November–December 2017, a mass die-off of Guiana dolphins (*Sotalia guianensis*) began in Rio de Janeiro, Brazil. Molecular and pathologic investigations on 20 animals indicated that cetacean morbillivirus played a major role. Our findings increase the knowledge on health and disease aspects of this endangered species.

Cetacean morbillivirus (CeMV; family Paramyxoviridae) is a highly infectious pathogen responsible for numerous cetacean mass die-offs worldwide. Currently, there are 3 well-characterized strains (*1*), the porpoise morbillivirus, the dolphin morbillivirus, and the pilot whale morbillivirus, and 3 less-known strains, including the novel Guiana dolphin strain (GD)–CeMV, recently identified in a single specimen from Brazil (*2*). CeMV was detected in Ireland, England, and the Netherlands in 1988–1990 (*3*,*4*), when the porpoise morbillivirus strain was identified in a small number of stranded harbor porpoises (*Phocoena phocoena*). Since then, CeMV has been implicated as the causal agent of numerous outbreaks and also endemic, sporadic deaths involving multiple cetacean species throughout the North Sea, north Atlantic Ocean, Mediterranean Sea, Black Sea, Indian Ocean (Western Australia), and Pacific Ocean (Hawaii, Japan, and Australia) (*1*).

To date, no epizootics linked to CeMV causing the death of large numbers of marine mammals has been detected in the South Atlantic. A Guiana dolphin (*Sotalia guianensis*) stranded in Espírito Santo, Brazil, which tested positive for CeMV by reverse transcription PCR (RT-PCR) and immunohistochemistry, has been the only confirmed fatal case in South Atlantic cetaceans (*2*). We describe the results of pathologic and molecular investigations on 20 deceased Guiana dolphins in the onset of the ongoing unusual mortality event in Rio de Janeiro, Brazil.

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The Study

During November–December 2017, a unusual mortality event involving 56 Guiana dolphins began in Ilha Grande Bay, Rio de Janeiro (Brazil; 23°4′45″–23°13′38″S, 44°5′30″–44°22′28″W). This area is a relatively well-preserved ecosystem, and Guiana dolphin population census size in this area was estimated at ≈900 animals (*5*). Stranding occurrence for the same period in previous years ranged from 0 to 3 specimens. During this event, carcasses were recovered adrift or washed ashore. We performed necropsies on 20/56 (37.7%) Guiana dolphins and recorded epidemiologic and biologic data (Table 1).

We collected representative tissue samples of major organs and fixed them in 10% neutral buffered formalin or froze them at –80°C. For PCR analysis, we extracted viral RNA from frozen lung, brain, spleen, liver, and kidney (Table 1) using Brazol Reagent (LGC Biotecnologia Ltda, São Paulo, Brazil), according to the manufacturer's instructions. We used random primers and M-MLV Reverse Transcription Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) to synthesize cDNA. We performed amplification using primers targeting highly conserved fragments of the phosphoprotein (P) gene (*6*) and RNA-dependent RNA polymerase protein coded by the L gene (primers RES-MOR-HEN) as previously described (*2*,*7*).

We detected CeMV genome in 15/20 (75%) animals for the P gene and 6/6 (100%) animals for the L gene. We sequenced amplified products and compared them with sequences of CeMV using blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). We obtained identical sequences for the L gene, and 2 sequences with variation in 1 nucleotide position for the P gene. Sequencing of 405-bp amplified fragments of the CeMV P gene revealed 99%–100% identity to GD-CeMV (*2*) and 78%–82% identity with other CeMV strains. A 443-bp amplified fragment of the CeMV L gene revealed 74%–75% identity to CeMV and other morbillivirus species. Partial P and L gene sequencing and analysis using MEGA7 (http://megasoftware.net/) corroborate that the GD-CeMV strain differed from other morbilliviruses and represented a distinct lineage (Figure 1).

For histologic examination, we embedded formalin-fixed tissues in paraffin wax, processed them as routine,

Table 1. Individual epidemiologic stranding data and biologic data of 20 Guiana dolphins stranded or retrieved from Ilha Grande Bay, Rio de Janeiro, Brazil, November 2017*

No.	Date found	Location coordinates	Sex	Body length, cm	Age class	Body condition	Decomp status	Main gross findings	CeMV RT-PCR, tissue/result
1	9	−23.16738, −44.13948	F	177	Adult	Poor	Fr	Lactation; verminous pneumonia; pulmonary edema; mediastinal empyema; gastrointestinal petechiae, gastrointestinal parasitosis; absence of ingesta	Lung/pos,† brain/pos, spleen/pos
2	14	−23.01327, −44.44241	M	94	Calf	Moderate	Fr	Cyanotic mucous membranes; pulmonary edema; hepatic lipidosis; gastrointestinal petechiae	Lung/pos,† liver/pos
3	14	−23.15123, −44.32286	F	124	Juvenile	Poor	MA	Pulmonary edema; gastric ulcers; absence of ingesta	Lung/pos,† liver/pos
4	23	−23.0319, −44.54259	ND	71.5	Calf	ND	AA	ND (AA)	Lung/pos†
5	23	−23.03725, −44.55784	ND	189	Adult	ND	AA	Verminous pneumonia	Lung/pos,† liver/pos
6	23	−23.08996, −44.35695	M	160	Juvenile	Poor	MA	Verminous pneumonia; pulmonary edema; absence of ingesta	Lung/pos,† brain/pos, spleen/pos
7	24	−23.00963, −44.35695	ND	170	ND	ND	AA	ND (AA)	Kidney/pos†
8	24	−22.07896, −44.23156	ND	132	Juvenile	ND	AA	Black stained ingesta (plastic)	Liver/neg, spleen/neg
9	26	−23.03688, −44.55140	M	167	Juvenile	ND	AA	ND (AA)	Kidney/neg
10	25	−23.04786, −44.57191	M	123	Juvenile	Good	MA	Proliferative pleuritis and peritonitis; gastrointestinal parasitosis; absence of ingesta	Lung/pos,† brain/pos, spleen/pos
11	25	−23.03637, −44.55041	F	123	Juvenile	ND	AA	ND (AA)	Lung/pos,† brain/pos, spleen/pos
12	27	−23.01980, −44.44088	F	124	Juvenile	ND	MA	ND (AA); absence of ingesta	Lung/pos,† brain/pos, spleen/pos
13	27	−23.04542, −44.59536	F	142	Juvenile	ND	AA	Verminous pneumonia; gastrointestinal parasitosis; absence of ingesta	Lung/pos, brain/pos†
14	28	−23.16538, −44.63874	M	118	Juvenile	ND	AA	ND (AA); absence of ingesta	Lung/pos, brain/pos, spleen/pos†
15	28	−23.1325, −44.62048	F	182	Adult	ND	AA	Hydrothorax and ascites; verminous pneumonia; absence of ingesta	Lung/pos,† brain/pos, spleen/pos
16	28	−23.12665, −44.622	M	89	Calf	ND	AA	Autolysis; absence of ingesta	Lung/neg, spleen/ neg
17	29	−23.11585, −44.66409	F	170	Adult	ND	MA	Pulmonary edema; absence of ingesta	Lung/pos,† spleen/pos
18	29	−23.12927, −44.66989	M	156	Juvenile	Moderate	Fr	Fishing gear lesions; hydrothorax and ascites; verminous pneumonia; pulmonary edema; hemopericardium; gastroenteritis; gastrointestinal parasitosis; urinary bladder petechiae; pterygoid and tympanic bullae trematodiasis	Lung/neg, spleen/neg
19	29	−23.12726, −44.67302	F	144	Juvenile	Good	Fr	Fishing gear lesions; hydrothorax and ascites; verminous pneumonia; pulmonary edema; gastric ulcer; gastrointestinal petechiae	Lung/neg, spleen/neg
20	30	−23.07919, −44.55559	M	125	Juvenile	ND	AA	ND (AA); absence of ingesta	Lung/pos,† brain/pos

*Collection period was November 9–December 29, 2017. AA, advanced autolysis; decomp, decomposition; Fr, fresh; MA, moderate autolysis; ND, not determined; neg, negative; no., animal no.; pos, positive; RT-PCR, reverse transcription PCR.
†Amplified fragment sequenced.

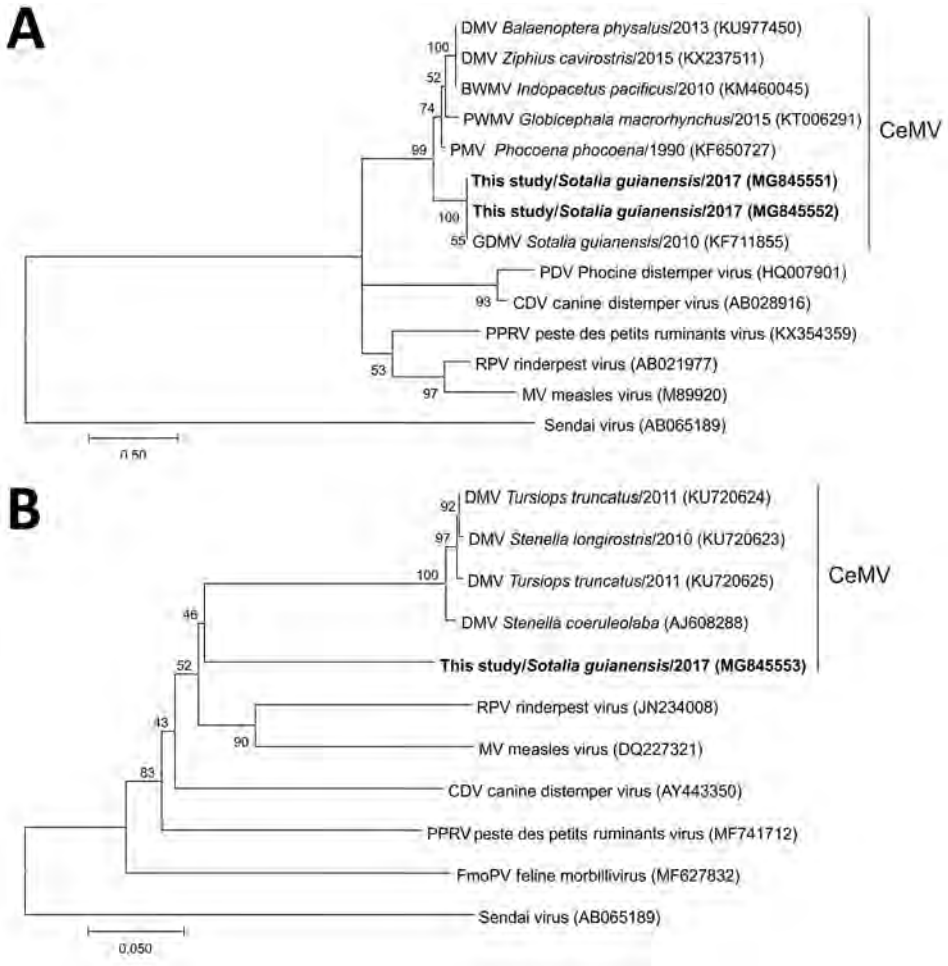

Figure 1. Phylogenetic tree showing partial sequence of A) morbillivirus phosphoprotein and B) large protein genes of cetacean morbillivirus (CeMV) isolates found in stranded Guiana dolphins (*Sotalia guianensis*) from Rio de Janeiro, Brazil (bold), 2017, and those of other previously described morbilliviruses. Sendai virus was added as an outgroup. Trees were generated by the maximum-likelihood method (A) and neighbor-joining method (B); bootstrap values (1,000 replicates) are indicated at the internal nodes. For comparison, recognized CeMV strains were included when available. Sequence names are followed by species, year of stranding (when available), and GenBank accession number. Scale bars indicate nucleotide substitutions per site. PMV, porpoise morbillivirus; DMV, dolphin morbillivirus; BWMV, beaked whale morbillivirus; PWMV, pilot whale morbillivirus.

and stained them with hematoxylin and eosin. We recorded detailed histopathologic findings of 6 animals positive for CeMV by RT-PCR (Table 2). One specimen had lesions consistent with CeMV infection, including marked multifocal, subacute bronchointerstitial pneumonia with type II pneumocyte hyperplasia, syncytia, and scattered intraepithelial, intranuclear, and intracytoplasmic inclusion bodies (INCIBs); mild to moderate multifocal histiocytic and lymphoplasmacytic mastitis with necrosis and epithelial INCIBs (Figure 2, panels A–C); and multicentric lymphoid depletion. In addition, most animals had moderate to severe verminous bronchopneumonia and pleuritis with morphologic evidence of pulmonary arterial hypertension, multicentric eosinophilic and necrotizing lymphadenitis, and chronic aortic endarteritis by adult nematodes and pulmonary endarteritis by migrating larval nematodes histomorphologically compatible with *Halocercus brasiliensis* (*8*). Other common findings included moderate to poor body condition and lack of ingesta with small amounts of feces. Two (10%) of the 20 animals (which were negative for CeMV by RT-PCR) showed typical external net markings and multiorgan acute hemodynamic alterations (congestion, edema, and hemorrhage) supporting asphyxia due to bycatch as the cause of death.

We performed immunohistochemistry studies using a monoclonal antibody against the nucleoprotein antigen of canine distemper virus (CDV-NP mAb; VMRD Inc., Pullman, WA, USA), as described (*2*). In lung tissue sections (cases 1, 2, 11, and 13), we evaluated number and distribution of immunopositive cells and immunolabeling intensity. Lung samples from all animals tested showed widespread and intense immunolabeling in bronchial, bronchiolar, and alveolar epithelium, alveolar macrophages, and syncytia (Figure 2, panels D,E).

In this investigation, typical histopathologic findings consistent with CeMV were evident in 1 animal, indicating a systemic infection. Although chronic bronchointerstitial pneumonia and multicentric lymphoid depletion observed in most animals are common findings in CeMV-infected cetaceans, these lesions were considerably overlapped by *H. brasiliensis* endoparasitosis. The pathologic signatures of GD-CeMV remain unknown. No other CeMV strain has been described in the South Atlantic Ocean. In subacute and chronic CeMV presentations, fatalities are often ascribed

Table 2. Summary of histopathologic findings for 6 Guiana dolphins recovered from Ilha Grande Bay, Rio de Janeiro, Brazil, 2017*

No.	Tissue	Histopathologic findings
1†	Lung	Marked, multifocal chronic bronchointerstitial pneumonia and proliferative pleuritis with sclerosis, type II pneumocyte hyperplasia, syncytia/multinucleated cells, rare INCIBs, calcified nematode debris and edema; multifocal tunica media hypertrophy/hyperplasia
	Mammary gland	Mild to moderate, multifocal, chronic lymphoplasmacytic and histiocytic mastitis with acinar ectasia, inspissated secretion, scattered necrosis, ceroid pigment and moderate INCIBs in epithelium
	Heart	Mild, focal, subacute fibrinous pericarditis; mild, multifocal myocardial fibrosis
	Kidney	Mild, multifocal, chronic membranous glomerulonephritis with glomerulocysts, tubular proteinosis, protein casts, and scattered tubuloepithelial necrosis
	Pulmonary lymph node	Mild, multifocal, chronic nodular eosinophilic and necrotizing lymphadenitis with fibrosis and hemosiderosis; diffuse lymphoid depletion
	Mediastinal lymph node	Mild, multifocal, chronic eosinophilic lymphadenitis; diffuse congestion
	Spleen	Diffuse congestion and multifocal, acute capsular hemorrhage; extramedullary hematopoiesis
	Adrenal	Mild, multifocal, acute corticomedullary hemorrhage
	Aorta	Mild, segmental, chronic proliferative endarteritis
	Liver	Moderate, multifocal, chronic bile duct adenomatous hyperplasia
	Uterus	Moderate, multifocal, chronic arteriosclerosis and arterial elastosis
	Glandular stomach	Mild, diffuse mucosal hyperplasia; multifocal arterial tunica media hypertrophy/hyperplasia
2‡	Lung	Mild, multifocal, acute interstitial pneumonia associated with marked alveolar edema, hemorrhage and alveolar histiocytosis, syncytia/multinucleated cells and keratin spicules
	Kidney	Mild, multifocal, acute tubular degeneration and necrosis; mild, multifocal, acute tubular proteinosis and protein casts; marked, focal, acute perirenal hemorrhage
	Pulmonary lymph node	Moderate, diffuse cortical and paracortical lymphoid depletion with scattered lymphocytolysis
	Prescapular lymph node	Diffuse congestion with focal acute hemorrhage and erythrophagocytosis; sinus vascularization
	Spleen	Moderate, diffuse, lymphoid depletion with sinus histiocytosis; extramedullary hematopoiesis
	Heart	Moderate, multifocal, acute subendocardial and epicardial hemorrhage
	Adrenal	Marked, multifocal, acute cortico-medullary hemorrhagic necrosis
	Esophagus	Focal acute hemorrhage in serosa
	Urinary bladder	Edema and focal acute hemorrhage in serosa
	Penis	Urethral luminal hemorrhage with single cell epithelial necrosis/apoptosis
	Cerebrum, cerebellum	Diffuse leptomeningeal congestion and perivascular edema in neuroparenchyma
10§	Lung	Mild to moderate, multifocal, chronic suppurative bronchopneumonia with adult nematodes (*Halocercus* sp.); multifocal alveolar, bronchiolar and bronchial mineralization
	Keratinized stomach	Mild, focal, chronic proliferative gastritis
	Skeletal muscle	Scattered acute hyaline myocyte degeneration and necrosis
11	Ascending aorta	Marked, segmental, chronic fibrosing and proliferative endarteritis with chondroid metaplasia and calcification; moderate, focal, chronic granulomatous periarteritis; mild, multifocal intimal fibroelastosis
	Aortic (semilunar) valve	Mild, multifocal, chronic intimal/endocardial fibroelastosis
12¶	Keratinized stomach	Mild, focal, chronic mononuclear gastritis
13#	Lung	Marked, multifocal, chronic suppurative to pyogranulomatous bronchopneumonia with bronchial/olar sclerosis, adult and larval nematodes (*Halocercus* sp.) and moderate, multifocal, chronic proliferative and fibrosing (villous) pleuritis; marked, multifocal, chronic tunica media arterial hypertrophy/hyperplasia with stenosis
	Skin	Mild, multifocal, chronic irregular epidermal hyperplasia
	Pyloric stomach	Moderate, focal, chronic granulomatous gastritis with numerous trematode ova (compatible with *Pholeter gastrophilus*)

*INCIBs, intranuclear and intracytoplasmic inclusion bodies; no., animal no.
†No significant lesions were observed for large intestine, thyroid, skin, trachea, cerebrum, cerebellum, spinal cord, or skeletal muscle.
‡No significant lesions were observed for keratinized and pyloric stomach, tongue, aorta, small intestine, pancreas, or trachea.
§Unable to observe lesions in liver, spleen, kidney, testicle, trachea, small and large intestine, skin, periaortic lymph node, cerebrum, or cerebellum because of advanced autolysis of animal.
¶Unable to observe lesions in ovary, skin, liver, skeletal muscle, heart, kidney, spleen, large intestine, bladder, lymph node, or adrenal gland because of advanced autolysis of animal.
#Unable to observe lesions in small intestine, liver, skeletal muscle, adrenal gland, bladder, kidney, or heart because of advanced autolysis of animal.

to secondary infections (e.g., toxoplasmosis, aspergillosis) (*9*,*10*). In our cohort, autolysis precluded microscopic examinations in some animals, so we could not draw further pathologic conclusions. Nonetheless, moderate to severe parasitosis by *H. brasiliensis* likely accounted for severe illness in most cases. Intense viral replication in the mammary acinar epithelium in a lactating female may imply a vertical transmission route, in addition to the horizontal aerogenous and direct contact routes (*10*). Therefore, future pathologic and epidemiologic studies in the South Atlantic should consider vertical transmission. Two cases from this cohort were bycaught, further supporting the multifactorial nature of the ongoing unusual mortality event.

The Guiana dolphin is a coastal and estuarine delphinid endemic from southern Brazil to Central America and one of the most threatened South Atlantic cetaceans, for which

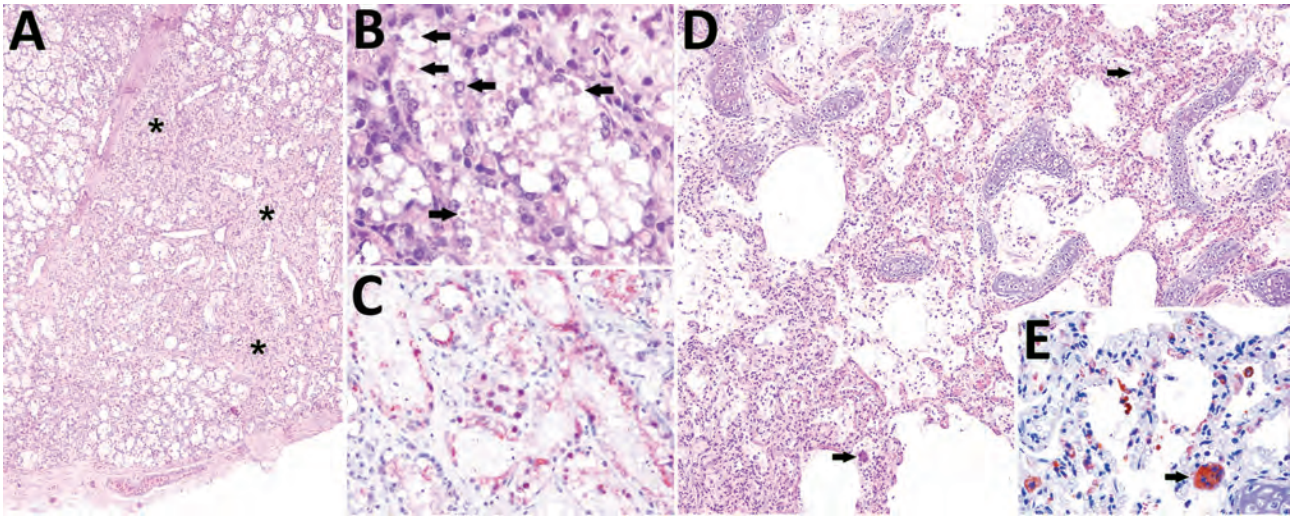

Figure 2. Cetacean morbillivirus–associated histopathologic findings in 2 Guiana dolphins (*Sotalia guianensis*), a female adult (case 1, panels A–C) and a male calf (case 2, panels D–E). A) The mammary gland parenchyma is focally disrupted by lymphohistiocytic inflammatory cells (not visible at this magnification) associated with collapsed and lost acini, and mild fibrosis (asterisks). Original magnification ×40; hematoxylin and eosin (H&E) stain. B) Swollen and degenerating mammary acinar epithelial cells have numerous intracytoplasmic and intranuclear inclusion bodies (arrows). Original magnification ×200; H&E stain. C) Degenerating and sloughed mammary acinar epithelial cells have intense granular cytoplasmic and intranuclear immunolabeling, identified by immunohistochemistry (IHC) for canine distemper virus (CDV), known to cross react with cetacean morbilliviruses. D) Pulmonary area displaying interstitial pneumonia with mildly thickened alveolar septa and alveoli containing proteinaceous edema, scattered fibrin strands, and small numbers of pleocellular inflammatory cells including occasional syncytia (arrows). Original magnification ×100; H&E stain. E) Degenerating and necrotic type I pneumocytes, sloughed and adhered type II pneumocytes, alveolar and septal macrophages, syncytia (arrow) and circulating (intravascular) mononuclear cells display intense immunolabeling. Original magnification ×400; IHC for CDV.

recent studies demonstrate severe population decline (*11*). Because of its near-shore distribution and site fidelity (*12*), the Guiana dolphin is susceptible to the effects of human activities (e.g., habitat degradation, chemical pollution, noise, and bycatch) (*13*). Many intricate and complex anthropic and natural factors interplay and modulate the decline of species. Human activities are by far the major threat and cause for decimation of cetacean populations (*14*); however, natural factors such as highly infectious pathogens, e.g., CeMV, may drive decimating events in susceptible hosts (*15*).

