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On the Cover

Pieter Claesz (1597–1660)
Still Life with Turkey Pie (1627)
Oil on wood panel
(75 cm × 132 cm)
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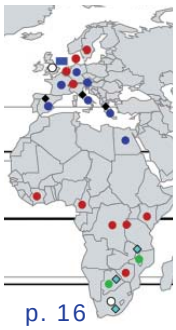
About the Cover p. 178

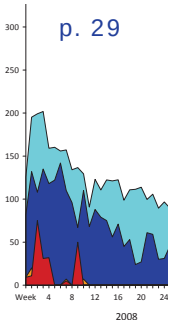
Synopses

CME ACTIVITY

Research



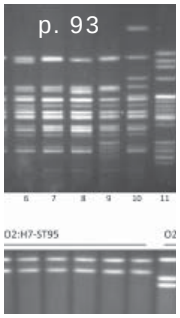
p. 16

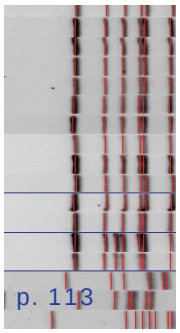


p. 29

EMERGING INFECTIOUS DISEASES
January 2010

Dispatches





Commentary

Another Dimension

Letters

Book Reviews

About the Cover

Erratum

Public Health Threat of New, Reemerging, and Neglected Zoonoses in the Industrialized World

Sally J. Cutler, Anthony R. Fooks, and Wim H.M. van der Poel

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Learning Objectives

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

- List animal hosts for different zoonoses
- Identify the type of zoonosis most likely to undergo genetic mutation
- Specify factors that increase the risk for zoonoses now and in the future
- Describe the epidemiology and clinical presentation of rickettsial diseases

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CME Author

Charles P. Vega, MD, Associate Professor; Residency Director, Department of Family Medicine, University of California, Irvine, California, USA. *Disclosure: Charles P. Vega, MD, has disclosed no relevant financial relationships.*

Authors

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Microbiologic infections acquired from animals, known as zoonoses, pose a risk to public health. An estimated 60% of emerging human pathogens are zoonotic. Of these pathogens, >71% have wildlife origins. These pathogens can switch hosts by acquiring new genetic combinations that have altered pathogenic potential or by changes in behavior or socioeconomic, environmental, or ecologic characteristics of the hosts. We discuss causal factors that influence the dynamics associated with emergence or reemergence of zoonoses, particularly in the industrialized world, and highlight selected examples to provide a comprehensive view of their range and diversity.

Author affiliations: University of East London, London, UK (S.J. Cutler); Veterinary Laboratories Agency, Addlestone, UK (A.R. Fooks); University of Liverpool, Liverpool, UK (A. R. Fooks, W.H.M. van der Poel); and Central Veterinary Institute of Wageningen University and Research Centre, Lelystad, the Netherlands (W.H.M. van der Poel)

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The World Health Organization/Food and Agriculture Organization/World Organisation for Animal Health joint consultation on emerging zoonotic diseases, held in Geneva in 2004, defined an emerging zoonosis as "a pathogen that is newly recognized or newly evolved, or that has occurred previously but shows an increase in incidence or expansion in geographical, host or vector range" (www.who.int/zoonoses/emerging_zoonoses/en). Through continued alterations in human and animal demographics and

environmental changes, new and recurring diseases are likely to continue to emerge.

The effects of zoonoses on human health and economics have recently been underscored by notable outbreaks such as those involving Nipah virus and severe acute respiratory syndrome (SARS) coronavirus (CoV). A recent retrospective study of 335 emerging infectious episodes over a 64-year period (1940–2004) emphasized the role of wildlife as a source of emerging infections. However, research efforts have typically been focused toward either humans or economically related species (*1*).

The frequency of these events increased substantially over the period of investigation (*2*). Such infections are now often perceived as agents of biologic warfare rather than infections with a long but insidious history in their appropriate ecologic niche. Why then are these infections becoming a serious public health concern? The answer is a complex multifactorial set of changing circumstances. To support the growing human population, we have an increasing demand for nutritional support, resulting in intensive agricultural practices, sometimes involving enormous numbers of animals, or multiple species farmed within the same region. These practices can facilitate infection to cross species barriers.

Additionally, we are witnessing increasing globalization, with persons (*3*), animals, and their products (*4*) moving around the world. This movement enables unprecedented spread of infections at speeds that challenge the most stringent control mechanisms. Furthermore, continual encroachment of humans into natural habitats by population expansion or tourism brings humans into new ecologic environments and provides opportunity for novel zoonotic exposure. Climatic changes have facilitated the expansion of compatible conditions for some disease vectors, remodeling dynamics for potentially new, emerging, and reemerging zoonoses (*5*). In the next 2 decades, climate change will be the most serious issue that dominates reemergence of pathogens into new regions.

Climate change also effects evolution of pathogens, and where relevant, their vectors. Continual mutation and recombination events give rise to variants with altered levels of fitness to persist and spread. Changing ecologic circumstances and pathogen diversity can give rise to variants with altered pathogenic potential. However, the host must not be ignored. Increased longevity and therapies for persons with diseases can modulate host susceptibility and concomitant infections and upset the evolving and dynamic infection balance.