Conclusions

We provide compelling molecular and pathologic evidence associating GD-CeMV infection with the ongoing Guiana dolphin mass die-off near Rio de Janeiro, Brazil. As of January 2018, this event had resulted in the deaths of >200 Guiana dolphins in southern Rio de Janeiro state, and the deaths appeared to be extending southward. The environmental consequences and conservation effects, coupled with the anthropogenic threats, are expected to be dramatic. The factors underlying the die-off are being investigated, but our results indicate that GD-CeMV plays a major contributory role. Our findings increase the body of knowledge on health and disease aspects of this endangered species.

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Use of Urea Wash ELISA to Distinguish Zika and Dengue Virus Infections

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Serologic testing remains crucial for Zika virus diagnosis. We found that urea wash in a Zika virus nonstructural protein 1 IgG ELISA distinguishes secondary dengue from Zika virus infection with previous dengue (sensitivity 87.5%, specificity 93.8%). This test will aid serodiagnosis, serosurveillance, and monitoring of Zika complications in dengue-endemic regions.

The rapid spread of Zika virus and its association with fetal microcephaly and other birth defects (congenital Zika syndrome) present a pressing need for sensitive and specific diagnostic tests (*1*,*2*). Centers for Disease Control and Prevention guidelines for laboratory diagnosis of Zika virus infection include a positive reverse transcription PCR as soon as possible after symptom onset to confirm Zika virus and a negative IgM test result to exclude Zika virus (*3*). Serologic testing remains a crucial component of Zika diagnosis because most Zika virus infections are asymptomatic, many persons seek Zika virus testing beyond the period during which RNA is detectable, and Zika virus can be transmitted sexually or after asymptomatic infection (*1*–*3*).

Zika virus belongs to the family *Flaviviridae*, in which several arboviruses, including the 4 serotypes of dengue virus (DENV-1–4), cause substantial disease in humans. Because of cross-reactivity of antienvelope antibody to Zika virus and other flaviviruses, positive or equivocal IgM results based on envelope protein require further testing with plaque-reduction neutralization tests (*3*–*5*). These tests can confirm acquisition of Zika virus as the first flavivirus infection (primary Zika virus [pZIKV] infection) but are more challenging to interpret for those who have experienced previous flavivirus infections.

Several studies have demonstrated that DENV–immune serum and monoclonal antibodies can enhance Zika virus replication in vitro and in vivo (*6*–*9*) and raised concerns that previous DENV infection might increase the risk for and severity of congenital Zika syndrome. A recent study reported that a nonstructural protein 1 (NS1)–based blockade of binding ELISA can distinguish Zika virus and other flavivirus infections (*10*). However, it cannot distinguish pZIKV, Zika virus infection with previous dengue (DENV-ZIKV), and secondary DENV (sDENV) infections, which is critical in Zika virus– and DENV-endemic regions.

The Study

The Institutional Review Board of the University of Hawaii approved this study of coded serum or plasma samples (CHS #17568, CHS #23786). Convalescent-phase samples from patients with confirmed Zika virus infection who were either DENV-naive (designated as pZIKV panel) or previously exposed to DENV (designated as DENV-ZIKV panel) were from a cohort study in Nicaragua (*11*) (Table). Convalescent-phase samples from patients who had symptoms compatible with Zika virus infection and detectable anti-DENV IgG during the acute phase (probable DENV-ZIKV panel) came from Bahia, Brazil (*12*). Convalescent-phase or post–convalescent-phase (3 months–6 years after symptom onset) samples from patients who had confirmed primary DENV (pDENV) or sDENV infection came from Taiwan, Hawaii (USA), and Nicaragua; 12 flavivirus-naive samples had been previously described (*12*,*13*).

The expression and purification of Zika virus NS1 protein (strain HPF2013) have been described (*12*). Purified DENV-1 NS1 protein was from the Native Antigen Company (Oxford, UK). NS1-IgG and NS1-IgM ELISAs as well as cutoff, positive, and negative controls in each plate have been described (*12*). The relative optical density (rOD) values were OD divided by the mean OD of positive controls. For the urea wash, we added 100 μL urea (4–8 mol/L) to each well at room temperature for 5 min between the second and third washings of NS1-IgG ELISA after the primary antibody (total 4 washings) (*14*). We used the 2-tailed Mann-Whitney test to determine p values comparing 2 groups (GraphPad Prism 6, https://www.graphpad.com/scientific-software/prism).

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Table. Sampling time, serotype, and sources of serum/plasma panels in study of use of urea wash ELISA to distinguish Zika and dengue virus infections*

Panel sample collection times	Category	Sampling time after symptom onset, mean (range)	No. patients	Source (no. patients) and year(s) of sample collection	Shown in
Single time point					
pDENV-1	Convalescent to postconvalescent	138 (19–263) d	16	Taiwan (4), 2001–2002; Hawaii, USA (12), 2015	Figure 1
pZIKV	Convalescent	17 (14–24) d	20	Nicaragua, 2016	Figure 1
sDENV	Convalescent	14 (8–35) d	24	Taiwan, 2001–2002	Figure 1
DENV-ZIKV	Convalescent	16 (14–19) d	20	Nicaragua, 2016	Figure 1
Probable DENV-ZIKV	Convalescent	10 (6–14) d	19	Brazil, 2015–2016	Figure 1
sDENV	Postconvalescent	3.2 (3–4) mo	6	Taiwan (2), 2006–2009; Nicaragua (4), 2006–2008	Figure 2
sDENV	Postconvalescent	12 (12–12) mo	18	Nicaragua, 2006–2008	Figure 2
sDENV	Postconvalescent	19.7 (18–24) mo	14	Taiwan (10), 2006–2009; Nicaragua (4), 2006–8	Figure 2
sDENV	Postconvalescent	71 (67–72) mo	5	Taiwan, 2006–2009	Figure 2
Sequential time points					
sDENV	Postconvalescent	10 (3–18) mo	3	Nicaragua, 2006–2008	Figure 2

*DENV-ZIKV, ZIKV infection with previous dengue; pDENV-1, primary dengue virus 1 infection; pZIKV, primary Zika virus infection; sDENV, secondary dengue virus infection.
†3–4 samples/patient.

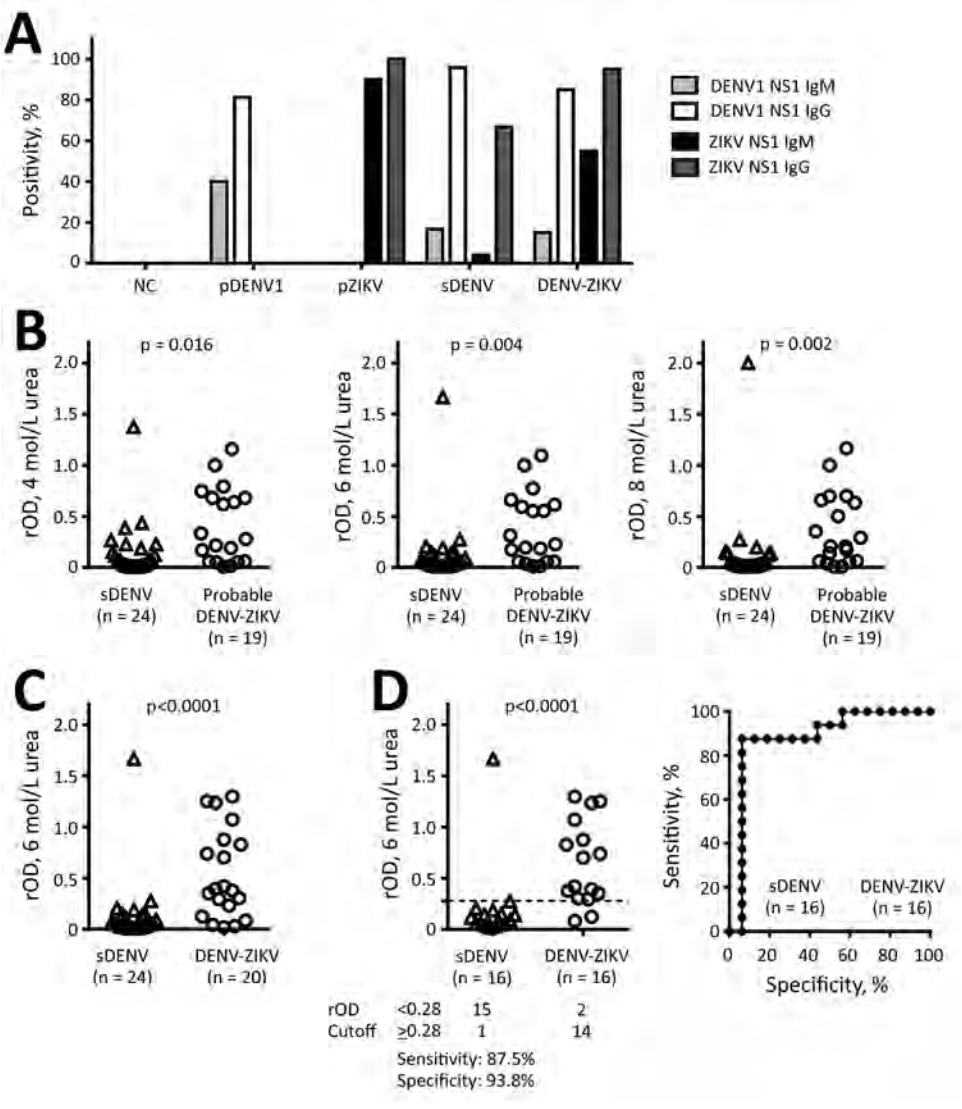

Figure 1. NS1 IgM and IgG ELISAs and urea wash in ZIKV–NS1 IgG ELISA. A) Positivity rates for each panel. Only samples collected <3 months after symptom onset were tested for IgM. B) sDENV infection and probable DENV-ZIKV panels were tested with different concentrations (4, 6, and 8 mol/L) of urea wash. C, D) sDENV and DENV-ZIKV panels were tested with 6 mol/L urea wash: C) all samples; D) samples positive for both DENV-1–NS1 and ZIKV-NS1 IgG ELISAs. Sensitivity and specificity are based on relative optical density cutoff at 0.28 (dashed line). Receiver-operating characteristics are shown in the graph on the right. Data are the mean of 2 experiments (each in duplicate). The 2-tailed Mann-Whitney test was used. DENV, dengue virus; DENV-ZIKV, confirmed Zika virus infection with previous exposure to DENV; NS1, nonstructural protein 1; pDENV1, primary DENV-1 infection; pZIKV, primary ZIKV infection; rOD, relative optical density; sDENV, secondary DENV infection; ZIKV, Zika virus.

To evaluate convalescent-phase samples from pDENV1, pZIKV, sDENV, and DENV-ZIKV panels, we used 4 ELISAs. The primary DENV1 and pZIKV panels recognized their own NS1 without cross-reactivity (Figure 1, panel A; online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/7/17-1170-Techapp1.pdf). The DENV-ZIKV panel recognized Zika virus and DENV NS1. The sDENV panel recognized not only DENV but also Zika virus NS1, especially in IgG ELISA, suggesting that cross-reactivity in NS1 IgG ELISA between sDENV and DENV-ZIKV panels is a challenge for NS1-based serologic tests for Zika virus infection.

We next investigated whether a urea wash in Zika virus NS1 IgG ELISA could distinguish sDENV and DENV-ZIKV infections. Different concentrations (4, 6, and 8 mol/L) of urea wash resulted in significantly lower rODs in the sDENV panel than in the probable DENV-ZIKV and DENV-ZIKV panels (Figure 1, panels B, C). We chose the 6 mol/L urea wash for further analysis, considering its optimal cutoff value (data not shown). Comparing the samples with positive Zika virus– and DENV-1–NS1 IgG ELISA results (Figure 1, panel D), a cutoff rOD of 0.28 can distinguish the 2 panels with 87.5% sensitivity and 93.8% specificity.

We further investigated whether a 6 mol/L urea wash could reduce IgG cross-reactivity to ZIKV-NS1 by sDENV panel at later times. For the 43 post–convalescent-phase samples, positivity rates in DENV-1–NS1 IgG ELISAs decreased from 100% (3–6 months after symptom onset) to 80% (5–6 years) and for ZIKV-NS1 IgG ELISAs from 83.3% to 40%, respectively (Figure 2, panels A, B). After 6 mol/L urea wash in ZIKV-NS1 IgG ELISA, rOD decreased greatly, resulting in 4.7% having an rOD ≥0.28 (Figure 2, panel C). Results for sequential samples from 3 patients with sDENV infection (Figure 3, panel C) were generally consistent with the results from cross-sectional samples; rODs were all <0.28 after 6 mol/L urea wash (Figure 2, panel D).

Although neutralization tests can confirm pZIKV infection, they remain difficult to interpret for patients who have previously experienced flavivirus infections, including sDENV and DENV-ZIKV infections. A recent study reported reduced cross-neutralization against Zika virus among samples from patients with sDENV infection >6 months after symptom onset; however, 23% still cross-neutralized Zika virus (*15*). Our findings suggest that a 6 mol/L urea wash in ZIKV-NS1 IgG ELISA can distinguish DENV-ZIKV and sDENV panels. It is conceivable that

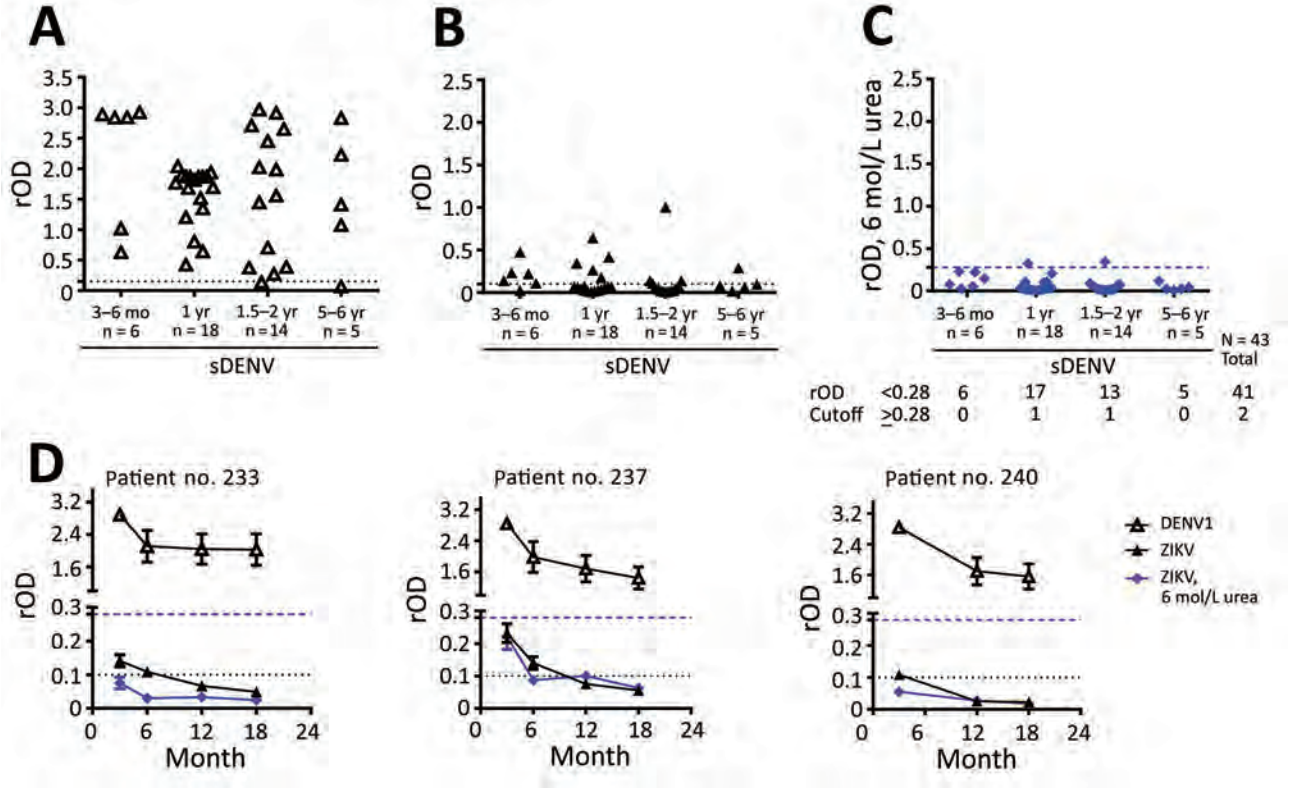

Figure 2. NS1 IgG ELISAs with urea wash for sDENV infection panel over time. A) DENV-1–NS1 IgG ELISA; B) ZIKV–NS1 IgG ELISA; and C) ZIKV-NS1 IgG ELISA with 6 mol/L urea wash for sDENV samples collected from 3 months to 6 years after symptom onset. D) Sequentially collected samples from 3 patients with sDENV infection. Dotted lines indicate relative optical density cutoffs of ELISAs; dashed lines indicate rOD cutoff (0.28) of ELISA with 6 mol/L urea wash. Data are expressed as mean ± SD (for panel D) of 2 experiments (each in duplicate). The 2-tailed Mann-Whitney test was used. DENV, dengue virus; NS1, nonstructural protein 1; rOD, relative optical density; sDENV, secondary DENV infection; ZIKV, Zika virus.

during sDENV infection, memory B cells recognizing NS1 residues that are conserved within the DENV serocomplex and between DENV and Zika virus expand greatly and generate high-avidity anti-NS1 antibodies through affinity maturation (*9*,*13*). During Zika virus infection among those with previous DENV infection, memory B cells recognizing NS1 residues conserved between DENV and Zika virus will expand and generate high-avidity antibodies. Moreover, naive B cells recognizing Zika virus–specific NS1 residues will also expand; the combination of these 2 types of anti-NS1 antibodies may contribute to anti-NS1 antibodies with higher avidity, which remain bound after urea wash, compared with those from the sDENV panel (Figure 1, panel C; online Technical Appendix Figure 1).

This study has limitations. First, we tested only convalescent- and post–convalescent-phase samples. Second, the sample size in each panel was small; future studies with larger samples, including acute-phase and more sequential samples, are needed to validate these observations. Third, because our previous study showed cross-reactivity of anti-DENV NS1 antibodies within the DENV serocomplex (*5*), we chose only DENV-1-NS1 IgG ELISA for this study; there was no difference in the positivity rates of DENV-1-NS1 IgG ELISA between primary DENV-1 and sDENV-2 panels and between sDENV-1, sDENV-2, and sDENV-3 panels (online Technical Appendix Table 2). Fourth, given the global spread of Zika virus to regions where different flaviviruses are prevalent, development of serodiagnostic assays to distinguish Zika virus and other medically relevant flaviviruses remains to be explored.

Conclusions

Our method of combined ELISAs plus 6 mol/L urea wash in Zika virus-NS1 IgG ELISA is simple, cost-effective, and applicable for use at field sites. This method could be used for routine serologic testing for Zika virus in dengue-endemic regions and for serosurveillance and Zika pregnancy studies to clarify epidemiology, transmission, and complications (*1*–*3*). Because congenital Zika syndrome may affect infants during growth and development, IgG-based NS1 ELISAs plus 6 mol/L urea wash could be used in retrospective studies to elucidate the contribution of pZIKV infection alone or Zika virus infection with previous DENV to the full spectrum of congenital Zika syndrome (*1*,*2*).

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A US provisional patent application (no. 62/534,654), entitled Distinguish Zika and Dengue Virus Infection in ELISA, has been filed through the University of Hawaii.

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Seroepidemiologic Survey of Crimean-Congo Hemorrhagic Fever Virus in Selected Risk Groups, South Africa

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Crimean Congo hemorrhagic fever virus (CCHFV) is endemic in South Africa, but whether mild undiagnosed cases occur is unclear. In a seroepidemiologic survey, only 2 of 387 adults considered at risk because of occupational or recreational activities had evidence of previous infection. Seroprevalence in South Africa remains low within the groups investigated.