Emerging, Reemerging, and Neglected Zoonoses

Data for this review were identified in PubMed searches and relevant journal articles and excluded those studies not published in English. Emerging or reemerging pathogens must be considered on multiple levels. First, pathogens not previously known have been identified. For example, alteration in the processing of cattle feed in the United Kingdom resulted in extended host range and emergence of bovine spongiform encephalopathy in cattle (*6*). Similarly, mixing of multiple species under stressful conditions can promote a species jump such as that witnessed with SARS-CoV (*7*). New opportunities can be created by climatic changes such as global warming and ecologic alterations facilitated through changed land use and movements of infected hosts, susceptible animals, or disease vectors.

In 1987, 1997–1998, and 2006–2007, outbreaks of infection with Rift Valley fever virus in Africa were associated with changes in river flow and flooding resulting from damming of rivers or heavy rainfall. Many zoonotic pathogens fall into the category of generalist agents exhibiting extensive host diversity, e.g., *Coxiella burnetii*, the etiologic agent of Q fever. This bacterium can successfully infect hosts ranging from domestic animals to wildlife, reptiles, fish, birds, and ticks.

Others agents have restricted specific transmission dynamics because of limited host ranges. These agents include simian immunodeficiency viruses 1 and 2, which are found in chimpanzees and sooty mangabees, and Rift Valley virus, which is transmitted by *Aedes* spp. and *Culex* spp. mosquitoes and found in sheep and goats. For many zoonotic agents, the potential to cause infection in accidental hosts, such as humans, exists, but often this represents a dead-end host. Pathogens such as *Anaplasma* spp., *Erhlichia* spp., *Rickettsia* spp., *Bartonella* spp., West Nile virus, and rabies virus can be included in this group.

From an epidemiologic point of view, "A reservoir should be defined as one or more epidemiologically connected populations or environments in which a pathogen can be permanently maintained and from which infection is transmitted to the defined target species" (*8*). Conversely, some zoonoses in specific conditions show remarkable ability for human-to-human transmission beyond the confines of natural sylvatic cycles. This ability was seen during a recent outbreak of plague among diamond miners in the Congo. This outbreak was initiated by an infection of a miner, which became pneumonic and resulted in 136 secondary cases of pneumonic plague and 57 deaths (*9*). Transmission of plague is complex and dynamic, with combinations of stochastic and adaptive mechanisms. As seen in this example, rapid transmission often occurs, but this is accompanied by slower, localized transmission among enzootic reservoir species, which often use vector-borne expansion among low-density hosts (*10*). Other zoonoses, given correct circumstances, can result in human-to-human transmission. These zoonoses include those that cause Ebola fever, influenza A, plague, tularemia, and SARS (*11*).

New or emerging virulence traits can evolve and result in large-scale transmission and concomitant alteration of pathogenicity. This new pathogenicity may include increased invasiveness, enhanced spread, toxin production, or antimicrobial drug resistance. *Y. pestis* has shown a resurgence in regions such as Madagascar, with isolates showing a marked increase in resistance to antimicrobial agents (*12*). Similarly, a recently evolved outer surface protein A serotype of a Lyme borreliosis spirochete (*Borrelia garinii* serotype 4), has shown particularly aggressive tendencies and is often associated with hyperinvasive infection (*13*). Concern has also been noted about increasingly frequent isolation of *Corynebacterium ulcerans* carrying the diphtheria toxigenic phage.

Mutation is the ultimate source of genetic variation, on which natural selection, genetic drift, gene flow, and recombination act to shape the genetic structure of populations. This factor is especially notable in viruses, which have relatively small genomes and short generation times, particularly among viruses with more error-prone RNA genomic replication (*14*). However, most mutations are deleterious and under pressure of innate and adaptive host immunity, viruses probably always experience selection for mutation rates >0. The upper limit on mutation rates will be determined by factors such as natural selection, genomic architecture, and the ability to avoid loss of viability or genetic information, albeit, that a loss of genetic information and increased specialization is observed in co-evolution with a host (*15*).

According to evolutionary theory, higher mutation rates should be favored in a changing environment, such as altered host immune defenses. However, in experimental settings, artificially increased mutation rates are often associated with lower virus titers. In addition, a complex relationship exists between underlying mutational dynamics and the ability to generate antigenic variation, which in turn has serious implications for the epidemiologic potential of the virus.

Evolutionary changes are not always a prerequisite for viral emergence in a new host. Some viruses (e.g., poxviruses), have a wide host range and show a relatively low mutation rate. However, in other viruses such as Venezuelan equine encephalitis virus, evolutionary change is essential for efficient infection and transmission to new hosts (*16*). Because most viruses replicate poorly when transferred to new hosts, greater variation is more likely to assist viral adaptation to its new host.

All too frequently, the diagnosis of zoonotic disease is delayed through lack of clinical suspicion or failure to obtain adequate clinical histories. Some zoonotic infections are unusual (e.g., scabies infection after handling of pet guinea pigs). Other infections may have a less obvious animal link. Mowing lawns is believed to be a risk factor for acquiring tularemia (caused by *Francisella tularensis)* in disease-endemic areas where lagomorph reservoirs may be killed by mowers or hedge trimmers (*17*).

For some infections, zoonotic transmission occurs indirectly through food. Human brucellosis is not usually acquired through animal contact but is transmitted more often by consumption of infected animal products such as unpasteurized dairy products (*18*). *Salmonella* spp. have repeatedly caused outbreaks of salmonellosis after persons have eaten uncooked eggs (*19*). Hepatitis E virus has been transmitted through consumption of uncooked deer meat (*20*).

Exposure routes may be airborne, as demonstrated for several outbreaks of Q fever (*21*). An ongoing airborne Q fever outbreak in the Netherlands related to goat farming has raised awareness of this previously neglected zoonosis (*22*). How humans were exposed to these animals would not have been apparent; the exposures were identified by epidemiologic mapping of the distribution of cases. These examples underscore the necessity of gathering comprehensive patient data to effectively diagnose zoonoses.

Recreational Zoonoses

Sporting activities can expose humans to zoonotic infections. Hunting wildlife has been associated with infections such as brucellosis and tularemia (*23*). Less obvious routes arise from activities such as water sports. *Leptospira* spp.–infected animals excrete viable organisms in their urine, which can persist in aquatic environments for prolonged periods. After a triathlon event in 1998, a total of 52 of 474 athletes tested were diagnosed with leptospirosis (*24*). Suspicion of water sport–related infections with hepatitis A and *Leptospira* spp. led to closure of an area of Bristol, United Kindom, where docks were used for recreational water activities (*25*).

Horses are now moved from countries in Europe to warmer regions (e.g., United Arab Emirates) to prolong the racing season during the winter. Hunting activities have promoted large-scale export of animals such as hares (possible reservoirs of tularemia and brucellosis) from Poland and the movement of potentially rabies-infected raccoons in the United States. In other countries such as the United Kingdom, pheasants are bred and released for shooting in the fall and provide plentiful hosts for questing ticks and increasing their abundance. Importation of pheasants into the United Kingdom from France was associated with introduction of a mild zoonotic infection (Newcastle virus disease) in 2007 (*26*).

Role of Companion Animals

Companion animals have many forms of contact and opportunities to transmit multiple zoonoses. The sexual stage of the life cycle of *Toxoplasma* spp. occurs in cats,

thus exposing humans to infection in situations in which hygienic measures have not been observed. Cats also serve as reservoir for *Bartonella henselae*, the etiologic agent of cat-scratch fever (*27*). Cowpox virus can also be transmitted to humans by contact with cats (*28*). Animal bites can result in zoonotic infections, typified by infection with *Pasteurella multocida*. Even in the absence of a bite, contact with animals (e.g., licking of wounds) can result in infection. More recently, attention has focused on transmission of *Rickettsia felis* into the human environment by cat fleas (*29*).

Dogs are the most likely source when humans become infected with rabies virus and are potential sources of *Toxocara* spp. This emerging threat is becoming apparent with importation of rescued dogs and global movement of dogs with their owners, which has resulted in several cases of leishmaniasis in the absence of sand fly vectors. Dogs can be a source of methicillin-resistant *Staphylococcus aureus* and could play a role in zoonotic spread of genetic elements responsible for antimicrobial drug resistance (*30*). Contact with dogs in Mediterranean regions has been implicated as a likely source of infection in recent cases of Mediterranean spotted fever reported in traveling humans (*31*).

Cats and dogs can introduce plague or rabies into human environments and have been associated with Q fever in humans and dermatophytosis (ringworm). Scavenger habits of these animals bring them into contact with many zoonotic agents, and close living relationships with humans such as sharing meal plates or beds offer many opportunities for disease transmission.

Pet rats have recently been incriminated as the source of *Leptospira icterrohemoragiae* infection in their owners. Psittacine birds are an established risk factor for acquisition of *Chlamydophila psittaci*. During recent years, the market for exotic pets has greatly increased. This increase has resulted in transmission of several unusual organisms, such as exotic *Salmonella* spp., which are often associated with pet reptiles. Media attention was captured after an outbreak of monkeypox in America that affected ≥70 persons in 2003. After infected African rodents had been imported for the pet trade, the infection spread into native North American black-tailed prairie dogs and was subsequently disseminated among humans (*32*).

Bush Meat

Zoonotic diseases associated with hunting and eating wildlife is of increasing global concern. Bush meat is considered a delicacy by many and has resulted in its growth as a commercial enterprise. Tracking, capturing, handling, butchering in the field, and transporting of carcasses involve risks of cross-species transmission. Particularly high risks are associated with hunting nonhuman primates. The act of butchering is a greater risk factor for acquiring bloodborne pathogens than transporting, selling, and eating the butchered meat (*33*).

Zoonotic pathogens from wildlife may infect humans with little or no human-to-human transmission (e.g., avian influenza virus and Hendra virus). Alternatively, increased travel or migration and increased between-person contacts have facilitated emergence of simian immunodeficiency virus/HIV/AIDS in Africa. Increased exposure to wild-caught animals and high mutation rates of many RNA viruses have increased their predominance among emerging zoonoses transmitted from human to human; RNA viruses from bush meat may therefore play a possible role in future disease emergence.

Globalization and Livestock Movement

Large-scale movement of persons, livestock, food, or goods is now commonplace and provides increasing opportunities for rapid spread of pathogens. Trichinellae in horsemeat have been transported across the Pacific Ocean and infected consumers in other parts of the world. Discarded tires provide new habitats for mosquitoes in addition to their usual ecologic niches. The World Organisation for Animal Health and the Food and Agriculture Organisation implement strict control of animal movement. Transport of animals can result in mingling of different species in crowded and stressful conditions. This mingling can suppress immune responses to persistent infections and increase pathogen shedding. Under such circumstances, susceptible species can readily become infected (*34*).