Crimean-Congo hemorrhagic fever virus (CCHFV; family *Nairoviridae*, genus *Orthonairovirus*) is a tick-borne virus that causes human disease (*1*). Humans can be infected through the bite of an infected tick, squashing of an infected tick, or contact with blood or tissues of infected humans or animals. Farmers, herders, veterinarians, hunters, abattoir workers, and persons engaged in informal slaughtering are thus at an increased risk (*2*).

CCHFV is endemic to Africa, the Middle East, Asia, and southeastern Europe (*2*). Its seroprevalence differs geographically between and within regions. In Greece, CCHFV seroprevalence among various prefectures ranges from 0% to 27.5% (*3*). In Turkey, seroprevalence ranges from 10% to 19.6%, with estimates of 88% subclinical infections (*4*,*5*). Studies among high-risk populations in Iran (*6*) and Oman (*7*) documented seroprevalences of ≈12% and 26.2%, respectively. The factors responsible for subclinical infections are unknown but have been suggested to include differences in host immune responses, viral load, and virus pathogenicity.

In South Africa, surveillance studies found a high prevalence of CCHFV in adult *Hyalomma* ticks and high antibody prevalence in wild and domestic animals (*8*). Two studies among farm workers conducted in the 1980s found a seroprevalence of 1.3%–1.5% (*8*,*9*).

We studied whether the low seroprevalence identified among farm workers reflects that in other high-risk groups. We selected groups on the basis of risk for exposure because of occupational activity, recreational activity, or both and included abattoir workers, horse handlers, recreational hunters, and large animal veterinarians. In South Africa, horse handlers frequently remove ticks from horses, and recreational hunters are exposed to ticks on animals and tissues from animals. The Free State and Northern Cape provinces are farming regions known to have *Hyalomma* ticks. In this study, we therefore aimed to determine the current seroprevalence among healthy persons in selected high-risk groups within CCHFV-endemic provinces of South Africa.

The Study

The Health Sciences Research Ethics Committee of the University of the Free State provided ethics approval for this study (HSREC34/2016 and ETOVS152/06). A questionnaire inquiring about demographic and occupational information and possible risk exposure was completed for each volunteer participant. We collected 374 blood samples from volunteers during April 2016–February 2017 and included 13 stored serum samples, collected mainly from large animal veterinarians in 2012.

Specific IgG against CCHFV was detected by using a commercial indirect immunofluorescence assay (IFA) (Crimean-Congo Fever Virus Mosaic 2 IFA; Euroimmun, Lubeck, Germany), according to the manufacturer's instructions. Each IFA slide contains biochips coated with transfected cells expressing either CCHFV glycoprotein (GP), nucleoprotein (NP), or untransfected cells. We screened serum samples at a dilution of 1:100 and retested positive or undetermined samples using serum diluted 2-fold from 1:100 to 1:800. Samples reacting against CCHFV NP only were retested using 2-fold dilutions from 1:10 to 1:80 for evidence of low reactivity against CCHFV GP. We tested all positive reactors for IgM using IFA.

Most (299 [77.3%]) participants were from the Free State province (Table 1; Figure). Most participants were male (343 [88.6%]), and most resided in urban areas (254 [65.6%]). Ages ranged from 18 to 76 years (median 33 years).

Abattoir workers formed the largest high-risk group sampled, accounting for 215 (55.6%) of participants. An additional 30 (7.8%) participants were involved in informal slaughtering. Most participants reported multiple potential routes of exposure, either currently or in the past,

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Table 1. Study participants at high risk for Crimean-Congo hemorrhagic virus infection, South Africa

Risk group	No. (%) participants, n = 387
Abattoir workers	215 (55.6)
Informal slaughterers	30 (7.8)
Veterinarians	11 (2.8)
Horse handlers	64 (16.5)
Recreational hunters	49 (12.7)
Farmers	12 (3.1)
Other*	6 (1.6)

*Tick exposure, livestock exposure, farm worker.

resulting in considerable overlap among the different groups. A total of 163 (42.1%) participants reported tick exposure; 27 (7%) participants reported an illness after a tick bite or exposure to animal blood or tissue, and 18 (4.7%) reported a confirmed diagnosis of tick-bite fever.

Of the 387 serum samples tested, 2 tested positive for CCHFV IgG. The seropositive samples were collected from men, both 27 years of age, who were abattoir workers at the same abattoir in rural Free State. Both participants had additional potential CCHFV risk exposures, including tick exposure and hunting (Table 2). Neither participant reported any illness after a tick bite or after exposure to animal blood or tissue, and both were healthy at the time the blood was collected.

IgG-positive samples for both men tested IgM negative, which excluded acute or recent infections. The IgG titers obtained for participant 1 were 1:100 against the NP and 1:80 against the GP antigen. The IgG titer for participant 2 was 1:400 against the NP antigen only. The variation in antibody titers against NP and GP is not unexpected and has been reported previously, although the reason is unknown. Evidence exists of serologic cross-reactivity between CCHFV and Hazara virus; however, previous serologic surveys suggest that Hazara virus is not circulating in South Africa (*10*).

CCHFV is considered an emerging virus with potential for spread to areas where *Hyalomma* ticks are present (*2*). In terms of which populations are particularly at risk for infection, a retrospective study in Iran found that 34% of confirmed CCHFV cases were in slaughterhouse workers and 28.5% were in farmers or livestock handlers (*11*). Similarly, a study in Kenya found that 19% of patients with a febrile illness were eventually confirmed to have CCHFV; the highest prevalence (29.3%) occurred among farmers (*12*). Many seroprevalence studies have documented the unanticipated finding of asymptomatic or mild disease. In Greece, the low number of cases of infection with the high seroprevalence has been suggested to indicate circulation of a strain that is potentially of lower virulence (*13*). The use of different serologic methods must be considered in comparing the results of surveys. However, a frequently used assay in recent

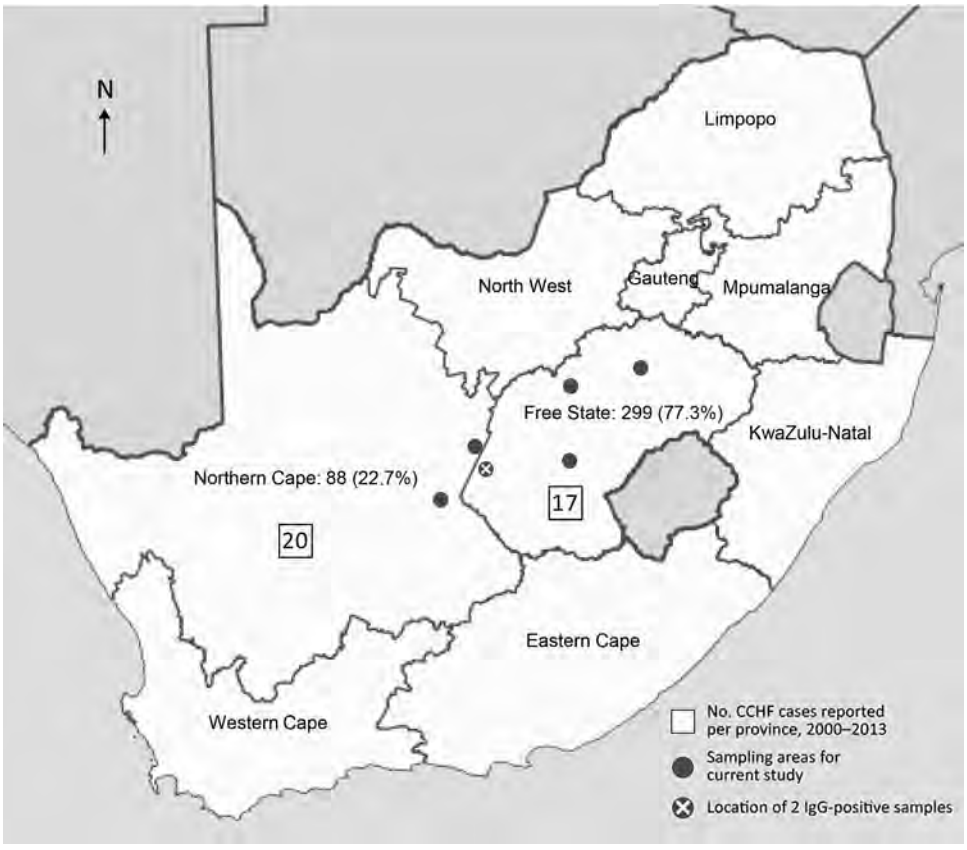

Figure. Number and percentage of participants in the Free State Province (177 abattoir workers, 30 informal slaughterers, 11 veterinarians, 32 horse handlers, 46 recreational hunters, 3 other) and Northern Cape Province (38 abattoir workers, 32 horse handlers, 3 recreational hunters, 12 farmers, 3 other) in a seroprevalence study of Crimean-Congo hemorrhagic fever virus in South Africa, April 2016–February 2017.

Table 2. Detailed risk exposure for the 2 Crimean-Congo hemorrhagic fever virus–positive study participants, South Africa

Risk exposure	Participant 1	Participant 2
Farmer	No	Yes
Farm worker	Yes	No
Tick exposure	Yes	Yes
Livestock exposure	No	Yes
Hunter	Yes	Yes
Abattoir worker	Yes	Yes
Horse handler	Yes	Yes
Veterinarian	No	No
Veterinary researcher	No	No
Laboratory worker	No	No
Major illness after tick bite	No	No
Major illness after exposure to animal blood or tissue	No	No

studies is the commercially available ELISA (Vektor-Best, Novosibirsk, Russia) that, when compared with IFA, showed reasonably comparable sensitivities of 80.4% and 86.1%, respectively.

Since 1981, when CCHFV was first identified in South Africa, sporadic cases have been reported mainly from the country's central farming areas. The principal vectors associated with transmission, *H. truncatum* and *H. rufipes* ticks, are widely distributed throughout South Africa but are most numerous in the interior of the country, where prevalence of CCHF antibody in cattle serum is high; up to 96% of cattle serum tested in some herds was positive. CCHFV was isolated in ≈20% of tick pools, representing both tick species, collected in the North West province (*10*,*14*). The Free State and Northern Cape provinces are considered CCHFV-endemic regions. During 1981–2013, a total of 192 CCHFV cases were laboratory confirmed in South Africa; 54 laboratory-confirmed cases were documented during January 2000–August 2013. Of these, 17 (31.5%) were from the Free State and 20 (37%) from the Northern Cape (Figure) (*15*).

Conclusions

Our seroprevalence results were similar to those obtained 30 years ago among farm workers (*8*,*9*), indicating that, even within high-risk groups, CCHFV remains uncommon in South Africa. The number of participants was low but focused on selected high-risk populations.

The 2 participants with CCHFV IgG tested negative for CCHFV IgM and recalled no previous illness resembling severe Crimean-Congo hemorrhagic fever, which might hint at possible mild CCHF in South Africa. However, in view of documented widespread CCHFV and antibodies in ticks and animals, respectively, in South Africa (*8*), more widespread mild infection would be anticipated. Our study conducted among groups at high risk for CCHFV in the endemic regions of Free State and Northern Cape provinces found that the seroprevalence of the virus remains low as previously shown, despite multiple potential routes of exposure in the cohort.

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Diagnosis of Methionine/Valine Variant Creutzfeldt-Jakob Disease by Protein Misfolding Cyclic Amplification

Daisy Bougard, Maxime Bélondrade, Charly Mayran, Lilian Bruyère-Ostells, Sylvain Lehmann, Chantal Fournier-Wirth, Richard S. Knight, Robert G. Will, Alison J.E. Green

A patient with a heterozygous variant of Creutzfeldt-Jakob disease (CJD) with a methionine/valine genotype at codon 129 of the prion protein gene was recently reported. Using an ultrasensitive and specific protein misfolding cyclic amplification–based assay for detecting variant CJD prions in cerebrospinal fluid, we discriminated this heterozygous case of variant CJD from cases of sporadic CJD.

Variant Creutzfeldt-Jakob disease (vCJD) is a neurodegenerative infectious disease caused by transmission of a cattle prion disease (bovine spongiform encephalopathy) to humans (*1*). Most vCJD cases have occurred in the United Kingdom, where an estimated 1 in 2,000 persons is potentially asymptomatically infected, although there is some uncertainty about interpretation of detection of abnormal prion protein (PrP^{TSE}) in appendix tissues on which this incidence is based (*2*) (Public Health England, https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/546883/hpr2616.pdf).

Until recently, all clinical cases of vCJD for which the prion protein gene has been analyzed have been shown to be methionine homozygous at codon 129, a genotype present in almost 40% of Caucasian populations. The report of the first definite heterozygous methionine/valine vCJD patient who died in 2016 (*3*) underlined previous concern about a possible second wave of vCJD cases (*4*). The clinical features of this patient were more similar to those of patients with sporadic CJD (sCJD) than to those with vCJD. This patient had met the agreed surveillance diagnostic criteria for probable sCJD (*5*). However, vCJD was diagnosed during an autopsy; florid plaques were observed by histologic examination of the brain and peripheral detection of PrP^{TSE} in lymphoid tissues. Western blot analysis of brain tissue confirmed a type 2B molecular profile of PrP^{TSE}, which is characteristic for vCJD.

A diagnostic test to identify methionine/valine heterozygous vCJD cases is urgently needed to enable discrimination between heterozygous vCJD and sCJD and in view of the potential reservoir of methionine/valine heterozygous asymptomatic vCJD carriers in the blood donor population. We developed a highly sensitive and specific assay that accurately detects vCJD prions in blood even before the occurrence of clinical signs (*6*). We adapted this assay, which was based on protein misfolding cyclic amplification (PMCA) (*7*), for specific detection of vCJD in cerebrospinal fluid (CSF) and confirmed the ability of this assay to differentiate patients with atypical heterozygous vCJD from patients with sCJD.

The Study

We blindly analyzed 98 CSF samples provided by the National CJD Research and Surveillance Unit (Edinburgh, Scotland, UK) and the Centre Hospitalier Universitaire de Montpellier (Montpellier, France) after obtaining appropriate consent. Clinicians distributed CSF samples into blinded panels from the United Kingdom and France; 41 from patients with vCJD; 23 from patients with sCJD; 1 from a patient with genetic CJD; and 33 from patients with non-CJD, including samples from patients with Alzheimer's disease and patients with nonneurodegenerative diseases.

CSF samples were thawed at room temperature and used directly in PMCA. We performed PMCA amplification by using brains from humanized transgenic mice as substrate for normal prion protein. After successive rounds of 160 cycles of PMCA for 15 min and sonication for 20 s, we detected PrP^{TSE} by using Western blot after digestion with proteinase K (*6*).

Of the 98 CSF samples analyzed, our assay identified 40 of 41 cases of clinical vCJD, including the methionine/valine heterozygous patient, thus showing a diagnostic sensitivity of 97.6% (95% CI 87.1%–99.9%) (Table). One CSF sample from a probable case of vCJD showed a negative result. After decoding by clinicians, we retested this sample in duplicate; it showed a positive result.

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Table. Analysis of CSF samples from patients with CJD and controls by PMCA*

Diagnosis	No. patients with positive detection of PrP^{TSE} in CSF and codon 129 genotype/no. tested				Analytical performance, % (95% CI)
	Total	MM	MV	VV	
Clinical CJD					
Variant CJD	40/41†	37/38	1/1	NA	Diagnostic sensitivity 97.6 (87.1–99.9)
Definite	29/29	28/28	1/1	NA	
Probable	10/11	8/9	NA	NA	
Possible	1/1	1/1	NA	NA	
Sporadic CJD	0/23†	0/7	0/12	0/3	Analytic specificity 100 (93.7–100)
Definite	0/14	0/2‡	0/10	0/1‡	
Probable	0/9	0/5	0/2	0/2	
Genetic CJD	0/1	0/1	NA	NA	Analytic specificity 100 (93.7–100)
Non-CJD					Analytic specificity 100 (93.7–100)
Alzheimer's disease	0/12	ND	ND	ND	
Other nonneurodegenerative diseases	0/21	ND	ND	ND	

*CJD, Creutzfeldt-Jakob disease; CSF, cerebrospinal fluid; MM, methionine homozygous; MV, methionine/valine heterozygous; NA, not available; ND, not determined; PMCA, protein misfolding cyclic amplification; PrP^{TSE}, abnormal prion protein; VV, valine homozygous.
†Genotyping of the prion protein gene at codon 129 was not conducted for 2 patients with variant CJD and 1 patient with sporadic CJD.
‡The protease-resistant protein subtype was available for 3 patients with definite sporadic CJD and showed an equal distribution of MM1, MM2a, and VV2a.

Our assay also showed high analytical specificity; 0 of 57 potentially cross-reacting CSF specimens from patients with sCJD, gCJD, Alzheimer's disease, and other nonneurodegenerative diseases showed a positive result (specificity 100% [95% CI 93.7%–100%]) (Table). The case-patient with methionine/valine heterozygous vCJD was specifically discriminated from the 12 methionine/valine heterozygous neuropathologically confirmed sCJD case-patients tested.

We then compared by using Western blot the PrP^{TSE} molecular signature obtained for the clinical vCJD amplified samples from classical methionine homozygous cases and the new methionine/valine heterozygous vCJD case with that of the reference brain sample from a patient with vCJD (Figure). As expected, the profile obtained after PMCA amplification of the CSF from the methionine/valine heterozygous vCJD patient was similar to those obtained for methionine homozygous vCJD patients. The characteristic type 2 mobility and clear predominance of the diglycosylated isoform was obtained for all vCJD patients before or after amplification.

Conclusions

We report a specific detection method that enables clinical diagnosis of a heterozygous methionine/valine heterozygous vCJD patient. This patient was the first definite heterozygous patient described since the start of the vCJD epidemic in the United Kingdom in 1996 (*3*). Clinical diagnosis was difficult because clinical signs and symptoms, particularly cerebral appearance by magnetic resonance imaging, were suggestive of sCJD (*3*). The vCJD blood test (direct detection assay) developed by the Medical Research Council Prion Unit (London, UK) (*8*) showed a negative result for this case-patient. We found characteristic vCJD prion protein amplification in the CSF, which led to a specific diagnosis of vCJD because sCJD samples did not show positive results by PMCA. This result also demonstrates the possibility of amplifying methionine/valine heterozygous vCJD prion protein by PMCA with a substrate from humanized transgenic mice that overexpress homozygous

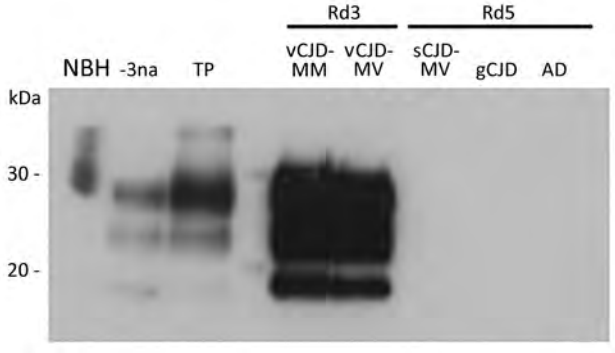

Figure. Western blot analysis of vCJD prions obtained after amplification by protein misfolding cyclic amplification (PMCA) of cerebrospinal fluid (CSF) from 2 patients with vCJD (MM and MV) and 3 control patients and a crude reference brain homogenate from a vCJD patient (National Institute for Biological Standards and Control [Ridge, UK] no. NHBY0/0003). Abnormal prion protein patterns were assessed by using antibody 3F4 after digestion of samples with proteinase K. A total of 20 μL of each sample was subjected to electrophoresis on a 12% polyacrylamide gel. Lane NBH, negative control brain homogenate from a person without CJD and no digestion with proteinase K (National Institute for Biological Standards and Control no. NHBZ0/0005); lane –3na, Western blot control (10^{-3} dilution of vCJD reference brain sample without amplification); lane TP, positive control for amplification (10^{-6} dilution of vCJD reference brain sample after 1 round of PMCA); lane vCJD-MM, CSF from a patient with MM vCJD after 3 rounds of PMCA; lane vCJD-MV, CSF from a patient with MV vCJD after 3 rounds of PMCA; lane sCJD-MV, CSF from a patient with MV sCJD after 5 rounds of PMCA; lane gCJD, CSF from a patient with gCJD after 5 rounds of PMCA; lane AD, CSF from a patient with Alzheimer's disease after 5 rounds of PMCA. CJD, Creutzfeldt-Jakob disease; gCJD, genetic CJD; MM, methionine homozygous; MV, methionine/valine heterozygous; Rd, round (1 round indicates 80 cycles of PMCA); sCJD, sporadic CJD; vCJD, variant CJD.

methionine prion protein (*9*). However, PMCA analysis should be performed in a Biosafety Level 3 laboratory and requires highly experienced personnel.

Iatrogenic transmission of vCJD by blood transfusion has been documented in 3 recipients of nonleukodepleted erythrocyte concentrates from blood donors during development of disease (*10*). One additional probable case of vCJD transmission by blood transfusion was identified during an autopsy of a methionine/valine heterozygous patient who died from a nonneurologic disorder and in whom vCJD prion protein was detected in the spleen (*11*). The presence of infectivity in blood of the definite methionine/valine heterozygous vCJD patient involved in our study is uncertain and requires further investigation.

From a clinical point of view, prion amplification technologies, such as PMCA and real-time quaking-induced conversion (RT-QuIC), have already shown their sensitive detection of disease-related prion protein in biologic fluids (*6,12–14*). Independent studies have shown that detection of prion protein seeding activity in CSF by RT-QuIC might have a specificity of 99%–100% for diagnosis of sCJD (*13,15*). Application of RT-QuIC and PMCA for CSF samples might represent a suitable strategy for premortem discrimination between sCJD and vCJD including methionine/valine heterozygous case-patients, particularly for cases with a heterozygous codon 129 genotype in which clinical distinction between sCJD and vCJD is problematic.