Tourism

Tourism has exponentially increased in recent years. This finding has resulted in increasing numbers of imported zoonoses, such as a variety of rickettsial spotted fevers, brucellosis, melioidosis, genotype I hepatitis E (*35*), tick-borne encephalitis (*36*), and schistosomiasis (*37*).A rapid increase in cases of African tick bite fever has been associated with travelers to sub-Saharan Africa and the eastern Caribbean. This disease, which is caused by *R. africae*, is transmitted by a particularly aggressive *Amblyomma* sp. tick; >350 imported cases have been observed in recent years (*31*). Infection sequalae, such as subacute neuropathy, may be found long after travel when tick bite fever eschars have disappeared (*37*). An estimated ≥1 million international journeys are made each day, and a staggering 700 million tourists travel on an annual basis. Detailed travel histories of patients who show clinical signs and symptoms of disease are needed.

Changed Land Use and Urbanization

Deforestation and development of natural habitats have been seen on a global scale to accommodate intensification of agriculture and living areas for humans. As a result, eco-

logic habitats have been disrupted, reservoir abundance has changed, and transmission dynamics have been altered. Reduced host abundance may force vectors to seek alternative hosts, increasing opportunities for disease transmission, as demonstrated by increases in human cases of Lyme borreliosis, ehrlichiosis, spotted fevers, and anaplasmosis. Development of forests to provide rubber plantations in Malaysia has been correlated with increases in schistosomiasis (*37*). Wildlife may modify feeding practices as a consequence of changing land use, bringing them closer to humans and livestock. This modification was suggested to have been instrumental in the Nipah virus outbreak that affected pigs and humans in Malaysia in 1999. Nipah virus persists as a serious problem in many rural areas of Bangladesh and India, where infected bats living near human dwellings, urinate in date palm sap, which is later consumed raw by humans (*38*).

Human population growth has been associated with reshaping of population demographics. Increasing from 1 billion at the beginning of the 20th century to 6 billion by the end of the century, current predictions forecast a human population of ≈10 billion by 2050. This prediction is accompanied by a staggering increase in urbanization of the population from 39% in urban environments in 1980 to 46% in 1997 and a predicted 60% by 2030. This high-density clustering of the human population paves the way for potential outbreaks on an immense scale (*5*).

Public Health Risks of Reemerging and Neglected Zoonoses

Many areas are now experiencing a reemergence of zoonotic pathogens, partly resulting from collapse of public health programs during political upheavals. Often, these areas increasingly appeal to those seeking adventurous or unusual holiday destinations.

Delay in development of clinical signs and often insidious onset can challenge appropriate diagnosis and patient management. Furthermore, movements of animals used for agricultural trade, sport, and as companions also offer opportunities for further dissemination of infections. Brucellosis-free countries have seen reintroductions associated with movement of infected livestock. Movement of pets throughout Europe has been associated with an alarming increase in diseases such as leishmaniasis. Moreover, pets can harbor ectoparasites such as ticks, fleas, and lice. All of these parasites, especially ticks, are notorious vectors of multiple zoonotic agents.

We are at risk for airborne transmission of zoonoses by many factors (e.g., from travel to farms, consumption of food, and mowing the lawn, which as been associated with tularemia). Visiting petting farms or having family pets increases the likelihood of potential zoonotic infections, especially if pets are exotic. Water sports may increase the risk for acquiring leptospirosis. Wilderness camping activities have been associated with hantavirus infection after inhalation of aerosolized urine excretions of rodents. Other sporting activities such as hunting have been associated with brucellosis and tularemia. Travel to other countries opens a range of new potential zoonotic exposures through direct contact or indirectly through fomites, food, or arthropod vectors. Increasingly exotic locations are being sought with associated exotic zoonoses. Some tourists consume local delicacies, such as aborted animal fetuses in Ecuador, which are a source of brucellosis (*39*).

Conclusions and Future Prospects

Many zoonoses can be considered opportunistic infections. Increasing demands for protein necessitate increased levels of farming. Food can provide a vehicle for spread of pathogens from animals to humans. Contact with animals during farming, hunting, or by animal bites can increase transmission of diseases (e.g., rabies and tularemia). Arthropod vectors can transmit diseases on an immense scale to other hosts as in cases of West Nile fever and plague.

Changing patterns of farming, life style, and transportation influence the dynamics of pathogen ecology. Pathogens are subjected to changes by many intrinsic and extrinsic factors. Mutation, recombination, selection, and deliberate manipulation can result in new traits acquired by pathogens and result in potential epidemic consequences.

Reemergence of diseases through opportunistic host switching is likely to continue as a major source of human infectious disease. Strategies to improve public health have focused on improved surveillance in regions of perceived high likelihood of disease (reemergence). These strategies include improved detection of pathogens in reservoirs, early outbreak detection, broad-based research to identify factors that favor reemergence, and effective control (i.e., quarantine and improved hygiene) (*40*).

To recognize and combat zoonotic diseases, the epidemiology of these infections must be understood. We need to identify pathogens, their vertebrate hosts, and their methods of transmission. Identification should include knowledge of spatiotemporal disease patterns and their changes over time. These features can be used to identify dynamic processes involved in pathogen transmission (Figure), which can be used to account for observed disease patterns and ultimately forecast spread and establishment into new areas.

Armed with information on expected disease patterns, we can address whether change has occurred beyond that which would normally be expected. However, this analysis may not be suitably responsive to control new and emerging zoonoses. Improved detection may be achieved through use of syndromic approaches rather than searching for specific pathogens.

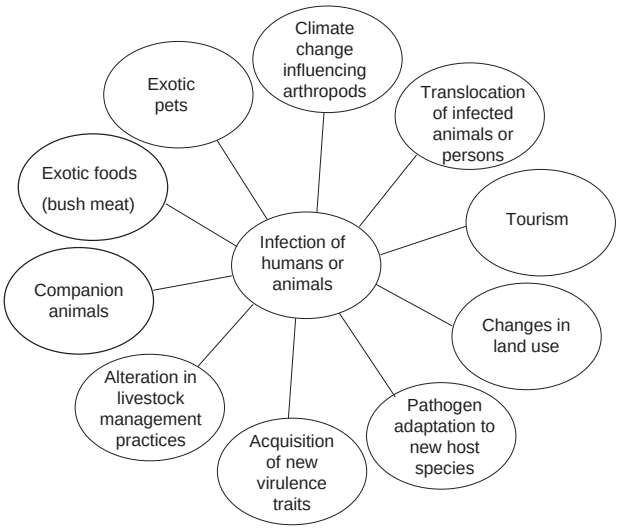

Figure. Factors influencing new and reemerging zoonoses.

Human disease surveillance must be associated with enhanced longitudinal veterinary surveillance in food-producing animals and wildlife. Prompt detection and instigation of control measures such as vaccination are pivotal to prevent disease spread. Novel molecular methods (e.g., DNA microarrays) offer unprecedented opportunities for rapid detection. However, these methods require optimization and validation before they can be used in routine microbiology laboratories. Cloned antigens or attenuated vaccines can be rapidly modified into appropriate antigenic forms. However, for identification of specific pathogens, more research will be needed to provide timely management of a new or emerging disease threat.

Approaches for identification of pathogen replication in vectors are more likely to offer substantial benefits for control of zoonoses. These methods are inappropriate for human vaccines, which must adhere to stricter legislative criteria. However, control of zoonotic infections in reservoir hosts has a pronounced protective effect in human populations. Use and development of antiviral drugs are other useful possibilities, but these drugs are likely to be too expensive for use in large disease outbreaks and emergence of drug resistance may result in concomitant loss of therapeutic options for these agents.

We do not know which zoonosis will be the next serious public health threat. However, as we increase efforts to improve the capacity to respond to this pathogen, we will also increase the likelihood that we can efficiently and effectively respond to new, reemerging, or neglected zoonoses in the future.

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Dr Cutler is a reader in microbiology and infectious disease at the School of Health and Bioscience of the University of East London. Her research interest is bacterial zoonoses, particularly Q fever, brucellosis, rickettsiosis, leptospirosis, and relapsing fever borreliosis.

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Address for correspondence Wim H.M. van der Poel, Central Veterinary Institute, Wageningen University and Research Centre, PO Box 65, 8200 AB Lelystad, the Netherlands; email: wim.vanderpoel@wur.nl

Laboratory Surge Response to Pandemic (H1N1) 2009 Outbreak, New York City Metropolitan Area, USA

James M. Crawford, Robert Stallone, Fan Zhang, Mary Gerolimatos, Diamanto D. Korologos, Carolyn Sweetapple, Marcella de Geronimo, Yosef Dlugacz, Donna M. Armellino, and Christine C. Ginocchio

The North Shore–Long Island Jewish Health System Laboratories serve 15 hospitals and affiliated regional physician practices in the New York City metropolitan area, with virus testing performed at a central reference laboratory. The influenza A pandemic (H1N1) 2009 outbreak began in this area on April 24, 2009, and within weeks respiratory virus testing increased 7.5 times. In response, laboratory and client service workforces were increased, physical plant buildout was completed, testing paradigms were converted from routine screening tests and viral culture to a high-capacity molecular assay for respiratory viruses, laboratory information system interfaces were built, and same-day epidemiologic reports were produced. Daily review by leadership of data from emergency rooms, hospital facilities, and the Health System Laboratories enabled real-time management of unfolding events. The ability of System laboratories to rapidly increase to high-volume comprehensive diagnostics, including influenza A subtyping, provided key epidemiologic information for local and state public health departments.

Local sentinel laboratories are a critical component of the Laboratory Response Network, providing frontline diagnostics and, in many instances, the initial reporting for infectious disease outbreaks. In the event of a major health crisis, the responses of a regional clinical laboratory can be central to the ability of civic authorities and healthcare systems to handle such emergencies. This report describes successful steps taken by a hospital-based regional reference laboratory in response to a 7.5× increase in respiratory virus testing during the first 3 weeks (April 27–May 15, 2009) of an outbreak of a novel influenza A (H1N1), now referred to as influenza A pandemic (H1N1) 2009, in the greater New York City metropolitan area.