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Highly Pathogenic Avian Influenza A(H5N8) Virus, Cameroon, 2017

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Highly pathogenic avian influenza A(H5N8) viruses of clade 2.3.4.4 spread into West Africa in late 2016 during the autumn bird migration. Genetic characterization of the complete genome of these viruses detected in wild and domestic birds in Cameroon in January 2017 demonstrated the occurrence of multiple virus introductions.

Since the first detection in China in 1996, highly pathogenic avian influenza (HPAI) viruses of the H5 subtype descendent of the H5N1 virus A/goose/Guangdong/1/1996 (Gs/GD/96) have evolved into multiple genetic clades (*1*,*2*) that have been threatening poultry worldwide. Since 2010, clade 2.3.4 has demonstrated an unusual propensity to replace its N1 subtype and acquire different neuraminidase (NA) genes from unrelated avian influenza viruses through reassortment, which has resulted in the emergence of new viral subtypes within the Gs/GD/96 H5 lineage (e.g., N2, N5, N6, N8). In late spring 2016, reassortant HPAI A(H5N8) clade 2.3.4.4 (group B) virus was detected in migratory wild birds in Qinghai Lake, China (*3*), and in the salt lake system of Uvs Nuur, on the border between Mongolia and the Russian Federation (*4*). Since then, this newly emerged virus has caused multiple outbreaks of the disease in poultry and wild birds across Europe, Asia, and the Middle East and has extended not only to countries in northern, central, and western Africa, as did the previous Gs/GD/96 H5 lineage outbreaks, but also to the eastern and southern parts of Africa (*5*). We investigated the epidemiology of the outbreaks in early 2017 of HPAI H5N8 virus in domestic and wild birds in Cameroon and determined the possible origin of this virus through whole-genome analyses.

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The Study

On January 2, 2017, high death rates were reported in Indian peafowl (*Pavo cristatus*) in a backyard exotic poultry farm located in the town of Makilingaye (Tokombéré district of Mayo-Sava division), a village neighboring Nigeria in the far-north region of Cameroon. Samples were collected by the Cameroon Epidemio-Surveillance Network (Reseau d'Epidemio-Surveillance au Cameroun [RESCAM]) of the Ministry of Livestock, Fisheries and Animal Industries and were sent to the National Veterinary Laboratory (LANAVET) in Garoua, where the H5N8 subtype was diagnosed. Almost all the peafowl (103/107) died within ≈2 weeks. Death in chickens (*Gallus gallus domesticus*, 24/24) housed in the same compound was delayed and appeared 5 days later than in peafowl. Following the laboratory confirmation of the first outbreak, the Cameroon government, through the Ministry of Livestock, Fisheries, and Animal Industries, implemented prompt and strong control measures to stop the spread of the virus and reduce the risk of human infections. Stamping out was deployed together with movement restrictions and virological surveillance; disinfection of premises and contact materials was intensified. The RESCAM team conducted a routine avian influenza survey in the Maroua, Yagoua, and Guidiguis central poultry markets in the far-north region. All the samples were analyzed at LANAVET Garoua and Annex Yaounde; H5N8 virus was detected in 5 birds (1 pigeon, 1 chicken, 2 guinea fowls, and 1 duck) out of 122 birds.

We sequenced the hemagglutinin (HA) and NA gene segments of the virus A/Indian peafowl/Cameroon/17RS1661-6/2017, identified from an Indian peafowl in Makilingaye, at the Istituto Zooprofilattico Sperimentale delle Venezie in Legnaro, Italy, along with the complete genomes of 2 positive samples collected from domestic birds (chicken and duck) and the partial genome (all segments except for polymerase acidic protein [PA] and polymerase basic protein 1 [PB1]) of a sample from

Table. Epidemiologic information of viruses characterized in study of highly pathogenic avian influenza A(H5N8) virus, Cameroon, January 2017*

Virus	Sample type	Location	EpiFlu accession numbers for 8 gene segments
A/chicken/Cameroon/17RS1661-1/2017	Tracheal swab	Maroua market	HA, MG650619; MP, MG650622; NA, MG650626; NP, MG650630; NS, MG650632; PA, MG650635; PB1, MG650638; PB2, MG650641
A/duck/Cameroon/17RS1661-3/2017	Tracheal swab	Yagoua market	HA, MG650620; MP, MG650623; NA, MG650627; NP,MG650629; NS, MG650633; PA, MG650636; PB1, MG650637; PB2, MG650639
A/pigeon/Cameroon/17RS1661-4/2017	Cloacal swab	Maroua market	HA, MG650621; MP, MG650624; NA, MG650628; NP, MG650631; NS, MG650634; PA, NR; PB1, na; PB2, MG650640
A/Indian peafowl/Cameroon/17RS1661-6/2017	Tracheal swab	Makilingaye	HA, MG650619; MP, NR; NA, MG650626; NP, NR; NS, NR; PA, NR; PB1, NR; PB2, NR

*HA, hemagglutinin; MP, matrix protein, NA, neuraminidase; NR, not reported; NP, nucleoprotein; NS, nonstructural proteins; PA, polymerase acidic protein; PB1, polymerase basic protein 1; PB2, polymerase basic protein 2.
†From GISAID EpiFlu database (online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/24/7/17-2120-Techapp2.xlsx).

a wild pigeon, all identified in the Maroua and Yagoua markets (Table). A detailed description of the methods used for sequencing and genetic analyses is provided in online Technical Appendix 1 (https://wwwnc.cdc.gov/EID/article/24/7/17-2120-Techapp1.pdf) and details on the HA gene segments used for the analysis are given in Technical Appendix 2 (https://wwwnc.cdc.gov/EID/article/24/7/17-2120-Techapp2.xlsx). We submitted consensus sequences to GenBank (accession nos. MG650618–41).

Topology of the phylogenetic tree based on the HA gene segment showed that the 4 H5N8 viruses detected in Cameroon in 2017 fell within genetic clade 2.3.4.4 group

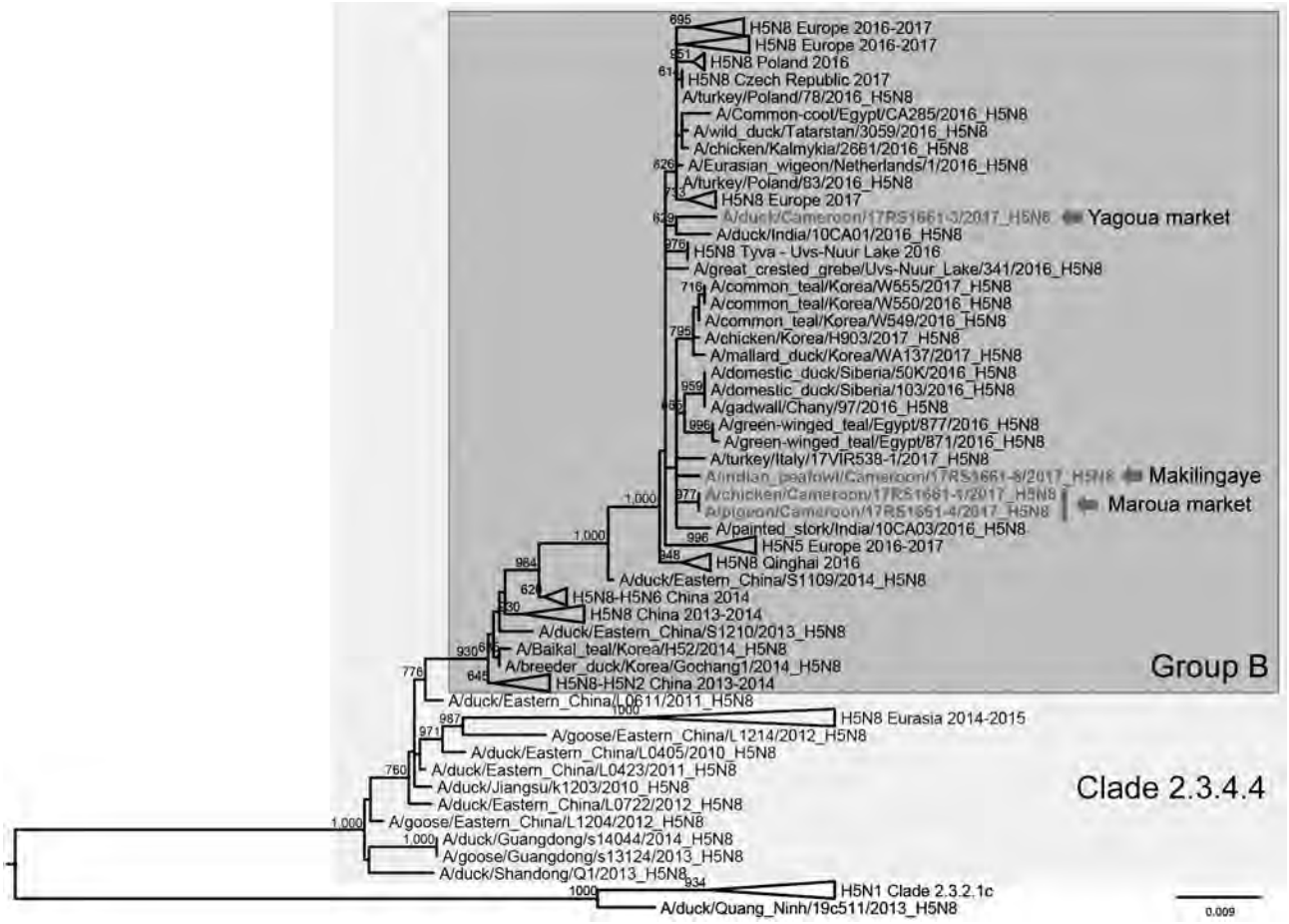

Figure. Maximum-likelihood phylogenetic tree of the HA gene of highly pathogenic avian influenza A(H5N8) virus from Cameroon (bold) and reference viruses. Arrows indicate location where Cameroon viruses were obtained. Bootstrap supports >600/1,000 are indicated next to the nodes. Scale bar indicates numbers of nucleotide substitutions per site.

B (Figure; online Technical Appendix 1 Figure 1). The HA gene segments of the 3 viruses from Makilingaye (A/Indian peafowl/Cameroon/17RS1661–6/2017) and Maroua market clustered together; similarity ranged from 99.5% (between the viruses collected from Makilingaye and Maroua markets) to 100% (between the 2 viruses from Maroua market). These segments grouped with H5N8 viruses collected during 2016–2017 in South Korea, Egypt, Italy, Siberia, and India (similarity between 99.3% and 99.6%). However, the virus A/duck/Cameroon/17RS1661-3/2017 identified in Yagoua market clustered separately and showed the highest similarity (99.3%) to a virus from India (A/duck/India/10CA01/2016) (Figure).

We confirmed the different clustering of the viruses collected from distinct markets in Cameroon by the analyses of the other gene segments, which suggests the occurrence of ≥2 distinct viral introductions in the country. Specifically, sequences of the 2 viruses from Maroua market were identical for all the available gene segments (Table) and clustered with viruses collected in Asia, Europe, and Egypt (online Technical Appendix 1 Figures 2–8). On the other hand, the virus A/duck/Cameroon/17RS1661-3/2017 grouped with the A/duck/India/10CA01/2016 virus (similarity 94.3–99.9%), except for the nucleoprotein (NP) gene, where it showed the highest identity (99.3%) with a virus collected in 2016 in the Russian Federation (Uvs Nuur Lake). The lack of genetic information on other H5N8 viruses detected in Africa makes it impossible to pinpoint how these viruses entered Cameroon and spread, nor is it feasible to determine where the 2 introductions occurred. The time to the most recent common ancestor estimated for the HA gene (online Technical Appendix 1) suggested that 2 introductions into Africa may have occurred almost simultaneously during March–December 2016 (online Technical Appendix 1 Figure 9). However, analyses of the amino acid sequences show that A/duck/Cameroon/17RS1661-3/2017 possesses the N319K mutation in the NP protein, which has been reported to enhance polymerase activity and stimulate vRNA synthesis in mammalian cells (*6*). In addition, A/chicken/Cameroon/17RS1661-1/2017 contains the V100A mutation in the PA protein, which is an amino acid signature typical of human influenza viruses (*7*).

Conclusions

Nigeria was the first country in West Africa to report the presence of the HPAI H5N8 virus in November 2016 (*8*,*9*). Less than 2 months later, in January 2017, the virus was detected in the far-north region of Cameroon in Makilingaye, close to the Nigerian border. Considering the extensive and porous frontier between Cameroon and Nigeria, trade and movement of poultry might have played a key role in the spread of the virus. However, the involvement of wild birds cannot be excluded. The region contains several wetlands (Domayo River, Mayo Kani River, and Maga Lake) where different wild birds congregate, in particular during the dry season (December–April), when the virus was first detected. The almost simultaneous detection (early January 2017) of the H5N8 virus in poultry and wild birds in distant locations in Africa, such as Tunisia and Uganda, suggests that the role of wild birds in the introduction and/or dissemination of the virus in the region should not be overlooked. Of note, the first outbreak caused by the Gs/GD/96 H5 lineage in Cameroon, in 2006, also occurred in the far-north region of Cameroon at about the same time. This finding might suggest a common pathway for introduction of the virus into this area and highlights the need to improve surveillance in this region.

Although the epidemiologic and genetic data are insufficient to establish definite pathways and time of introduction of H5N8 virus into West Africa, this study demonstrates that ≥2 distinct H5N8 viruses entered Cameroon. This finding, together with the evidence that this event represents the third incursion of a Gs/GD/96-lineage H5 HPAI virus into Cameroon, again underlines the need to perform avian influenza surveillance on an ongoing basis for rapid identification and response to outbreaks in this area.

Acknowledgments

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About the Author

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Highly Pathogenic Avian Influenza A(H5N8) Virus, Democratic Republic of the Congo, 2017

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In 2017, highly pathogenic avian influenza A(H5N8) virus was detected in poultry in the Democratic Republic of the Congo. Whole-genome phylogeny showed the virus clustered with H5N8 clade 2.3.4.4B strains from birds in central and southern Asia. Emergence of this virus in central Africa represents a threat for animal health and food security.

The detection of highly pathogenic avian influenza (HPAI) infections in poultry has greatly increased in the past decades, in particular as a consequence of the spread of the HPAI virus subtype H5, descendent of the H5N1 virus A/goose/Guangdong/1/1996 (Gs/GD), which was detected in China in 1996 (*1*). The evolution of the Gs/GD H5 lineage has resulted in the emergence of multiple clades characterized by distinct antigenic properties and zoonotic potential (*2*). Among them, the HPAI H5 clade 2.3.4.4 has stood out for its concerning ability to reassort and combine with different neuraminidase (NA) subtypes and to spread rapidly to and within multiple continents (*3*).

In late 2016, a reassortant HPAI H5N8 virus (clade 2.3.4.4 group B) began to spread from China (*4*) and the Russian Federation (*5*) to Asia, the Middle East, Europe, and western Africa and for the first time reached central, eastern, and southern Africa. Egypt, Tunisia, and Nigeria reported HPAI H5N8 virus in late autumn 2016, and virus detection continued to occur across Africa in the winter, spring, and summer of 2017 (*6*). This study provides insights from the epidemiologic and viral genome analysis on the outbreaks in the Democratic Republic of the Congo (DRC).

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The Study

In late April 2017, high death rates in domestic chickens and ducks were reported in 4 localities of the Ituri province (Bunia territory) of DRC, which is situated at the edge of Albert Lake between the Rwenzori Mountains and the Republic of Uganda (Figure 1). Because this outbreak followed an HPAI H5N8 outbreak in Uganda in January 2017 (*7*,*8*), this alert led to a strong suspicion of HPAI.

Clinical signs in the affected poultry included prostration, dyspnea, yellowish-colored diarrhea, generalized weakness, torticollis, and, in some cases, recumbency before death. Necropsies on carcasses revealed petechiae, hemorrhage, or both in all organs; hemorrhagic liver with soft consistency; and an empty gizzard with epithelial hemorrhage.

We sampled 22 birds (9 duck carcasses, 12 live ducks, and 1 live chicken) in the 4 infected villages. We collected tracheal and cloacal swabs from living birds showing clinical signs and collected organs including lung, intestine, trachea, and heart from dead birds.

We performed a rapid test for avian influenza virus (AIV) type A detection in the field using the AIV Ag Test Kit (BioNote, Hwaseong-si, South Korea). Of the 22 birds sampled, 6 ducks tested positive with the rapid test; real-time reverse transcription PCR analysis confirmed 11 H5-positive ducks. The Central Veterinary Laboratory of Kinshasa (Kinshasa, DRC) submitted the samples to the World Organisation for Animal Health (OIE) Reference Laboratory and the Food and Agriculture Organization of the United Nations (UN-FAO) Reference Center for Animal Influenza at the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy) for confirmatory diagnosis and genetic analysis.

Using an Illumina MiSeq platform (Illumina, San Diego, CA, USA), we obtained whole-genome sequences for 4 viruses selected as being representative of the 4 affected areas in Ituri province (Table 1; online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/7/17-2123-Techapp1.pdf). We submitted the full genomes to GenBank (accession nos. MG607401–32) (Table 1; online Technical Appendix 1 Table 1) and used the

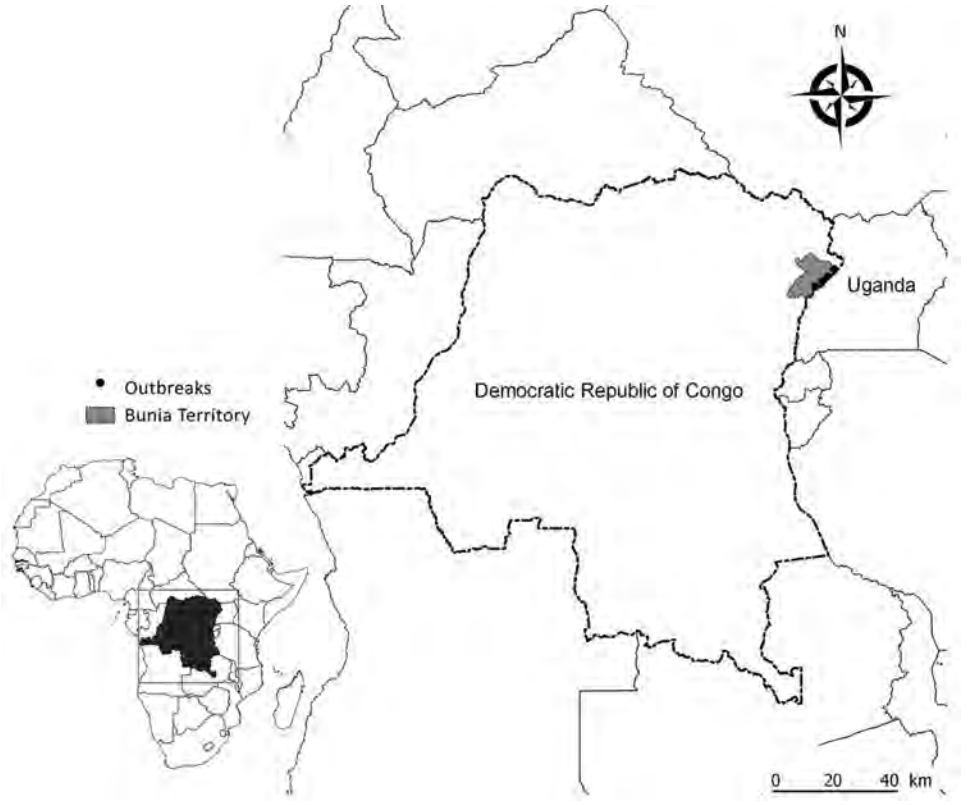

Figure 1. Location of confirmed highly pathogenic avian influenza virus A (H5N8) infection in Bunia territory, on the border with Uganda, Democratic Republic of the Congo, 2017. Inset shows location of Democratic Republic of the Congo in Africa.

maximum-likelihood method to generate phylogenetic trees for each gene segment using PhyML 3.1 (http://www.atgc-montpellier.fr/phyml/versions.php).

Among the 4 H5N8 viruses sequenced, A/duck/Democratic_Republic_of_the_Congo/17RS882-5/2017 and A/duck/Democratic_Republic_of_the_Congo/17RS882-40/ 2017 had identical hemagglutinin (HA) genes; these 2 sequences displayed a similarity of 99.9% with the HA sequences of A/duck/Democratic_Republic_of_the_Congo/17RS882-33/2017 and 99.6% similarity with the HA sequences of A/duck/Democratic_Republic_of_the_Congo/17RS882-29/2017. The topology of the phylogenetic tree based on the HA gene segment showed that the H5N8 viruses from DRC belonged to clade 2.3.4.4 group B (*9*) and grouped together with viruses collected in Qinghai, China; southern Russia; and India in 2016. The highest similarity (99.2%) was with an Indian virus (A/duck/India/10CA01/2016) (Figure 1; online Technical Appendix 1 Figure 1). For the NA gene, the sequences of the viruses A/duck/Democratic_Republic_of_the_Congo/17RS882-5/2017, A/duck/Democratic_Republic_of_the_Congo/17RS882-33/2017, and A/duck/ Democratic_Republic_of_the_Congo/17RS882-40/2017 were identical (100% similarity); these 3 sequences displayed 99.6% similarity with the NA sequence of A/duck/ Democratic_Republic_of_the_Congo/17RS882–29/2017(H5N8) (online Technical Appendix 1 Figure 2). The phylogenetic trees based on the NA and the internal gene segments (online Technical Appendix 1 Figures 1–8), except for the nucleoprotein (NP) gene segment, reflected the same topology of the HA tree, indicating that the H5N8 viruses from DRC were closely related to the virus A/duck/India/10CA01/2016. The topology of the phylogenetic tree based on the NP gene segment (online Technical Appendix 1 Figure 4) revealed a different clustering, with the viruses grouped with H5N8 viruses collected from wild birds in Qinghai and southern Russia in 2016. As discussed by Nagarajan et al. (*10*), it is possible that the Indian virus has been involved in a reassortment event that resulted in NP gene distinct from that described in the Qinghai and southern Russian viruses.

Table. Details of highly pathogenic avian influenza A(H5N8) viruses isolated from birds, Democratic Republic of the Congo, 2017

Date of sample collection	Sampling site	Isolate	GenBank accession no. for hemagglutinin gene
May 14	Tchomia	A/duck/ Democratic_Republic_of_the_Congo/17RS882-5/2017	MG607416
May 15	Joo	A/duck/Democratic_Republic_of_the_Congo/17RS882-29/2017	MG607413
May 14	Mahagi	A/duck/Democratic_Republic_of_the_Congo/17RS882-33/2017	MG607414
May 13	Kafe	A/duck/Democratic_Republic_of_the_Congo/17RS882-40/2017	MG607415

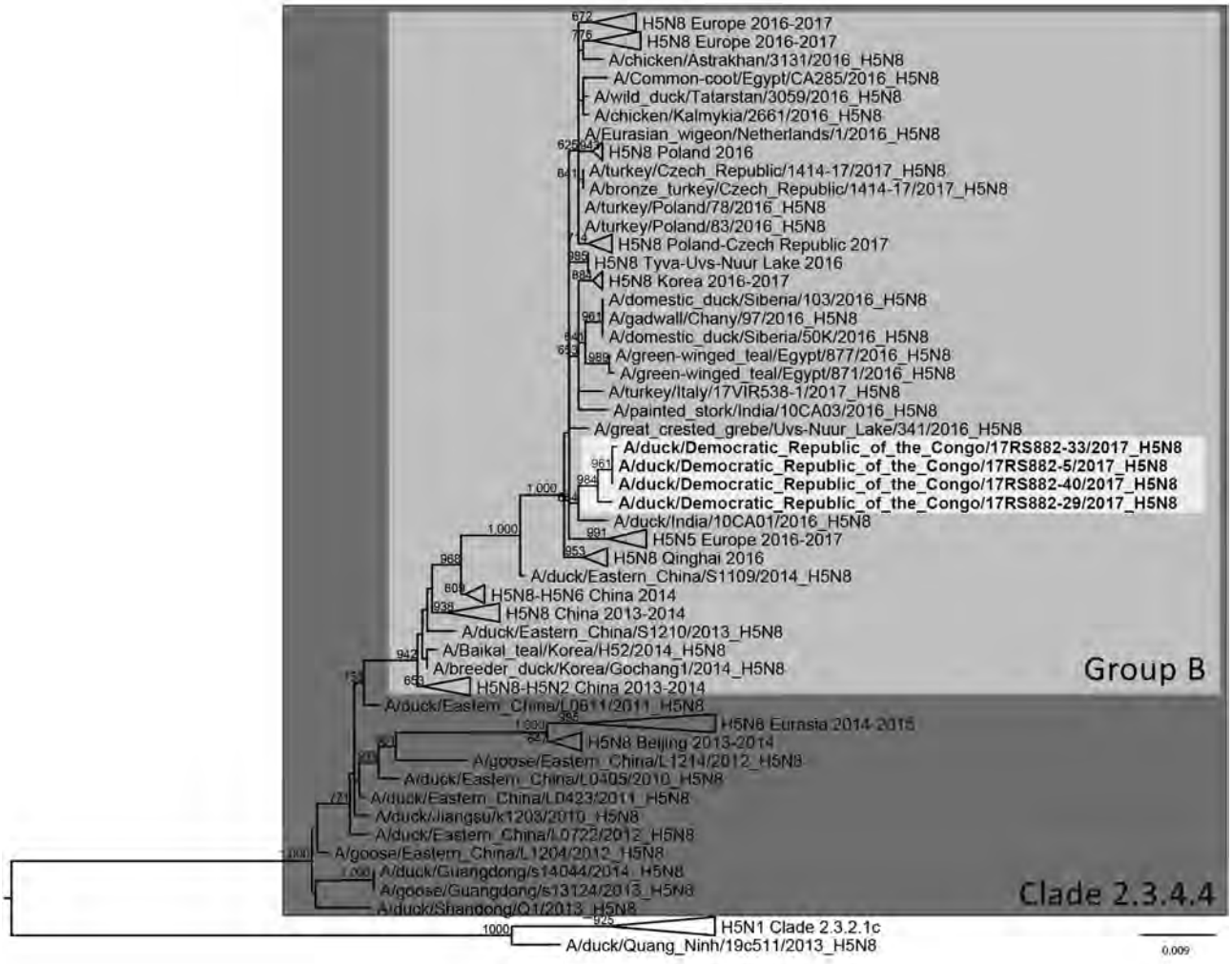

Figure 2. Phylogenetic tree constructed by the maximum-likelihood method of the hemagglutinin gene segment of 4 isolates of highly pathogenic avian influenza A(H5N8) viruses from the Democratic Republic of the Congo (light gray shading) and reference viruses. Bootstrap supports >600/1,000 are indicated above the nodes. Scale bar indicates number of nucleotide substitutions per site.

The time to the most recent common ancestor estimated for the HA gene suggested that the H5N8 virus may have reached DRC during March 2016–February 2017 (online Technical Appendix 1 Figure 9). However, the paucity of data in the public databases reduces the accuracy of the evolutionary analyses and limits the possibility to reconstruct the early transmission dynamics of the H5N8 virus in DRC.

Conclusions

Before 2017, no HPAI H5 goose/Guangdong lineage viruses had been reported in DRC. We have attempted to retrace the origin of the H5N8 outbreaks identified in the Ituri province through the evolutionary analysis of viral gene sequences. Considering the close phylogenetic relationship identified between the DRC viruses and those detected in wild and domestic birds in Asia and the overlap of the West Asian–East African flyway with the zones affected by the H5N8 infection, it is reasonable to assume that migratory birds may have been involved in the introduction of the virus in the eastern and central parts of Africa. The inter-African movements of wild birds and the commercial trade between countries could also have favored the spread of AIVs across the region. The outbreaks in Uganda in January 2017 and in DRC in April 2017 could exemplify this scenario because of the close contact between the 2 countries, even though no public information about the genetic characteristics of the Ugandan viruses is available for comparison. According to reports from the DRC veterinary service (www.au-ibar.org/2012-10-01-13.../348-newcastle-disease), different regions of the country have previously reported mortalities in wild and domestic birds; however, these were considered Newcastle disease cases because of the endemic status of this

disease in the country and therefore were not investigated further. For the HPAI H5N8 outbreak, the awareness of DRC veterinary services, as well as of the population, was raised following the Uganda HPAI outbreak notification, highlighting the crucial role of sharing information in the control of this transboundary disease.

Because DRC hosts many sites for residential and migratory wild birds and is considered a stopover point along the West Asian–East African flyway, surveillance in wild and domestic birds should be implemented for early detection of the virus and efficient control of its spread. However, the challenges for the sustainable development of strategies for the effective prevention and control of this disease are vast and deeply ingrained. Investments to overcome infrastructure obstacles hindering the implementation of a true early-warning system are urgently needed to reduce the risk of onward spread of the virus in the region.

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Poultry Infection with Influenza Viruses of Wild Bird Origin, China, 2016

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Migratory birds may play a role in transmission of avian influenza virus. We report the infection of black-tailed gulls and chickens in eastern China with avian influenza (H13N2) and (H13N8) viruses. We found that these H13 viruses were transmitted from migratory birds to domestic poultry.

Avian influenza virus with 10 hemagglutinin (HA) subtypes has emerged in poultry (*1*), and the potential role of migratory birds in transmission of avian influenza virus has caused concern (*2*). We report infection with low pathogenicity avian influenza (LPAI) virus of HA subtype 13 (H13) among migratory birds (black-tailed gulls [*Larus crassirostris*]) and domestic poultry (chickens) in Weihai, Shandong Province, eastern China (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/7/17-1220-Techapp1.pdf).

Weihai is a breeding center for black-tailed gulls that congregate from northern Asia, eastern Asia, Southeast Asia, and North America. These gulls reside along the coastlines of the East China Sea and Japan and have been found as vagrants in Alaska, North America, and the Philippines (*3*). In China, black-tailed gulls perch at the Longxudao wharf (37°23′24.05″N, 122°41′26.16′E), located in the northeastern corner of Weihai. In December 2016, we collected 149 fecal samples from black-tailed gulls at Longxudao wharf and screened them for evidence of influenza virus by reverse transcription PCR, DNA sequencing, and BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) analysis in the GenBank database. After independently inoculating positive fecal samples into the allantoic cavities of specific pathogen-free embryonated chicken eggs, we obtained 6 influenza H13N2 and 60 influenza H13N8 virus isolates.

To assess the epidemiologic characteristics of these H13 isolates, we completely sequenced an H13N2 isolate (A/black-tailed gull/Weihai/115/2016) and an H13N8 isolate (A/black-tailed gull/Weihai/17/2016) (GenBank accession nos. MF461177–92). Phylogenetic analysis indicated that their HA and neuraminidase (NA) segments were derived from the Eurasian lineage, in accordance with their geographic distribution (online Technical Appendix Figures 2, 3). Moreover, the H13N2 and H13N8 isolates possessed high nucleotide sequence identity to the avian influenza virus subtypes previously isolated from Europe, Asia, and North America (online Technical Appendix Table 1). We speculate that avian influenza virus subtypes H13N2 and H13N8 are reassortants between the Eurasian and North American lineages (online Technical Appendix Figures 4, 5).

We next analyzed the timing of the reassortment events that led to the emergence of subtype H13N2 (Figure, panel A). During July 2009, June 2012, July 2009, and June 2015, the following genes, respectively, were transferred from seagulls in Europe: HA, nucleocapsid protein (NP), matrix (M), and nonstructural (NS). During 2004, November 2011, and October 2014, the following genes, respectively, originated from waterfowl in Asia: polymerase basic (PB) 1, polymerase acidic (PA), and NA. In November 2007, the PB2 gene was transferred from avian influenza viruses circulating among wild waterfowl in North America.

We also estimated the timing of the reassortment events that led to the emergence of subtype H13N8 (Figure, panel B). During June 2012, July 2013, July 2013, and June 2015, the following genes, respectively, were transferred from seagulls in Europe: NP, NA, M, and NS. During 2004, November 2011, and September 2012, the following genes, respectively, originated from waterfowl in Asia: PB1, PA, and HA. In November 2007, the PB2 gene was transferred from avian influenza viruses circulating among wild waterfowl in North America.

According to these data, the generation of influenza virus subtypes H13N2 and H13N8 in seagulls seems to have been a complex process and was probably completed in the middle of 2015 (Figure). We also found that subtypes H13N2 and H13N8 possessed some molecular markers associated with increased virulence and transmission among mammals (online Technical Appendix Table 2).

In April 2017, we analyzed serum samples collected from 48 chickens at a chicken farm at Songcun town (37°04′39.96″N, 122°00′38.83″E) in Weihai for serologic evidence of exposure to H13 viruses. We found detectable hemagglutinin inhibition (HI) antibody titers against H13N2 virus in 4 (8.3%) samples and detectable HI antibody titers against H13N8 virus in 14 (29.2%) samples (online Technical Appendix Table 3). When we evaluated reference serum samples known to contain HI antibodies against each of the virus subtypes for potential cross-reactivity, we observed no apparent cross-reactivity of H13

[1]All authors contributed equally to this article.

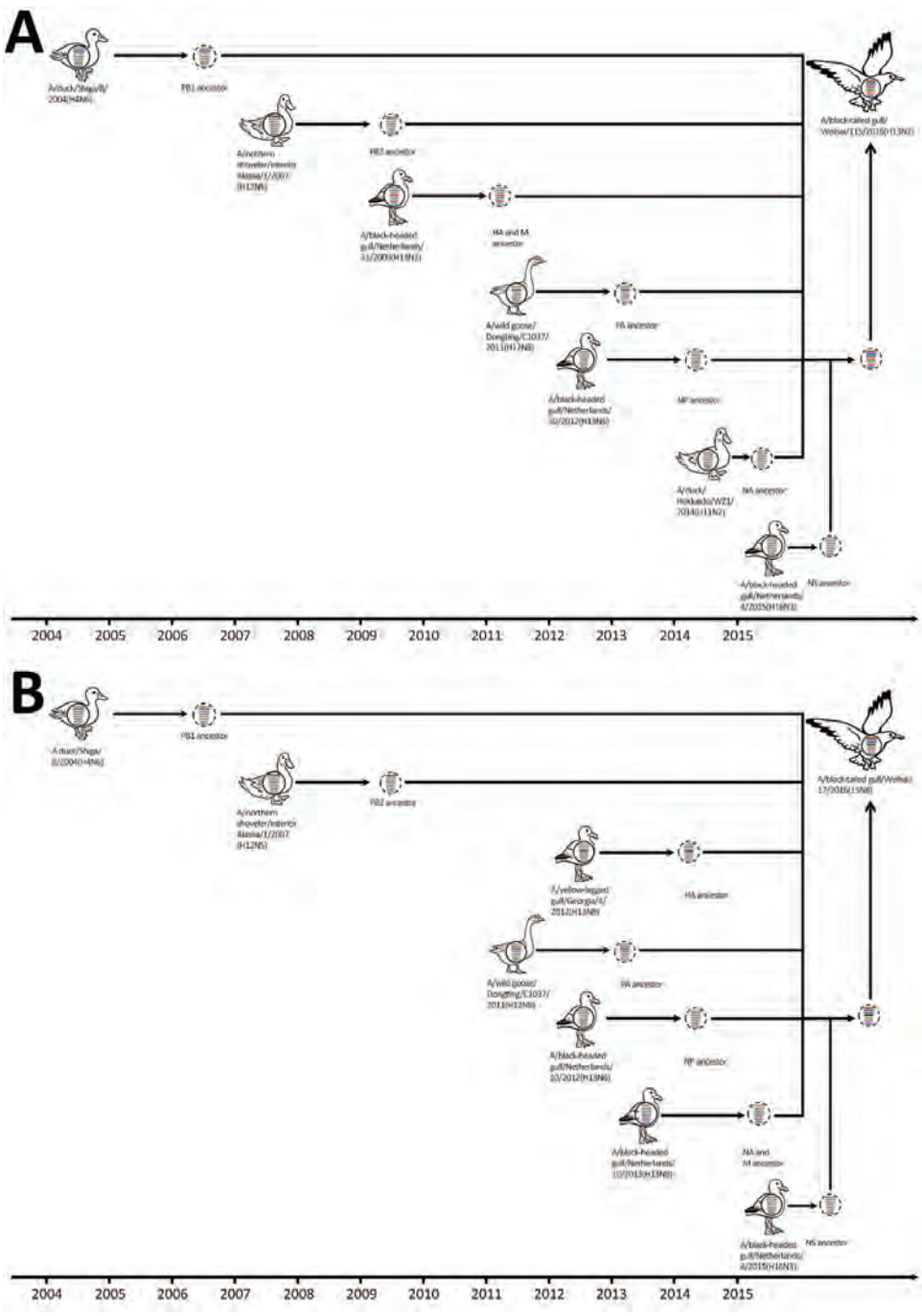

Figure. Hypothetical evolutionary pathway of avian influenza viruses of H13N2 (A) and H13N8 virus (B) subtypes isolated from black-tailed gulls in eastern China, 2016. Dashed virions indicate unidentified viruses. HA, hemagglutinin; M, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural protein; PA, polymerase acidic protein; PB, polymerase basic protein. A color version of this figure showing gene segments origins is available online (https://wwwnc.cdc.gov/EID/article/24/7/17-1220-F1.htm).

antibodies against 7 other HA subtype viruses (online Technical Appendix Table 6). Therefore, although the serum samples' HI antibody titers against H13 viruses were not high, we cannot exclude the possibility that these antibodies were generated in response to independent exposure to H13 viruses.

In March 2013, the novel LPAI H7N9 virus causing serious human infections was detected in eastern China (*4*–*7*); after circulating among domestic poultry, this virus evolved into a highly pathogenic virus (*8*). Therefore, enhanced surveillance is needed to determine whether other LPAI viruses could be introduced into domestic poultry and pose a threat to public health.

In this study, we isolated a large number of LPAI H13 viruses from seagulls at the Longxudao wharf and detected H13-specific seroconversion in chickens at a chicken farm, which is ≈100 km west of this wharf and lies on the migratory route of black-tailed gulls. These findings indicate

that H13 viruses may have been introduced into domestic poultry from migratory birds and that they may have the potential to become a global cross-species threat.

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Rat-Bite Fever in Human with *Streptobacillus notomytis* Infection, Japan

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We report a case of rat-bite fever in a 94-year-old woman with *Streptobacillus notomytis* infection. We established an epidemiologic link between exposure to rats and human infection by performing nested PCRs that detected *S. notomytis* in the intraoral swab specimens obtained from rats captured in the patient's house.

Streptobacillus is a genus of gram-negative, filamentous, rod-shaped bacilli belonging to the family *Leptotrichiaceae*. Since 2014, four novel species other than *S. moniliformis* have been reported: *S. hongkongensis* was isolated from 2 human patients, *S. felis* from the lung of a cat, *S. ratti* from black rats, and *S. notomytis* from a spinifex hopping mouse (*1*–*4*). We report a case of a human infection with *S. notomytis*.

A 94-year-old woman sought treatment at our hospital for general malaise, anorexia, and bilateral knee pain. At admission, her body temperature was 38°C; physical examination revealed swelling in both knees. Her skin was intact, with no rashes or animal bites. Laboratory tests revealed high leukocyte count (1.42×10^9 cells/L) and elevated level of C-reactive protein (19.5 mg/dL).

Bilateral knee arthrocentesis yielded 25 mL of purulent fluid; Gram stain demonstrated the presence of few, thin, gram-negative bacilli with pyrophosphate calcium crystals and neutrophils (Figure). Bacterial culture yielded transparent, small, smooth colonies on 5% sheep blood agar (Kyokuto, Tokyo, Japan) incubated at 37°C under 5% CO_2 for 48 h. However, the automated bacterial identification method (Vitek 2; bioMérieux, Tokyo, Japan) failed to identify the isolate. We evaluated the isolate (NR2245) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using Bruker MALDI BioTyper software version 4.001 library database (Bruker Daltonik GmbH, Bremen, Germany) employing ethanol–formic acid extraction. We identified the isolate as *S. moniliformis*

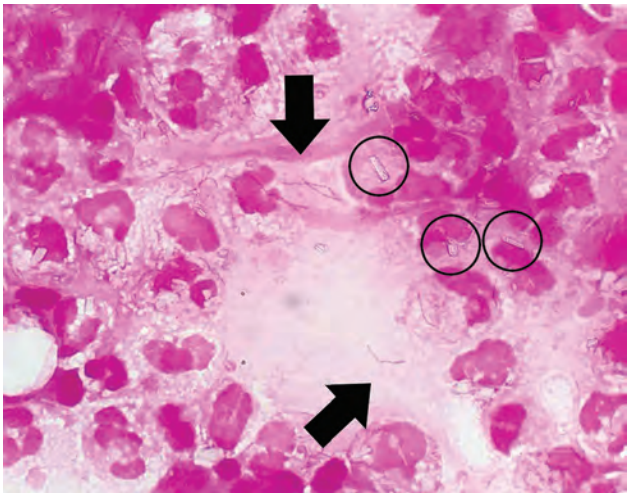

Figure. Gram staining of pus obtained from a patient with rat-bite fever. Circles indicate pyrophosphate calcium crystals. Arrows indicate chain-shaped gram-negative bacilli. Original magnification ×1,000.

(score: 1.608, 24 h). The database included only 1 entry from *S. moniliformis*, DSM 12112T.

We administered ceftriaxone. Subsequent results of arthrocentesis and blood cultures (BacT/ALERT; bioMérieux) were negative; however, the patient's fever and bilateral knee pain persisted. Transthoracic echocardiography showed no evidence of infective endocarditis. We replaced ceftriaxone with sulbactam and ampicillin on hospital day 16, followed by intraarticular administration of dexamethasone on day 17 for pseudogout (diagnosed by the presence of pyrophosphate calcium crystals). On day 20, we performed bilateral knee lavage; thereafter, the patient's fever and knee pain resolved. The surgery specimen was serous fluid; results of Gram stain and aerobic and anaerobic culture were negative. On day 30, we replaced sulbactam/ampicillin treatment with oral minocycline (100 mg every 12 h) as maintenance therapy; however, pneumonia developed, and the patient died of respiratory failure on day 56. We detected *Acinetobacter baumannii* complex and *Enterococcus faecium* from the sputum; however, we did not detect *Streptobacillus* species.

To identify the isolate from the patient's synovial fluid, we performed 16S rRNA gene sequencing using a universal primer pair: 27F (5′-AGAGTTTGATCC TGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The sequence (GenBank accession no. LC360808) showed 100% identity (1,380/1,380 bp) to *S. notomytis* AHL_370–1^T (GenBank accession no. KR001919) and 98.55% (1,360/1,380 bp) identity to *S. moniliformis* DSM12112^T (GenBank accession no. CP001779) in the EzBioCloud 16S database (http://www.ezbiocloud.net/eztaxon). We performed PCR and sequencing of housekeeping genes (*groEL* and *gyrB*) using *Streptobacillus* species–specific primers (*5*). BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the *groEL* (GenBank accession no. LC371754) and *gyrB* (GenBank accession no. LC371753) sequences showed 100% identity to the gene sequence of *S. notomytis* KWG2 (522/522 bp and 758/758 bp, respectively) and 99.6% 5(20/522 bp) and 99.9% (757/758 bp) identity, respectively, to the gene sequence of *S. notomytis* AHL_370–1^T.

We determined antimicrobial susceptibility pattern by broth microdilution. MIC of penicillin was ≤0.06 μg/mL; cefazolin, ≤0.5 μg/mL; ceftriaxone, 0.25 μg/mL; vancomycin, ≤0.25 μg/mL; clarithromycin, 8 μg/mL; minocycline, ≤0.12 μg/mL; and levofloxacin, ≤1 μg/mL.