The North Shore–Long Island Jewish Health System (NSLIJHS) is the third largest nonsectarian not-for-profit health system in the United States and serves Nassau and Suffolk Counties, New York, and the Queens and Staten Island boroughs of New York City. The NSLIJHS Central Laboratories serve 15 hospitals and affiliated regional physician practices; virus testing is performed at the Centralized Laboratories in the Clinical Virology Laboratory (CVL). In the aftermath of the anthrax event of September 2001, NSLIJHS developed an extensive system-wide emergency preparedness plan to deal with potential biothreat and bioterrorism events that could greatly affect a health system. This plan was tested, beginning Friday evening, April 24, 2009, when 20 students 14–17 years of age with symptoms of an influenza-like illness sought evaluation at the pediatric emergency room of Schneider Children's Hospital at Long Island Jewish Medical Center, one of the hospitals served by NSLIJHS. The students were among those attending a preparatory school in Queens, New York, who began experiencing influenza-like symptoms April 22–23, 2009 (*1*). Some students had recently traveled to Mexico, raising immediate concern about pandemic (H1N1) 2009 (*2*). The following day, Saturday, April 25, an additional 67

Author affiliations: North Shore–Long Island Jewish Health System Laboratories, Manhasset, New York, USA (J.M. Crawford, R. Stallone, F. Zhang, M. Gerolimatos, D.D. Korologos, C.C. Ginocchio); Krasnoff Quality Management Institute of the North Shore–Long Island Jewish Health System, Manhasset (C. Sweetapple, M. de Geronimo, Y. Dlugacz); and North Shore University Hospital, Manhasset (D.M. Armellino)

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persons 11–18 years of age and 16 children <8 years of age were evaluated in NSLIJHS emergency rooms. Over the next 3 days, influenza-related cases at NSLIJHS emergency rooms increased rapidly. Specimens from index patients at Long Island Jewish Medical Center on April 24–25 were screened for influenza A/B antigen, using the 3M Rapid Detection Flu A+B test (3M Medical Diagnostics, St. Paul, MN, USA). In conjunction with the New York City Department of Health (DOH), the Medical Center shipped 35 specimens with test results positive for influenza A to the Centers for Disease Control and Prevention (CDC). Testing at CDC confirmed that 28 of the 35 influenza A–positive samples were influenza A pandemic (H1N1) 2009 (*1*). The remaining 68 samples from the Long Island Jewish Medical Center (i.e., those with negative rapid test results) were referred to CVL for direct fluorescent antibody (DFA) testing and viral culture, according to the usual protocol.

On Monday, April 27, 2009, an emergency operations status was declared for NSLIJHS. Herein, we detail the specific steps taken to increase the surge capacity at the NSLIJHS Central Laboratories, thereby enabling timely reporting of respiratory virus test results.

Standard Testing for Respiratory Viruses and Test Capacity

During the normal influenza season, the clinical laboratories of NSLIJHS hospitals perform rapid influenza A+B antigen testing, using either BinaxNOW A+B test (Inverness, Scarborough, MA, USA) or the 3M test. Nasopharyngeal swab samples in Universal Transport Media (Diagnostic Hybrids Inc., Athens, OH, USA) and nasopharyngeal wash and aspirate samples are tested. Specimens with test results positive for influenza A or B are not processed further unless warranted by underlying patient conditions. Due to the suboptimal sensitivity of rapid antigen tests, all samples with negative test results are forwarded to CVL for detection of adenovirus, human metapneumovirus, influenza A and B, parainfluenza viruses 1, 2, and 3, and respiratory syncytial virus by DFA testing, using D3 Ultra Respiratory Virus reagents (Diagnostic Hybrids Inc.) and by rapid respiratory virus culture, using R-Mix cells (Diagnostic Hybrids Inc.).

NSLIJHS laboratories routinely encounter a seasonal increase in respiratory virus testing, peaking in mid-to-late February and waning by May. The historic maximum test volume occurred in February 2008, when CVL tested 6,021 samples and clinical laboratories system-wide performed 2,901 rapid influenza tests, for a combined daily average of 308 tests. During April 1–23, 2009, CVL tested 1,955 samples and clinical laboratories system-wide performed 676 rapid influenza tests, for a combined daily average of 119 tests. These volumes were similar to those for preceding years.

Molecular Detection of Respiratory Viruses

During 2008, the central NSLIJHS Molecular Diagnostics Laboratory performed extensive validation studies of the Luminex xTAG Respiratory Virus Panel (RVP) assay (Luminex Molecular Diagnostics, Toronto, Canada) (*3,4*). The version of the RVP assay that has been cleared by the US Food and Drug Administration detects adenovirus, human metapneumovirus, parainfluenza viruses 1, 2, and 3, rhinovirus/enterovirus group, respiratory syncytial virus, and influenza A and B. This RVP assay can subtype influenza A as seasonal human H1 or H3 virus. The research-use-only version of the RVP assay also detects parainfluenza 4 and coronaviruses OC43, NL64, 229E, and HKU-1. Prior to the outbreak of pandemic (H1N1) 2009, the RVP assay was used for selected clinical cases and research studies, with the intention of converting to use of the RVP assay for all respiratory virus testing during the off-peak 2009 summer months.

Laboratory Testing during the Novel Influenza (H1N1) Outbreak

By Monday, April 27, 2009, it was clear that an unusual event was occurring (*5,6*). Rapid influenza testing at all NSLIJHS clinical laboratories and the centralized laboratories increased dramatically (Figure 1). On April 29, daily test volumes peaked at 903 tests, representing a 7.5× increase over the prior average daily volume for April and a sustained 3× increase over the February 2008 record daily volume of 308 tests.