S. moniliformis is known to cause rat-bite fever in humans (*6*). To study the association between exposure to rats and *S. notomytis* infection, we visited the patient's house after her death and captured 2 rats (*Rattus rattus*), from which we collected stool and intraoral and rectal swab samples. On the same day, we brought the specimens at room temperature to our laboratory and performed bacteriological cultures in 5% sheep blood agar, incubated at 37°C under 5% CO_2; the specimens did not grow *Streptobacillus*. We performed nested PCR with DNA extracted from each specimen, amplified the 16S rRNA gene using the universal primer pair 27F and 1492R, and performed nested PCR using the amplicons from the first PCR as templates, with the *Streptobacillus*-specific primers sbmF (5′-GAGAGAGCTTTGCATCCT-3′) and sbmR (5′-GTAACTTCAGGTGCAACT-3′) (*7*). Only 1 rat's intraoral specimen yielded PCR products, and the sequence of the amplicon by nested PCR showed 100% identity (1,089/1,089 bp) to *S. notomytis* AHL_370–1^T.

Since 2014, a total of 4 novel *Streptobacillus* species have been reported. Whether these new species have recently emerged or existed previously is uncertain. In 2014, Eisenberg et al. identified 2 isolates recovered from rats in 2008 as *S. notomytis* (*2*); it is possible that *S. notomytis* may have been prevalent but underrecognized in Japan because identification is difficult by conventional methods (*2*). Fukushima et al. reported that 16S rRNA sequencing identified an isolate obtained from a rat-bite fever patient as *S. notomytis,* not *S. moniliformis* as originally identified (*8*). By detecting *S. notomytis* from the rats captured in this patient's house, we support a potential epidemiologic link between rat exposure and human infection.

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Perceptions of Zika Virus Risk during 2016 Outbreak, Miami-Dade County, Florida, USA

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We conducted a survey on Zika virus perceptions and behaviors during the 2016 outbreak in Miami-Dade County, Florida, USA. Among women, Zika knowledge was associated with having a bachelor's degree. Among men, knowledge was associated with knowing someone at risk. Interventions during future outbreaks could be targeted by sex and education level.

Misconceptions about arboviruses transmitted by *Aedes* spp. mosquitoes, such as Zika virus, can lead to misplaced reactions and affect local public health officials' abilities to contain outbreaks (*1*–*3*). Despite media campaigns on Zika virus, misperceptions persisted during the 2016 outbreak among some subgroups in Miami, Florida, USA (*4*). More than 4 in 10 Americans mistakenly thought that Zika virus infection was fatal and that symptoms were noticeable (*5*).

We conducted a structured bilingual (English, Spanish) telephone survey with a random sample of adults in late spring (May 1–June 30, 2016), when the Zika virus outbreak began in Florida. We applied the basic concepts of the Health Belief Model (HBM) in an attempt to understand perceptions of Zika virus risk and prevention practices in Miami-Dade County, Florida, the epicenter of the 2016 Zika virus outbreak (*6*).

The HBM provided the framework enabling effective structuring of messages to influence behavioral change in the context of health communication strategies for Zika virus prevention and control. According to the HBM, persons are influenced by their perceived susceptibility to a disease and the severity of that disease (*7*). To use the HBM, participants must have the ability to implement a desired behavior, self-efficacy (i.e., confidence in their ability to implement that action), and cues to action (which could lead to health behavior changes) (*7*). Because Zika virus infection mainly affects pregnant

women (*8*,*9*), we report differences in perception and behavior by sex. Our target sample size was 421, with a power of 0.90 and margin of error of 0.4. The survey took 10–30 minutes to complete, and ≈62% (262/421) of the target population participated.

We determined predictive factors of Zika virus knowledge (dependent variable, values 0 or 1) by using multivariate logistic regression with a log-link function adjusted for demographics (age, sex, employment status, education level, income level) and all other variables of the HBM. We presented data as adjusted odds ratios (aORs) with 95% CIs. A low score (0–7 points) on the Zika virus knowledge test indicates the participant correctly answered 0–7 questions and suggests the respondent had simply heard of Zika and knew that mosquitoes could transmit Zika virus. A high score (8–12 points) indicates the participant correctly answered 8–12 questions and suggests the respondent had a good understanding of microcephaly and Guillain-Barré syndrome.

Table. Multivariate logistic regression analysis of variables associated with high Zika virus knowledge among Miami-Dade County residents, by sex, Florida, USA, 2016*

	aOR (95% CI)		
Category	Total, n = 262	Female, n = 149	Male, n = 113
Constant†	0.07 (0.01–0.37)‡	0.05 (0.004–0.647)§	0.066 (0.004–1.094)¶
Self-efficacy			
Confidence to protect household from Zika virus infection			
Medium	1.29 (0.59–2.77)	1.36 (0.45–4.12)	1.97 (0.50–7.68)
High	1.26 (0.52–3.05)	1.15 (0.32–4.13)	2.81 (0.59–13.14)
Took action to protect against Zika virus			
No	Referent	Referent	Referent
Yes	2.39 (1.24–4.61)‡	2.30 (0.882–5.999)¶	3.18 (1.07–9.44)§
Severity of disease			
Severity of Zika virus infection			
Less severe	Referent	Referent	Referent
Somewhat severe	1.09 (0.38–3.16)	1.24 (0.27–5.67)	0.84 (0.13–5.38)
Very severe	1.35 (0.46–3.96)	2.62 (0.61–11.08)	0.53 (0.061–4.54)
Severity of microcephaly			
Not severe	Referent	Referent	Referent
Somewhat severe	1.07 (0.51–2.27)	1.26 (0.45–3.58)	1.04 (0.31–3.51)
Very severe	0.79 (0.34–1.87)	1.07 (0.32–3.58)	0.52 (0.12–2.12)
Susceptibility to disease			
How likely are you to contract Zika virus			
Very unlikely	Referent	Referent	Referent
Somewhat unlikely	1.56 (0.82–2.96)	1.34 (0.557–3.226)	2.45 (0.83–7.26)
Likely	2.36 (0.896–6.25)¶	1.36 (0.323–5.795)	3.21 (0.70–14.63)
Benefits of action			
Taking action against Zika virus			
Beneficial	Referent		
Not beneficial	−0.91 (−2.55 to 0.73)	NA	NA
Possible cues to action			
Knowing someone at risk for Zika disease (pregnant or planning on being pregnant)			
No	Referent	Referent	Referent
Yes	2.13 (0.95–4.77)¶	1.15 (0.41–3.22)	11.73 (2.28–60.28)‡
Demographics			
Age	0.99 (0.97–1.01)	0.99 (0.96–1.02)	1.00 (0.97–1.04)
Sex			
M	Referent		
F	1.18 (0.63–2.20)	NA	NA
Employment status			
Not in the workforce	Referent	Referent	Referent
In the workforce	1.23 (0.579–2.605)	1.02 (0.35–2.97)	1.15 (0.33–4.02)
Education level			
Less than bachelor's degree	Referent	Referent	Referent
Bachelor's degree or higher	2.37 (1.25–4.47)‡	2.92 (1.199–7.12)§	1.54 (0.53–4.42)
Income level			
<$50,000	Referent	Referent	Referent
$50,000-$100,000	0.98 (0.46–2.09)	1.15 (0.44–2.98)	0.65 (0.18–2.25)
>$100,000	2.06 (0.88–4.78)¶	2.51 (0.72–8.73)	1.75 (0.42–7.32)
Don't know or NA	0.86 (0.33–2.21)	1.73 (0.52–5.78)	0.04 (0.006–0.304)‡

*aOR, adjusted odds ratio; NA, not applicable.
†The constant is the expected mean value of *y* when *x* equals zero.
‡$p \leq 0.01$.
§$p \leq 0.05$.
¶$p \leq 0.10$.

Of the 262 survey participants, 149 (56.9%) were women and 113 (43.1%) were men; age range was 18–94 (mean 49, SD 19) years. More than half (56.9%) of participants were foreign born, 185 (70.6%) considered themselves Hispanic or Latino, and 138 (52.7%) were married. More women (36.9%) than men (31.0%) scored high (8–12 points) for Zika virus knowledge (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/7/17-1650-Techapp1.pdf).

A total of 53.0% of women and 49.6% of men felt somewhat confident they could protect their households from contracting Zika (online Technical Appendix Table). Personal protective measures included window and door screens, checking for and draining standing water, and using repellents. A higher percentage of women (53.7%) than men (42.5%) perceived Zika to be a severe disease, and women (50.4%) were more likely than men (43.6%) to report fear of contracting Zika.

Taking action to protect oneself against Zika virus infection (aOR 2.39, p = 0.01) and knowing someone pregnant (cue to action) (aOR 2.13, p = 0.10) were associated with a higher knowledge of Zika virus (Table). This high level of knowledge might be attributable to the Florida Department of Health's aggressive information campaign and a Zika virus information hotline created to help inform the public about Zika virus and procedures to avoid infection. Participants with bachelor's degrees (aOR 2.37, p = 0.01) were also more likely to be knowledgeable about Zika virus than those without bachelor's degrees.

Among women, Zika virus knowledge was higher among those who had taken action to prevent Zika virus infection (aOR 2.30, p = 0.10) and those with bachelor's degrees (aOR 2.92, p = 0.05). However, among men, Zika virus knowledge was higher among those who knew someone at risk for Zika (aOR 11.73, p = 0.01) and those who took action to prevent Zika virus infection (aOR 3.18, p = 0.05).

Our analysis indicates that women were more concerned about Zika than were men in Miami-Dade County and that those with bachelor's degrees were more knowledgeable than were those without. Therefore, targeting prevention and treatment interventions by sex and education level should be considered to maximize positive outcomes in high-risk areas during outbreaks (*10*). For local governments, planning and implementing effective interventions aimed at preventing and controlling mosquito-borne disease outbreaks require ongoing assessments of knowledge, attitudes, and practices that are sensitive to local residents' health practices and concerns. These findings have critical implications for future studies that seek more accurate and confirmatory evidence on the association between socio-demographics and Zika virus–related health practices.

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Recovery of *Cryptococcus gattii* from an Infected Ventriculo-Peritoneal Shunt, Illinois, USA

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Cryptococcus gattii is a fungal pathogen endemic in tropical and subtropical regions. Isolated cases and outbreaks have been reported in areas of North America and Europe, expanding the distribution pattern beyond warmer regions. We describe a case of ventriculo-peritoneal shunt infection by *C. gattii* in an immunocompetent person in Illinois.

Cryptococcus gattii is a fungus found in soil and decaying organic materials (*1*–*3*). *C gattii* infections have been reported in tropical and subtropical regions worldwide. In the United States, *C. gattii* human infection is rare; <300 cases have been documented, of which 169 were reported to the Centers for Disease Control and Prevention (CDC) during 2005–2013 (*4*). Most cases were reported in southern California before a rise in cases occurred after 1999 in the Pacific Northwest (*2*,*5*). We report a case of ventricular shunt infection by *C. gattii* in an immunocompetent person in Illinois.

A 40-year-old man from Lake County, Illinois, with no known medical problems was admitted in October 2015 for evaluation of hydrocephalus. The patient reported 4 months of throbbing frontal headaches, nausea, and vomiting. Progressive confusion, altered memory, intermittent gait, and balance disturbances also were present. No visual changes, fevers, chills, or seizures were reported. The patient had no travel outside of Illinois and no ill contacts. Computed tomography (CT) scan of the brain demonstrated hydrocephalus, which was concerning because it indicated possible abnormalities in the flow of cerebrospinal fluid (CSF). A right frontal ventriculostomy catheter was placed. Repeat CT imaging of the head showed a possible mass within the right cerebellar hemisphere and surrounding vasogenic edema. To determine whether an infectious pathogen was the cause, we performed a workup that included HIV screening, *Echinococcus* serologic testing, interferon gamma release assay, cysticercosis serologic testing, and 3 CSF cultures; all results were negative. Results of a complete blood count with differential and comprehensive metabolic panel were unremarkable. The patient was not receiving immunosuppressive therapy nor had any other known risk factors associated with immunosuppression. A right ventriculo-peritoneal (VP) shunt with a programmable valve was placed, and the patient was discharged to home in stable condition.

One month later, the patient was readmitted with recurring symptoms. CT imaging of the head showed stable ventricular size. Contrast-enhanced magnetic resonance (MR) of the brain showed abnormalities above the tentorium, possibly representing a cystic mass obstructing the foramen of Monroe bilaterally, with pronounced distention of both lateral ventricles. CSF studies showed a leukocyte count of 3/μL (reference range 0–5/μL) with lymphocytic predominance (93% [reference range 40%–80%]) and protein level of 42 mg/dL (reference range 15–45 mg/dL). We observed large round yeasts on Gram stain of CSF. The VP shunt was externalized. Cryptococcal antigen (Immy; Norman, OK, USA) was positive in the CSF (1:160 titer). Cultures from the CSF grew yeast that we identified as *Cryptococcus neoformans* by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Vitek MS IVD Database version 2; bioMérieux, Durham, NC, USA). We then subcultured the organism to CGB Agar (L-canavanine, glycine, bromothymol blue; Hardy Diagnostics, Santa Monica, CA) to differentiate *C. neoformans* from *C. gattii*. The organism, which produced blue coloration on CGB agar, was determined to be *C. gattii* and was confirmed as *C. gattii* molecular biotype VGI by multilocus sequence typing performed at CDC (Atlanta, Georgia, USA). Induction with amphotericin B and flucytosine was given for 14 days and high-dose fluconazole (800 mg/d) was subsequently given as consolidation therapy for 8 weeks. The dose was then decreased (to 200 mg/d) for maintenance therapy. The patient was lost to follow-up after his first outpatient clinic visit.

We postulate that our patient likely had a cryptococcoma with a low organism burden on initial presentation. We found no cases of VP shunt infection attributable to *C. gattii* in the literature. Only 10 cases of VP shunt infections attributable to *C. neoformans* have been reported; the time from shunt placement to symptom onset ranged from 10 days to 20 years (*6*,*7*). Six of 10 cases resulted from shunt placement in persons previously infected (*6*,*8*). The patient we report had onset of symptoms 4 weeks after VP shunt placement, likely reflecting an underlying infection before VP shunt placement.

Only 4 isolates of *C. gattii* have been identified from the Midwest region of the United States; these isolates were identified as VGI and VGIII types (S. Lockhart, CDC, pers. comm., 2017 Jul 18) (*9*). A recent study demonstrated that a large subset of isolates from throughout the United States

were VGI, including a cluster of isolates with a single multilocus sequence type originating in the southeastern United States (*10*). The isolate in this case was identified as molecular type VGI and by multilocus sequence typing was shown to have the same sequence type as isolates from patients in Florida and Georgia and isolates from the environment in Washington.

Infections attributable to *C. gattii* are not confined to tropical and subtropical regions. The case we describe serves to extend the known range of this organism to include Illinois. Infections might be missed, given that many laboratories do not routinely differentiate *C. gattii* from *C. neoformans*. Mortality rates can range from 13% to 33% (*4*). Thus, clinicians and laboratorians must have increased awareness of this emerging infectious disease.

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Late or Lack of Vaccination Linked to Importation of Yellow Fever from Angola to China

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During March and April 2016, 11 yellow fever cases were identified in China. We report epidemic and viral information for 10 of these patients, 6 of whom had been vaccinated before travel. Phylogenetic analyses suggest these viruses nested within the diversity of strains endemic to Angola, where an outbreak began in 2015.

In December 2015, the first case of a major yellow fever outbreak was reported in Angola; the outbreak spread to Democratic Republic of the Congo (DRC) (http://www.who.int/csr/don/13-april-2016-yellow-fever-angola/en/). In addition to 965 confirmed cases in DRC during

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December 2016–February 2017 (https://reliefweb.int/report/democratic-republic-congo/yellow-fever-outbreak-angola-and-democratic-republic-congo-ends), the outbreak led to exportation to other countries. In China, 11 imported cases were reported during March and April 2016 (*1*); 1 case-patient subsequently died (*2*). We conducted a clinical-epidemiologic study on 10 of the 11 case-patients. The study was approved by the Review Board of Beijing Ditan Hospital (Beijing, China) and the Ethics Committee of State Key Laboratory of Pathogen and Biosecurity. We also obtained informed consent from recruited patients.

We confirmed the yellow fever diagnoses according to criteria established by the China National Health and Family Planning Commission, in agreement with the recommendations of the World Health Organization (*3*). All case-patients were citizens of China who had stayed in Angola for a period of 5 months to 7 years before returning to China; 7 (70%) were male, the median age was 41 (range 17–50) years (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/7/17-1868-Techapp1.pdf). Six case-patients reported a history of yellow fever vaccination; 4 of those were vaccinated <14 days before symptom onset. Case-patient 7 received vaccination in China 5 years before symptoms occurred, and case-patient 10 was vaccinated in Namibia 10 months before onset of illness. None had received fractional doses of yellow fever vaccine as advocated in some studies (*4*).

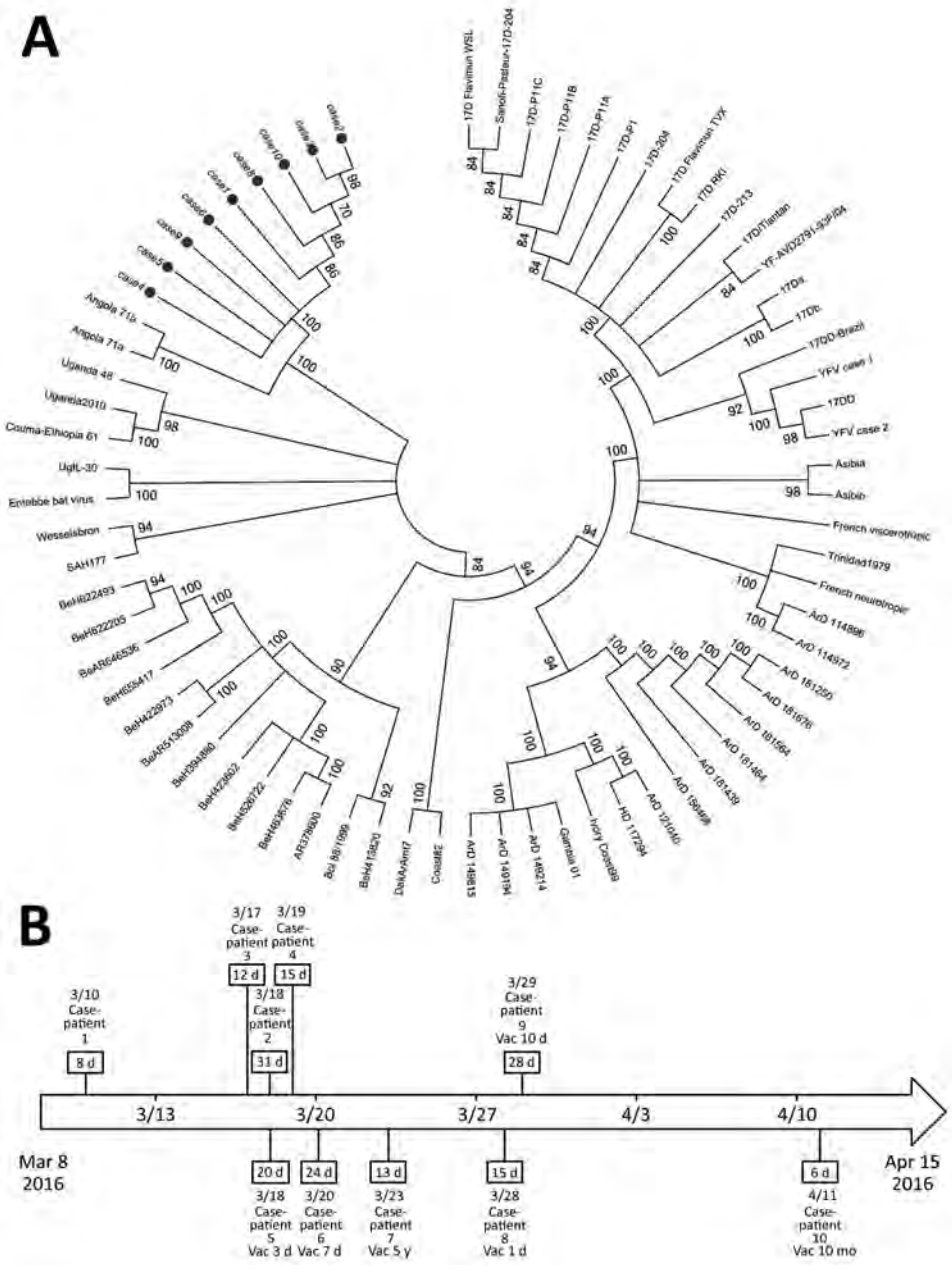

Figure. Phylogenetic analysis of yellow fever viruses and clinical courses for persons with yellow fever cases imported from Angola to China. A) Phylogenetic relationships among the yellow fever viruses from samples obtained from 9 case-patients (black circles). An unrooted dendrogram with maximum-likelihood by genome sequences represents the phylogenetic relationships. Clusters with bootstrap support values <70 were integrated; bootstrap values are shown on the branches. B) Dates of hospital admission, detection of virus in urine, and vaccination status for the 10 case-patients investigated. Case-patients 1–4 were not vaccinated. Numbers in boxes indicate longest interval from symptom onset to urine virus sequence detection. Vac, interval from previous vaccination to symptom onset.

We tested blood samples from all case-patients for yellow fever–specific antibodies by ELISA. We also extracted RNA from blood and urine samples by using the QIAamp MinElute Virus Spin Kit (QIAGEN, Valencia, CA, USA) for reverse transcription PCR virus detection. Phylogenetic analysis (Figure, panel A) of samples from 9 case-patients showed that the sequences were very closely related to each other and resembled the wild-type strain implicated in the Angola outbreak (*2*). We evaluated virus sequences of 5 of the 6 vaccinated case-patients; all showed the same wild-type strain. For the remaining patient (case-patient 7), RNA extraction was unsuccessful. She had been vaccinated 5 years before her illness, excluding the possibility of a vaccine-related adverse event.