The weekend of April 25–26, prior to declaration of a system-wide emergency, scheduled CVL staff responded with a round-the-clock effort to keep up with testing needs. On April 27, despite full staffing, considerable overtime was required to perform testing. On April 28, a command meeting was held at the NSLIJHS central laboratories to delineate an action plan to respond to the crisis. The major issues were staffing, testing algorithms, laboratory space, laboratory information systems and biostatistical reporting, leadership roles, and client service functions. The action plan was immediately authorized by system leadership. A key consequence of the plan was immediate deployment of an enhanced and diversified work force, including non-licensed support staff, licensed research staff, laboratory information services personnel, and biostatistical reporting staff. Beginning April 28, these actions enabled CVL to expand its weekday working hours from 6:00 AM–6:00 PM to 5:00 AM–1:00 AM and its weekend working hours from 8:00 AM–4:00 PM to 6:00 AM–10:00 PM. With this personnel strategy, CVL was able to report DFA assay and R-Mix culture results on a real-time basis throughout the crisis. To accommodate the high volume of R-Mix cultures, CVL reduced the normal test algorithm of screening at 24 hours, 48 hours, and 7 days to a single screening and confirmatory

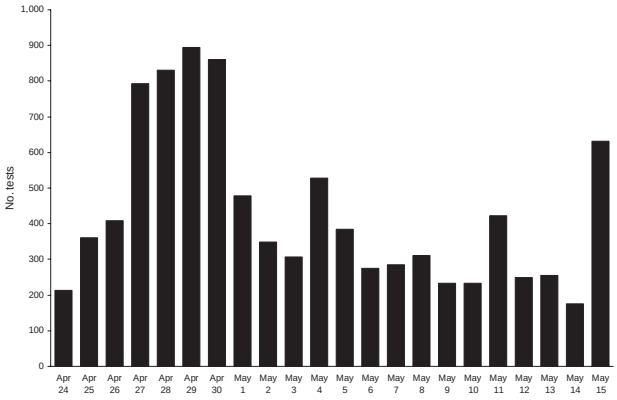

Figure 1. Daily clinical virology test volumes in the North Shore–Long Island Jewish Health System, New York City metropolitan area, USA, April 24–May 15, 2009. General clinical laboratories performed influenza A/B rapid antigen testing only. The central Clinical Virology Laboratory performed direct immunofluorescence antibody testing and R-Mix viral culture, and beginning May 2, the central Molecular Diagnostics Laboratory performed molecular testing for respiratory viruses (xTAG Respiratory Virus Panel, Luminex Molecular Diagnostics, Toronto, Ontario, Canada).

testing at 48 hours. Under these unusual circumstances, this change was considered acceptable as historic laboratory data had demonstrated that 97%–98% of all respiratory viruses are detected in 48 hours.

A second key element of the April 27–May 1 work week was the initiation of RVP testing for influenza A subtyping. The assay was needed to 1) identify which patients were possibly infected with virus subtype H1N1 rather than circulating seasonal H1 or H3 strains and 2) track the magnitude of the outbreak. By May 1, in consultation with the New York State and New York City DOHs, the NSLIJHS Molecular Diagnostics Laboratory began testing, with the RVP assay, the remaining samples for index patients screened on April 24–25 (i.e., the 68 archived samples with negative rapid influenza A/B antigen screening results) as well as all incoming samples with test results positive for influenza A by DFA assay and/or culture.

Due to the large volume of incoming and archived samples, RVP testing was prioritized for hospitalized patients, followed by persons known to be at risk as a result of the school exposure or recent travel to Mexico. May 2–3, the laboratory identified, by RVP assay, 141 samples with nonsubtypeable influenza A, 78 samples with seasonal virus subtype H3, and 2 samples with seasonal virus subtype H1. Including the initial 28 samples that were sent to CDC, the NSLIJHS Molecular Diagnostics Laboratory had identified 169 confirmed or probable pandemic (H1N1) 2009 cases. On May 5, 101 samples with nonsubtypeable influenza A were tested by the New York State Wadsworth Center Laboratory of Viral Diseases: 99 had test results positive for pandemic (H1N1) 2009 and 2 had inconclusive test results due to low virus titers (*7*). These data indicated that during the outbreak, the predictability of a nonsubtypeable influenza A virus identified by RVP assay to be pandemic (H1N1) 2009 was high (*7*). In July 2009, the NSLIJHS Molecular Diagnostics Laboratory obtained New York State approval to confirm cases of pandemic (H1N1) 2009 by using the published CDC method. The ability to subtype influenza A, ruling out seasonal subtypes H1 and H3, and to detect additional respiratory viruses by RVP assay (*8*) within 24 hours enabled NSLIJHS leadership to know whether high-risk patients, inpatients, or members of the System's workforce had probable pandemic (H1N1) 2009.

From that point forward, the Molecular Diagnostics Laboratory provided RVP subtyping results (seasonal H1, H3, or nonsubtypeable) within 24 hours for critically important cases identified by infection-control or civic authorities, especially health officials making public health decisions about regional school systems, and within 48–72 hours for lower priority cases. The laboratory documented that the sensitivity of the RVP assay for detecting all influenza A types was far superior to that for other test methods (*8*), justifying RVP testing for admitted patients with negative influenza A/B rapid test results. This simplified protocol was instituted May 11, 2009, in consultation with system and regional civic authorities.