Clinically, all 10 case-patients had acute onset of fever lasting 1–7 days; the highest temperature was 39.5°C (online Technical Appendix Table 2). The most common symptoms were fatigue, headache, dizziness, and myalgia. Of 10 case-patients, 8 had been treated for malaria before yellow fever was confirmed. Eight case-patients, including 2 of the 4 unvaccinated case-patients, had relatively mild symptoms. Case-patients 1 and 2, both unvaccinated, had severe disease; signs and symptoms were jaundice, vomiting, hemorrhaging (petechiae, ecchymosis, and gastrointestinal bleeding), and oliguria, as well as high levels of liver enzymes (alanine aminotransferase 11,425 and 3,710 u/L, respectively) and total bilirubin (>100 μmol/L). We also noted bleeding tendency, reflected by a high international normalized ratio and thrombocytopenia. Case-patient 1 deteriorated rapidly as a result of severe kidney and liver damage; biopsy showed evidence of panlobular and confluent hepatocyte necrosis (*2*). His CD4 T cell count was low at 155 cells/μL, but we excluded HIV co-infection. Despite continuous hemofiltration and hemodialysis and mechanical ventilation support, he died 9 days after symptom onset. The remaining 9 patients recovered after hospitalization, which lasted for a median of 16 (range 11–52) days.

Case-patients 1 and 2 also showed strong inflammatory responses as reflected by high plasma level of interleukin 6. Yellow fever virus nucleic acid continued to be detectable in urine during week 1 after symptom onset; case-patient 2 had the longest interval of detection at 31 days (Figure, panel B).

Overall, the clinical courses of these imported yellow fever cases in China were similar to others reported in the published literature: mild diseases in most case-patients, but high fatality rates among severe cases (*5*). China is not a yellow fever–endemic area, but importation of the virus was not surprising, considering the high number of travelers between Angola and China (*6*). Unfortunately, vaccination coverage was not high for Chinese travelers bound for Angola; among this cohort, only 2 had been vaccinated before travel to a yellow fever–endemic area. Travelers may be worried about vaccine-associated viscerotropic disease (*7*), but this condition did not occur among the vaccinated case-patients in our study, who all had a moderate disease, requiring hospitalization of 11–29 days. The World Health Organization had recommended vaccination at least 10 days before entering a yellow fever–endemic area, but late vaccination in travelers is common (*8*).

Our observations highlight the importance of timely immunization to achieve protection during an outbreak within a yellow fever–endemic area. Vaccination efficacy and long-term protection are other concerns highlighted in this study: 2 patients were infected despite vaccination received 1–5 years previously. Although most international guidelines did not recommend booster administration of vaccine, its possible role in outbreak settings demands further research (*9*,*10*).

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Enterovirus A71 Infection, Thailand, 2017

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An outbreak of hand, foot and mouth disease among children in Thailand peaked in August 2017. Enterovirus A71 subgenogroup B5 caused most (33.8%, 163/482) cases. Severe disease (myocarditis and encephalitis) was observed in 1 patient. Coxsackievirus A6 was detected in 6.0% (29/482) of patients, and coxsackievirus A16 was detected in 2.7% (13/482) of patients.

Hand, foot and mouth disease (HFMD) and herpangina, caused primarily by enterovirus A, commonly affect children and result in painful blisters in the buccal cavity and on the soles of the hands and feet. In rare situations, enterovirus infection can lead to severe neurologic complications, notably aseptic meningitis, encephalitis, acute flaccid paralysis, and death in young children (*1*).

A nationwide outbreak of HFMD caused by coxsackievirus A6 (CV-A6) affected many children in Thailand in 2012 (*2*). Since that time, ≈50,000 cases of HFMD have been reported annually to the Thailand Ministry of Public Health (*3*); CV-A6 and CV-A16 are the main causative agents. Most reports were based on clinical symptoms; laboratory-based confirmations are rare. Consequently, the incidence increased awareness and the need for diagnostic-based epidemiologic surveillance. As of September 25, 2017, a total of 59,071 cases of HFMD have been reported (*4*). We determined the prevalence and viral etiology of enterovirus infections among patients with clinical HFMD and herpangina in Thailand during 2017.

The study was approved by the Chulalongkorn University Faculty of Medicine Institutional Review Board (institutional review board no. 002/60). We tested clinical specimens from 482 children requiring hospital care for HFMD (n = 435) and herpangina (n = 47) that were submitted to King Chulalongkorn Memorial Hospital (Bangkok, Thailand) during January 1–October 30, 2017, from 12 provinces with reported HFMD outbreaks (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/7/17-1923-Techapp1.pdf). The study population was 273 boys and 209 girls (sex ratio M:F 1.3:1; age range 4 days to 45 years; mean ± SD age 2.9 ± 4.0 years; median age 2.0 years).

We subjected specimens to 2 real-time reverse transcription PCRs (RT-PCRs). The first RT-PCR identifies enterovirus A71 (EV-A71), CV-A6, and CV-A16 (*5*). We then subjected virus-positive samples to full-length virus capsid protein 1 (VP1) gene amplification by using a conventional RT-PCR and nucleotide sequencing to identify EV-A71 subgenogroups (*6*). The second RT-PCR is a pan-enterovirus real-time RT-PCR, which also detects the glyceraldehyde-3-phosphate dehydrogenase gene (internal control). We subjected samples to a conventional RT-PCR that used CODEHOP degenerate primers to identify enterovirus serotypes other than EV-A71, CV-A6, and CV-A16 (*7*). We also performed molecular typing by using phylogenetic analysis and nucleotide sequence comparisons of strains. Sequences were deposited in GenBank (accession nos. MG843892–844136).

Enterovirus infections were detected throughout the year; increased frequency was observed in the rainy season (June–September) (online Technical Appendix Figure 1). Enteroviruses were identified in 67.4% (325/482) of samples. EV-A71 was most frequent (33.8%, 163/482), followed by CV-A6 (6.0%, 29/482), and CV-A16 (2.7%, 13/482). Analysis of partial VP1 sequences showed that almost all (99.4%, 162/163) of EV-A71 was subgenogroup B5: only 2 samples were subgenogroup C4 (Figure 1A). Full-length VP1 analysis also confirmed our EV-A71 subgenogroup assignment (online Technical Appendix Figure 2).

Comparison of full-length VP1 nucleotide sequences of subgenogroup B5 viruses between strains isolated in Thailand during this study and those previously isolated in

Thailand showed 93.3%–100.0% identity (online Technical Appendix Table). Strains isolated in this study also had similar sequence identity with strains previously isolated (92.7%–98.6%). CV-A4 accounted for 6.6% (32/482) of isolates, followed by CV-A2 (1.5%, 7/482) and CV-A8 (1.0 %, 5/482). Minor genotypes (CV-A5, CV-A10, and echovirus 9) accounted for 0.4% (2/482). One sample each was positive for CV-A9, CV-B2, echovirus 4, and echovirus 5. The remaining 13.5% (65/482) of isolates positive for pan-enterovirus were not typeable.

A severely ill 9-month-old boy in Nakhon Sawan Province was given a diagnosis of HFMD and required prolonged hospitalization because of sequelae caused by severe brain damage from encephalitis and myocarditis. Laboratory tests on rectal swab samples identified EV-A71 subgenogroup B5. An 18-month-old girl from Uttaradit Province who suddenly died during development of HFMD was positive for CV-A9.

Enteroviruses associated with HFMD in Thailand during 2012–2016 are usually CV-A6 or CV-A16 (*5*). EV-A71 has been associated with major outbreaks in China and Vietnam (*8*), and infections have been linked to more severe symptoms and neurologic complications in patients requiring hospitalization. In the HFMD outbreak in Thailand during 2012, deaths were primarily associated with EV-A71 subgenogroup B5 (*9*). The relatively high frequency of HFMD attributed to EV-A71 in this study was unexpected because our previous studies showed that <10% of enterovirus-positive samples were EV-A71 (*2*,*9*).

Our study had limitations. Because samples were obtained only from provinces with reported HFMD outbreaks, we might have missed HFMD-associated enterovirus in other regions. Limited availability of demographic and clinical data also prevented more in-depth analysis of disease severity and clinical associations. Additional deaths caused by EV-A71 infections might have been missed. Thus, without accurate and complete reporting of severe complications associated with EV-A71 infections, clinical implications from such infections could not be determined.

Effective prevention of widespread HFMD is needed. The approved EV-A71 vaccine containing subgenogroup C4 and ongoing development of B2- and B4-based vaccines can elicit cross-neutralizing antibodies against circulating EV-A71 subgenogroups, which might prove to be effective in preventing subgenogroup B5 infection, which is predominant in Thailand (*10*). However, molecular epidemiologic surveillance of HFMD is needed to monitor enterovirus transmission.

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Influenza D Virus Circulation in Cattle and Swine, Luxembourg, 2012–2016

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We detected antibodies against influenza D in 80.2% of the cattle sampled in Luxembourg in 2016, suggesting widespread virus circulation throughout the country. In swine, seroprevalence of influenza D was low but increased from 0% to 5.9% from 2012 to 2014–2015.

Influenza D virus (IDV), a new orthomyxovirus distantly related to influenza C virus, was described in pigs with respiratory symptoms in 2011 (*1*). Although mild symptoms only were reported in experimental pig and calf infections, the virus has been implicated in bovine respiratory disease complex (*1*–*3*). Cattle are currently considered the main host of the virus, but other livestock species are also susceptible (*4*). In Europe, IDV circulation has been reported in France (*5*), Italy (*6*,*7*), and Ireland (*8*). Recent serosurveys in Italy showed extremely high seroprevalence rates in cattle (92.4% seropositive) (*9*) and a low but increasing seroprevalence in swine, from 0.6% in 2009 to 11.7% in 2015 (*7*). We investigated the presence of IDV in cattle and swine farms in Luxembourg.

In 2016, we collected serum samples from 450 asymptomatic cattle from 44 farms throughout Luxembourg (Figure, panel A; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/7/17-1937-Techapp1.pdf). We screened the samples for IDV antibodies by using hemagglutination inhibition (HI) assays. We also screened serum samples collected from pigs at 2 slaughterhouses in 2012 (n = 258, 27 farms) and 2014–2015 (n = 287, 29 farms). Because HI titers as low as 20 were measured in farms with demonstrated influenza D circulation (*7*), we considered HI titers ≥20 positive. In addition, we screened nasal swab specimens from asymptomatic pigs sampled at slaughter in 2009 (n = 232, 56 farms) and 2014–2015 (n = 427, 36 farms) by real-time reverse transcription PCR (*1*). No cattle samples were available for molecular screening.

We found an overall seroprevalence of 80.2% in cattle (361/450; HI titer range 20–1,280) (online Technical Appendix Figure); 97.7% of herds (43/44) had ≥1 seropositive animal. Average within-farm seroprevalence was 83.0% (range 20%–100%; Figure, panel A). These results suggested that IDV affects most animals in nearly all farms (Figure, panel A). All animals were much older than 6 months (average 70.5 mo, range 23–209 mo), so it is unlikely that the antibodies were maternally derived (*10*). The median age of seropositive animals (61 months) was significantly higher than the median age of seronegative animals (41 months; $p<0.001$). Seroprevalence was higher in beef cattle (88.0%, 95/108) than in dairy cattle (75.6%, 133/176; meat or dairy production type was not known for 166 animals), but beef cattle were also on average older than dairy cattle. A binary logistic regression model including herd as a random effect and age and production type as fixed effects revealed that only age substantially affected IDV seropositivity.

Most of the cattle investigated were born in Luxembourg (90%, 405/450), but IDV antibodies were found regardless of country of birth (others were born in Germany, France, Belgium, and Italy). This information demonstrates that our results cannot be explained by importation of seropositive animals alone and that IDV transmission takes place in Luxembourg. Within-herd seroprevalence of cattle herds was similar for herds located near the borders as well as those further inland, suggesting that the virus could also spread to and from the neighboring countries (Belgium, France, and Germany), for example, through cross-border grazing.

In Luxembourg, IDV seroprevalence was low in swine compared with cattle but has increased during recent years (0% in 2012 to 5.9% [17/287] in 2014–2015), as it has in Italy (*7*). We detected seropositive animals in 6/29 (20.7%) swine herds (Figure, panel B). The low virus prevalence from nasal swabs (0% in 2009, 0.7% [3/427] in 2014–2015) and the low viral RNA concentration (9.7–94.5 copies/μL) were not conducive to amplification of genetic material for sequencing. The low levels of virus circulation in pigs shown by seroprevalence data, the absence of symptoms at the time of sampling (*3*), and the short shedding period under experimental infection (*1*) probably contributed to the low detection rates observed in swine nasal swab samples. The IDV RNA–positive nasal swab samples originated from 2 different herds, 1 of which was also seropositive (9/10 pigs with HI titer ≥20; no samples were available from the second herd). Although we collected all 3 PCR-positive samples on the same day at the same slaughterhouse, it is unlikely that the pigs were infected during their short stay there.

Taken together, our results suggested that IDV circulates widely throughout cattle farms in Luxembourg and can be considered hyperenzootic in the country. Once

[1]These senior authors contributed equally to this article.

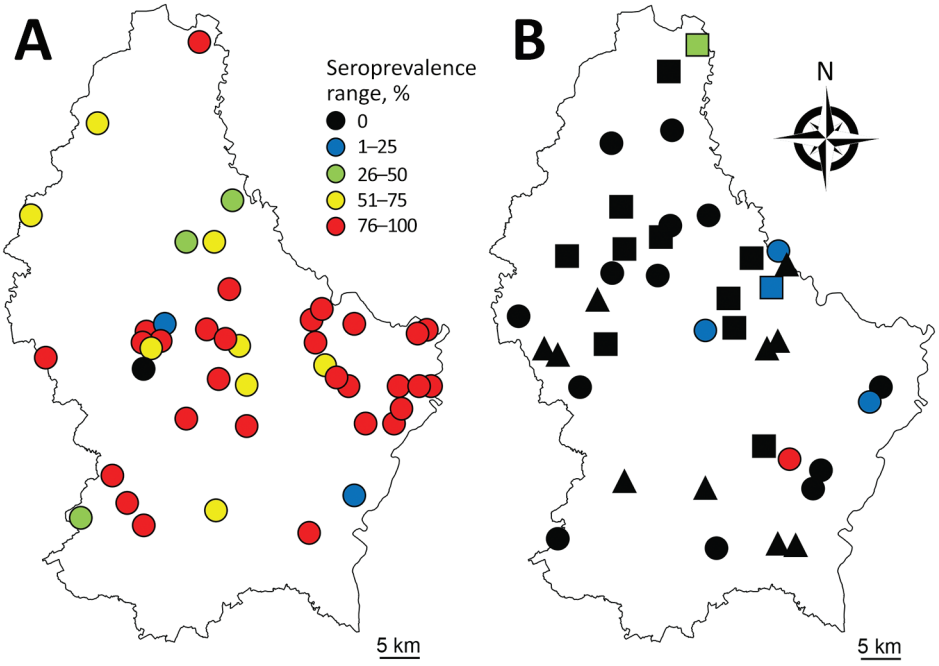

Figure. Within-farm seroprevalence range in A) cattle herds (n = 44) sampled in 2016 and B) swine herds sampled in 2012 (n = 10, triangles), 2014–2015 (n = 12, squares) or both (n = 17, circles), Luxembourg.

introduced into a herd, IDV seems to spread very efficiently, given the high within-farm seroprevalence rates. In light of cross-border trade and grazing, the region beyond Luxembourg's borders may be also hyperenzootic for IDV. Although IDV mainly affects cattle, we detected IDV antibodies in pigs and an increased seroprevalence in pig herds. We are planning systematic serologic and virologic screenings along with epidemiologic surveys to investigate the genetic diversity of IDV strains in Luxembourg, to evaluate the effect of IDV infection on cattle and pig health and productivity, and to study IDV interaction with other pathogens.

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Spillover of Swine Coronaviruses, United States

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Porcine epidemic diarrhea virus, a pathogen first detected in US domestic swine in 2013, has rapidly spilled over into feral swine populations. A better understanding of the factors associated with pathogen emergence is needed to better manage, and ultimately prevent, future spillover events from domestic to nondomestic animals.

Pathogen spillover mechanisms vary, but one route involves pathogens moving from heavily infected domestic animal hosts to nondomestic hosts (*1*). These spillover and emergence events create a dynamic landscape for pathogen transmission.

Porcine epidemic virus (PEDV) is an emergent pathogen in the United States. It can cause 90%–95% mortality in young, naive pigs and substantial weight loss and dehydration in adult swine. The virus was first documented in the United States in April 2013 and spread rapidly, leading to loss of 10% of the US commercial swine population in 31 states within 18 months (*2*), which cost the industry >US $400 million. Horizontal transmission of the virus on shared agricultural resources (*3*) most likely aided its rapid spread among facilities, demonstrating the difficulty of slowing the spread of robust pathogens.

During October 2012–September 2015, we collected serum from feral swine and analyzed it for PEDV exposure. The United States has ≈5–6 million feral swine, and their populations are expanding rapidly (*4*). Although opportunities for direct contact between feral swine and pigs in biosecure swine operations are limited, interactions have been documented with smaller backyard operations, and a recent multistate brucellosis outbreak was linked to backyard pigs infected by feral swine (*5*). Disease spillover into nondomestic hosts can serve as a continuous source for reintroduction into domestic animals, complicating international trade (*6*).

Of the 7,997 feral swine samples tested (Figure), 253 tested positive by PEDV ELISA (seroprevalence 3.2% [95% CI 2.8%–3.5%]). Those 253 samples underwent additional screening, and 8 (seroprevalence 0.1% [95% CI 0.03%–0.16%]) were confirmed to be PEDV antibody positive (online Technical Appendix Tables 1, 2, https://wwwnc.cdc.gov/EID/article/24/7/17-2077-Techapp1.pdf). Two additional samples were considered suspected positives. The remaining 245 positive samples (seroprevalence 3.1% [95% CI 2.7%–3.4%]) probably represent exposure to transmissible gastroenteritis virus (TGEV) rather than PEDV (online Technical Appendix Table 1).

The 8 PEDV-seropositive feral swine samples were from Hawaii and California (Figure). PEDV was first confirmed in California domestic swine in December 2013. The 4 positive feral swine samples from California were collected in September 2014 from adult animals in Santa Clara County. In Hawaii, seropositive feral swine were detected on Oahu and Kauai (Figure). Hawaii confirmed its first case of PEDV in domestic swine on Oahu in November 2014, but our findings identified a PEDV-positive feral swine sample collected in April 2014, before detection in domestic swine on the same island. This finding suggests initial PEDV introduction into domestic pigs in Hawaii might have gone undetected for 7 months before the first confirmed case. The 4 PEDV-positive feral swine samples from Hawaii were collected at 4 different times.

Results indicate that this newly introduced virus spilled over from domestic livestock to a nondomestic species during a relatively short period (<1 year). Prior research suggests directionality (*7*,*8*), with the virus moving from domestic swine to feral swine, rather than the reverse. Data presented here support this finding because positive feral swine were not detected until a year after detection in US domestic swine.

Biosecurity in the US commercial swine industry is comprehensive; however, the spread of PEDV demonstrates that a modern and precisely managed livestock industry is still susceptible to emergent pathogens. PEDV is relatively hardy, persisting on fomites for up to 20 days at low (4°C) temperatures (*9*). Biosecurity designed to prevent transmission of labile pathogens or to prevent introduction of a new pathogen through traditional routes may be insufficient for nonlabile pathogens introduced through new mechanisms.

The transmission pathway from infected facilities to feral swine is unknown. Previous research has detected PEDV in the environment (*3*,*7*) but did not differentiate viral RNA from infectious virus. Swine facilities often move waste to holding ponds, and these ponds could be a source of infectious virus. Infected swine in backyard operations also could facilitate spillover.

Our data also demonstrate that 3.1% of feral swine had been exposed to another coronavirus, probably TGEV. TGEV, like PEDV, is found only in swine, can survive on fomites, and can cause high mortality rates in pigs <2

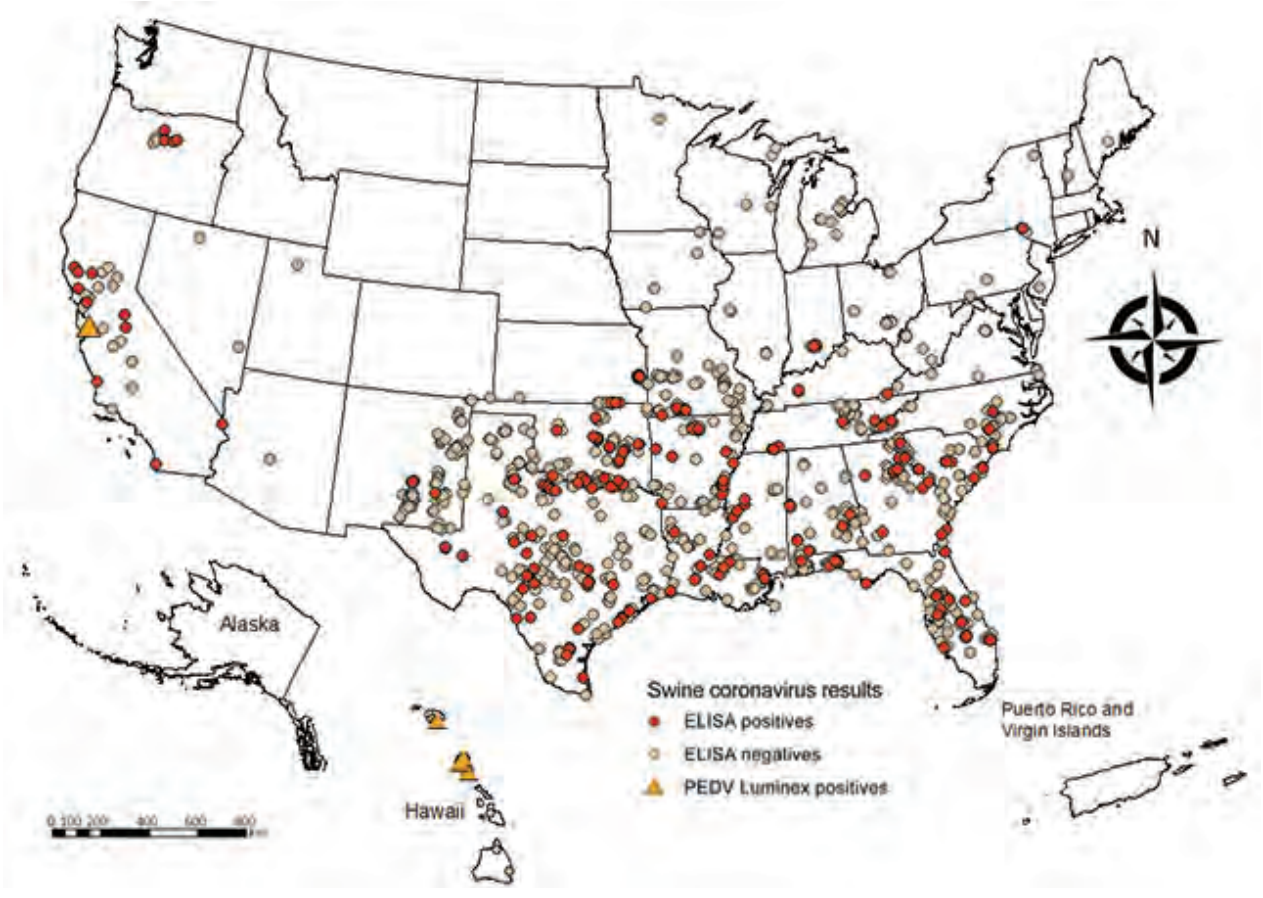

Figure. Collection locations of feral swine samples tested for exposure to swine coronaviruses, United States. In California, 4 PEDV-positive samples were detected at the same location. Samples that were ELISA-positive but PEDV-negative probably indicate exposure to transmissible gastroenteritis virus.

weeks of age. TGEV has been found in the US domestic swine industry since the 1940s. We found TGEV-positive feral swine throughout the entire sampling period and throughout the United States (Figure; online Technical Appendix Tables 1, 2), suggesting that TGEV is probably being persistently transmitted among feral swine, although continual spillover from domestic swine cannot be ruled out. Whether PEDV will display a similar pattern of endemicity over time is unknown; however, our data did not suggest continual transmission or high seroprevalence. For example, the most recent PEDV-seropositive feral swine in Hawaii was detected in January 2015. Seventy-six feral swine sampled from the same island after that date were seronegative, suggesting that either seroprevalence was low enough to evade detection or that viral transmission burned out, most likely after initial deaths of susceptible piglets. Research in Asia, however, has found higher PEDV exposure in wild boar, reinforcing that animals can survive infection and raising the possibility of continual transmission in nondomestic swine populations (*6*).

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LETTERS

Adenovirus Type 4 Respiratory Infections among Civilian Adults, Northeastern United States, 2011–2015

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To the Editor: We read with interest the article by Kajon et al. (*1*), which highlighted that human adenovirus type 4 might be an underrecognized cause of acute respiratory disease (ARD) outside military settings. We report that human adenovirus B7 (HAdV-B7) might also be a cause of this disease.

HAdV-B7 is well recognized as a causative agent of neonatal disease and infections in immunocompromised patients. However, we identified an unusual cluster of 4 cases of severe ARD caused by this pathogen in immunocompetent adults in Dublin, Ireland. These patients had acute respiratory illness when they came to the emergency department of Mater Misericordiae University Hospital in Dublin. The patients came to the hospital over a 4-week period during the summer of 2017, and each patient required intensive care support for single-organ failure. Three patients required intubation and ventilation; all 4 patients recovered.

Three patients reported gastrointestinal and respiratory symptoms, as seen in Oregon, USA (*2*). Although coinfection with other viruses or bacteria has been described (*3*), only 1 patient in our cluster had a possible concomitant pathogen. None of the 4 patients were given antiviral therapy but all received antimicrobial drugs.

All 4 case-patients were either homeless or in temporary accommodations for homeless adults, but we did not identify any epidemiologic link. The Department of Public Health and temporary accommodation sites were notified to raise awareness and offer early testing of symptomatic persons. However, no additional cases were identified.

HAdV-B7 was identified by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of viral hexon genes (*4*). Each virus had 100% identity within the region sequenced to a strain previously associated with respiratory illness in a military training camp in China (GenBank accession no. KP896481).

This cluster of HAdV-B7 causing severe ARD in immunocompetent adults appears to have no clear epidemiologic link. We agree that HAdV might be an underrecognized pathogen in severe communityonset ARD. Testing for viral respiratory pathogens should be considered in all patients and not just the immunocompromised.

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Novel Highly Pathogenic Avian Influenza A(H5N6) Viruses in the Netherlands

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DOI: https://doi.org/10.3201/eid2407.180635

To the Editor: In their recent article, Beerens et al. (*1*) reported detection of a novel clade 2.3.4.4 group B highly pathogenic avian influenza (HPAI) H5N6 subtype virus in wild birds and poultry in the Netherlands in December 2017. This novel virus is a reassortant of the HPAI H5N8 subtype virus with polymerase basic 2 (PB2) and neuraminidase genes from the Eurasian gene pool. This study highlighted concerns over the recent emergence and spread of these viruses. H5N6 subtype viruses have been identified in multiple Eurasian regions and have shown diverse genotypes since the beginning of 2017. Clade 2.3.4.4 group B H5N6 subtype viruses were reported to have affected migratory birds and poultry in South Korea in December 2017 (*2*). The viruses from South Korea have PB2 and polymerase acidic genes distinct from those of viruses from the Netherlands. H5N6 subtype viruses were also detected in Greece, Japan, and Taiwan in 2017. Viruses from these regions have the same genotype, but PB2, polymerase acidic, and neuraminidase genes differ from those in the viruses from the Netherlands.

Clade 2.3.4.4 group A H5N8 subtype viruses emerged in East Asia in early 2014 and subsequently spread, along migratory bird routes, to Europe and North America by the end of 2014. H5N8 subtype and reassortant viruses, including H5N1 and H5N2 subtypes (*3*), resulted in the deaths of 7.5 million turkeys and 42.1 million chickens in the United States alone. Since June 2016, these H5N8 subtype viruses have circulated in Europe and reassorted with local low pathogenicity avian influenza viruses. Group B H5N8 subtype viruses caused the long-lasting second wave of outbreaks in wild birds and poultry in 30 countries in Europe, including the Netherlands, Belgium, Luxembourg, and the United Kingdom (*4*). It is hard to predict whether novel clade 2.3.4.4 group B H5N6 subtype viruses might trigger a similar new wave of massive HPAI H5Nx outbreaks in wild and domestic birds and even cause infections among poultry workers. However, more surveillance data are needed to investigate the genetic diversity and evolution of these viruses and how widely they are circulating in wild birds.

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ANNOUNCEMENT

World Health Organization Regional Office for Africa Weekly Bulletin on Outbreaks and Other Emergencies

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The basis for effective public health action is accurate and timely information (*1*). During emergencies, a fundamental public health tool is rapid, proactive, and transparent communication. Information gathered and disseminated during emergencies not only guides public health authorities but also encourages communities to adopt protective behaviors, triggers a heightened level of disease surveillance across borders, and reduces confusion among national authorities and communities (*2*). Although the International Health Regulations (2005) have provided a strong system for urgent communications (*3*), these alerts often remain siloed among a few parties. For several decades, lack of information sharing or incompatible communication systems have remained paralyzing factors for complex emergency responses (*4*). Indeed, among the lessons from the 2014–2016 Ebola virus disease epidemic was the value of effective communication and transparency in reporting (*1*).

The Transformation Agenda of the World Health Organization (WHO) Secretariat in the African Region 2015–2020 has 4 major objectives, 1 of which is improved strategic and effective communication (*5*). In keeping with this objective, in March 2017, the WHO Regional Office for Africa launched the Weekly Bulletin on Outbreaks and Other Emergencies (the Bulletin). The Bulletin (Figure) is not intended to compete with or replace the more traditional communication strategies, which range from targeted risk communications and media to peer-reviewed scientific publications. Rather, the Bulletin aims to bridge the gaps within this spectrum, providing real-time actionable updates on the status of new and ongoing events, while highlighting actions taken and gaps that need addressing by Member States and partners.

However, providing regular communications on emergencies in the African region is no easy task. The sheer scope and magnitude of emergencies presents an enormous challenge. Capacity on the ground is often limited, and investigations and response efforts often take precedence over information dissemination. Communications must also proceed amid a great deal of uncertainty; events are often rapidly evolving or subjected to political sensitivities. For too long, these challenges have precluded timely communications about emergencies in the African region; information often remains unpublished, is published in retrospect, or is credited to authors outside the region.

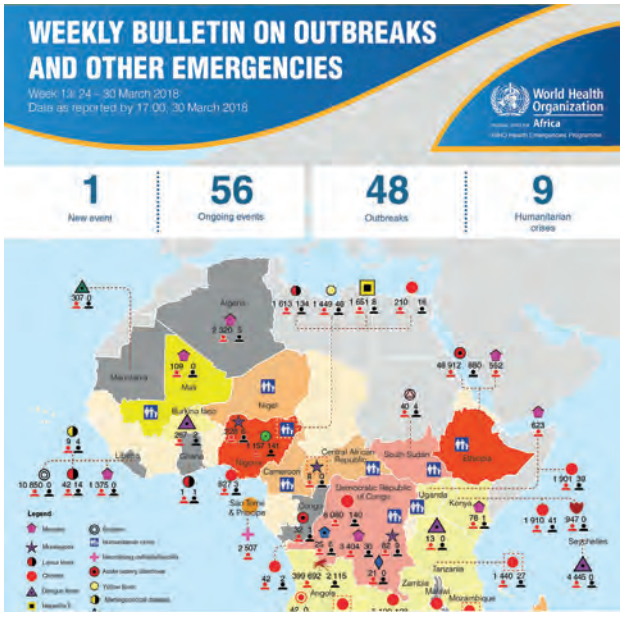

Figure. Cover of recent edition of the Weekly Bulletin on Outbreaks and Other Emergencies, published by the World Health Organization Regional Office for Africa.

The Bulletin provides a platform for overcoming these challenges. Through participation in the writing process, it provides a mechanism for WHO country offices to rapidly communicate updates to a wide audience. In 2017, the Bulletin published 43 editions, including 245 articles, and disseminated them directly to a growing readership of ≈2,000 members and posted them on social media and public health information websites, including but not limited to ProMED-mail, Outbreak News Today, and ACAPS (Assessment Capacities Project).

With the support of WHO Member States, it is our hope that the Bulletin will continue to play a major role in improving communication in the region. We welcome readers to join our mailing list.

Acknowledgments

The regular publication of the Bulletin would not be possible without the open contributions of WHO staff across the African Region; generous sharing of information by ministries of health and partners; and a team of writers, editors, graphics designers, and other staff in the WHO Health Emergencies Programme.

About the Author

Dr. Impouma is program manager of Health Emergency Information & Risk Assessment for the WHO Health Emergencies Programme within the Regional Office for Africa. His primary research interests include prevention and control of infectious disease outbreaks, global public health, monitoring and evaluation, and health information systems.

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Pieter Bruegel the Elder (c.1525–1569), *The Wine of Saint Martin's Day* (1565–1568) (detail). Tempera on linen. 58.2 in × 106.5 in/147.8 cm × 270.51 cm, Museo del Prado, Madrid, Spain, Copyright of the image Museo Nacional del Prado/Art Resource, NY.

A Masterwork of Art, a Metaphor for Prevention

Byron Breedlove and Anne Schuchat

Pieter Bruegel the Elder, the best known member of a large family of artists who were active in the 16th and 17th century Netherlands, is acclaimed as one of the most important 16th century Dutch and Flemish Renaissance painters. Art critic Johnathan Jones notes that "The genius of Bruegel shows itself not just in his wild imagination—in which he resembles the southern Dutch visionary Hieronymus Bosch—but his acute feeling for landscape and human behaviour."

The Wine of Saint Martin's Day is the largest of the 41 surviving paintings from this Flemish master and the last to be included among his oeuvre. In 2010, staff from the Museo del Prado verified that this painting was an authentic Bruegel. According to the museum's documents, this work is a *tüchlein*, painted in glue-size tempera on unprimed linen. Although many artists in 15th and 16th century Flanders used this technique, few examples have survived.

Bruegel's vivid, detailed representations of Flemish village life—the festivals, feasts, celebrations, weddings, and hunts—offer a durable record of a folk culture lost to time. The festival associated with Saint Martin's Day on November 11 was an occasion for feasting and revelry ahead of the oncoming winter. Central to the celebration was the new wine of the year, known as Saint Martin's wine. Art critic Michael Kimmelman describes Bruegel's sprawling painting as "a panoramic canvas showing a mountain of revelers drinking the first wine of the season, and a few of them suffering its consequences." Bruegel's calculated focus on details permeates his unfolding spectacle

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of avarice. As Kimmelman notes, "You can admire the delicacy of faces and hands and feet, alive and varied, making a jigsaw of humane detail, Bruegel's trademark. . . ."

The Museo del Prado describes revelers clustered at the center of the painting: "Around the barrel the artist has arranged a varied crowd of figures: old and young men, women, some with children, peasants, beggars and thieves, all trying to obtain the largest possible quantity of wine. Those who have been successful and have filled their containers with wine are back on the ground while others are still clinging onto the wooden supports, lying on the barrel or leaning over perilously to catch the wine as it spurts from the barrel in whatever recipients they have to hand, including their hats and shoes."

In addition to the consequences from overindulgence of from wine and food, this crowd of revelers, regardless of social status or age, could have shared something more insidious at the festival. Respiratory infections, which are easily spread among people who gather in close proximity, can be transmitted from person to person through respiratory droplets in coughs or sneezes or through contact with fomites contaminated by the respiratory droplets. Late autumn, when the November celebration of St. Martin's Day occurs, typically heralds the onset of cool, dry weather in temperate zones in the Northern Hemisphere and the season when pneumonia and influenza infections begin to increase.

Bruegel liked to place key actions away from the viewer's focus, as is evident in the more familiar work *The Fall of Icarus* captured in the W.H. Auden poem *Musée des Beaux Arts*. For *The Wine of Saint Martin's Day*, if the viewer's eyes wander to the blue-topped towers dwarfing a collection of small figures, scan the just discernible horizon, alight on the smattering of trees, or glance at the figure astride a white horse placed in the periphery, they invariably return to the 100 or so figures splayed across the canvas and that teeming bolus of activity around the huge red barrel.

The Heilbrunn Timeline of Art History notes that Bruegel's paintings ". . . demonstrate the artist's attentive eye for detail and attest to his direct observation of village settings, they are far from simple re-creations of everyday life. The powerful compositions, brilliantly organized and controlled, reflect a sophisticated artistic design." The Museo del Prado stresses that "The artist creates a deliberate contrast between the central group around the barrel and the much more stable, pyramidal group that depicts the charity of Saint Martin on the right."

St. Martin's act of cutting his cape in half to help provide warmth to a poor man is nearly overlooked, taking place at the lower right corner of the canvas. St. Martin's cape could be a metaphor for the protection from disease afforded by vaccines, the similarity being that prevention rarely garners the spotlight and sometimes may seem overlooked. However, immunization can provide protection against many respiratory and other vaccine-preventable diseases.

Although many emerging infections derive from exotic wildlife reservoirs or invasive mosquito and tick species, most respiratory and vaccine-preventable infectious diseases represent the mundane, previously common infections that do not generate headlines. Nonetheless, their control and even elimination represent a masterwork of public health.

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- Treating Myanmar Refugees with *Ancylostoma ceylanicum*, Thailand–Myanmar Border, 2012–2015
- Clonal Expansion of Macrolide-Resistant Sequence Type 3 *Mycoplasma pneumoniae*, South Korea
- Toxoplasmosis in Transplant Recipients, Europe, 2010–2014
- Susceptibility of Human Prion Protein to Conversion by Chronic Wasting Disease Prions
- Enhanced Surveillance for Coccidioidomycosis, 14 US States, 2016
- Therapeutic and Transmission-Blocking Efficacy of Dihydroartemisinin/Piperaquine and Chloroquine against *Plasmodium vivax* Malaria, Cambodia
- Dual Genotype Infection of *Orientia tsutsugamushi* in Patient with Rash and Eschar, Vietnam
- *Coxiella burnetii* Endocarditis and Meningitis in Man
- *Anncaliia algerae* Microsporidial Myositis, South Wales, United Kingdom
- Hospitalized Patient as Source of *Aspergillus fumigatus*, 2015
- Probable Locally Acquired *Babesia divergens*–like Infection in Woman, Michigan, USA
- Variation in Influenza B Virus Epidemiology by Lineage, China
- Fatal Nongroupable *Neisseria meningitidis* Disease in Vaccinated Patient Receiving Eculizumab
- Piperaquine Resistance in *Plasmodium falciparum*, West Africa
- Progressive Multifocal Leukoencephalopathy after Treatment with Nivolumab
- Dapsone Resistance in Leprosy Patients Originally from American Samoa, USA
- Babesiosis Misdiagnosed as Malaria, Equatorial Guinea, 2014
- Phylogeny of Yellow Fever Virus, Uganda, 2016

Complete list of articles in the August issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 26–29, 2018
ICEID
International Conference on Emerging Infectious Diseases
Atlanta, GA, USA
https://www.cdc.gov/iceid/index.html

September 23–26, 2018
ASM Conference on Rapid Applied Mirobial Next-Generation Sequencing and BIoinformatic Pipelines
Tysons, VA, USA
https://www.asm.org/

October 1–3, 2018
ASTMH
International Conference on Migration Health
Rome, Italy
http://istmsite.membershipsoftware.org/icmh2018

October 3–7, 2018
ID Week
San Francisco, CA, USA
http://www.idweek.org/

October 14–18, 2018
Keystone Symposia Conference
Hong Kong, China
http://www.keystonesymposia.org/18S2

October 28–30, 2018
International Society for Vaccines Annual Congress
Atlanta, GA, USA
http:// www.ISVCongress.org

October 28–November 1, 2018
ASTMH
American Society of Tropical Medicine and Hygiene
New Orleans, LA, USA
http://www.astmh.org/

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International Society for Infectious Diseases
7th International Meeting on Emerging Diseases and Surveillance
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Article Title

Large Outbreaks of Fungal and Bacterial Bloodstream Infections in a Neonatal Unit, South Africa, 2012–2016

CME Questions

1. Which of the following statements regarding infection with *Candida krusei* during the neonatal period is most accurate?

A. The mortality rate of infection exceeds 70%
B. *C. krusei* is highly sensitive to fluconazole
C. Established risk factors for *C. krusei* infection among neonates include low birthweight, central venous catheter use, and necrotizing enterocolitis
D. NICU outbreaks of *C. krusei* are more common than those associated with *Candida parapsilosis*

2. Which of the following statements regarding cases of *C. krusei* fungemia in the current study is most accurate?

A. Nearly all cases had *C. krusei* isolated just once
B. Approximately half of isolates were resistant to amphotericin B
C. Most cases had been exposed to human immunodeficiency virus (HIV)
D. The case-fatality rate was only slightly higher compared with the mortality rate among infants without candidemia

3. Which of the following variables was the most significant risk factor for the development of *C. krusei* candidemia in the current study?

A. The presence of necrotizing enterocolitis
B. Female sex
C. Receiving 3 vs. 2 antibiotics
D. Exposure to HIV

4. Which of the following statements regarding infection prevention and control practices in the current study is most accurate?

A. General cleanliness was a major problem
B. Isolation facilities were inadequate
C. Staff hand hygiene compliance was 98%
D. The ventilation system was found to be the source of *C. krusei*

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Article Title

Epidemiology and Geographic Distribution of Blastomycosis, Histoplasmosis, and Coccidioidomycosis, Ontario, Canada, 1990–2015

CME Questions

1. Your patient is a 67-year-old man with antibiotic-refractory pneumonia who recently returned from vacation in Ontario, Canada. According to the case series by Brown and colleagues, which of the following statements about the epidemiology of microbiology laboratory-confirmed cases of blastomycosis in Ontario, Canada, from 1990 to 2015 is correct?

A. Blastomycosis was the most common infection, with an incidence of 0.41 cases/100,000 population
B. The incidence of blastomycosis decreased from 1995 to 2001
C. There were localized hotspots of blastomycosis in the southeast region
D. Blastomycosis was most often diagnosed in the spring

2. According to the case series by Brown and colleagues, which of the following statements about the epidemiology of microbiology laboratory-confirmed cases of histoplasmosis and coccidioidomycosis in Ontario, Canada, from 1990 to 2015 is correct?

A. Coccidioidomycosis cases outnumbered histoplasmosis cases
B. The highest proportion of cases of histoplasmosis was in hotspots bordering the Great Lakes and the St. Lawrence Seaway
C. Coccidioidomycosis cases were presumed to be unrelated to travel
D. Coccidioidomycosis cases did not increase from previous reports

3. According to the case series by Brown and colleagues, which of the following statements about clinical and public health implications of the epidemiology of microbiology laboratory-confirmed mycoses in Ontario, Canada, from 1990 to 2015 is correct?

A. The investigators do not advocate mandatory disease reporting and surveillance for blastomycosis
B. Overall provincial and hotspot rates of blastomycosis are likely overestimated
C. There is no evidence for environmental presence of *Blastomyces* spp. in the northeast and south-central regions of Ontario
D. The differential diagnosis of antibiotic-refractory pneumonia in patients traveling to or living in Ontario should include blastomycosis and histoplasmosis

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Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words in the main body of the text or include more than 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Research Letters Reporting Cases, Outbreaks, or Original Research. EID publishes letters that report cases, outbreaks, or original research as Research Letters. Authors should provide a short abstract (50-word maximum), references (not to exceed 10), and a short biographical sketch. These letters should not exceed 800 words in the main body of the text and may include either 1 figure or 1 table. Do not divide Research Letters into sections.

Letters Commenting on Articles. Letters commenting on articles should contain a maximum of 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.

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