A detailed scientific analysis of the virology of the outbreak, especially the sensitivities and specificities of the tests, is described elsewhere (*8*); for this publication, summary results are given. Figure 2 shows the total number of positive and negative RVP influenza A test results during April 24–May 15, 2009. Of the total 979 RVP test results, 320 were negative and 677 were positive for any identifiable respiratory virus. A variety of viruses were identified in the 677 samples, including nonsubtypeable influenza A (345 samples), seasonal influenza A subtype H3 (126 samples), seasonal influenza A subtype H1 (5 samples), influenza B (3 samples), rhinovirus/enterovirus group (112 samples), human metapneumovirus (24 samples), parainfluenza viruses 1–4 (40 samples), adenovirus (9 samples), coronaviruses (8 samples), and respiratory syncytial virus (5 samples). Multiple viruses were identified in 41 patients. The outbreak began to subside at the end of June 2009; 8,766 rapid influenza A tests, 8,754 rapid influenza B tests, 8,858 DFA assays, 5,786 viral cultures, and 4,853 RVP assays (36.9% with nonsubtypeable influenza A results) had been performed. This finding represented a total of 34,017 tests for 11,624 patients, a volume that would normally equal the amount of testing performed over a 1-year period.

Physical Plant Construction

The decision was made on April 28 to immediately expand the CVL into contiguous space. Although the se-

verity and duration of the outbreak were unknown, failure to be proactive in expanding the surge capacity of the laboratory was unacceptable. Over 4 days, a negative-pressure laboratory was completed; biohazard hoods, vacuum, and CO_2 lines were installed; incubators and ancillary equipment were ordered; and a specimen-processing area with computer terminals was completed. The additional laboratory space enabled a sustained higher RVP testing capacity and was well-justified because the laboratory processed 700–970 tests a day during the second wave of the outbreak (May 15–31).

Laboratory Information System and Biostatistical Reporting

The Laboratory Information System team accomplished the following tasks over 3 days (April 28–30): 1) created 2 new tests (RVP, novel H1N1 confirmatory) and 3 Laboratory Information System environments (Cerner Classic and Cerner Millennium [Cerner Corp., Kansas City, MO, USA] and Meditech [Medical Information Technology, Inc., Westwood, MA, USA]); 2) validated tests and billing for 11 health information systems; 3) set up a CVL workstation dedicated to influenza specimens; 4) reported daily to infection-control and senior system leadership; 5) reported to the New York State DOH Electronic Clinical Laboratory reporting system; and 6) established logic rules to automatically print positive test results to Client Services. The NSLIJHS Krasnoff Quality Management Institute provided daily biostatistical reports to system leadership. Data assembly was automated by building an Oracle database with interfaces to the Laboratory Information System, with crosschecking to confirm the accuracy and validity of data.

Leadership and Ancillary Personnel

The medical director of CVL and the Molecular Diagnostics Laboratory (C.C.G.), the overall director of System laboratories (J.M.C.), and the chief operating officer of System laboratories (R.S.) divided responsibilities for oversight of staffing, physical plant resources, supplies, the laboratory information system, courier services, and finance; for daily briefings of laboratory staff, medical staff, infection control, and System leadership; and for DOH notification. This included daily system-wide conference calls at 7:00 AM, 3:00 PM, and 11:00 PM. A critical focus was the protection of the healthcare workforce and inpatients from nosocomial spread of pandemic (H1N1) 2009. The medical director reported regularly to the New York City, New York State, and Nassau and Suffolk County DOHs, keeping civic officials apprised of the epidemiology of the outbreak.

An additional key element of the laboratory response was communication with physicians and patients. From April 27 through May 15, the central laboratories' Client Services, in addition to their usual 8,000–9,000 calls a week, handled an additional 1,000 telephone calls a week pertaining specifically to the outbreak. Laboratory leadership provided scripts to Client Services, including answers to frequently-asked questions. Client Services made strategic calls to physician offices to provide updates on testing protocols and priorities. In addition, the NSLIJHS sales force was redirected to support physician offices, including communication of protocols and procedures and deliveries of supplies.

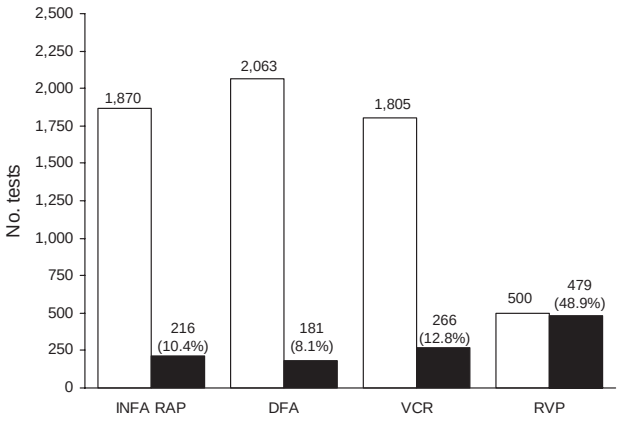

Figure 2. Cumulative virology test volumes and influenza A–positive results, North Shore–Long Island Jewish Health System, New York City metropolitan area, USA, April 24–May 15, 2009. INFA RAP, rapid antigen test for influenza A; DFA, direct immunofluorescent antibody test; VCR, rapid respiratory virus culture by R-Mix (Diagnostic Hybrids Inc., Athens, OH, USA); RVP, Luminex xTAG Respiratory Virus Panel (Luminex Molecular Diagnostics, Toronto, Canada). White bars, number of tests with negative results for influenza A.; black bars, number of test results positive for influenza A. Actual numbers are included above the bars. For influenza A, the percentages of samples positive for influenza A are shown in parentheses.

Discussion

The fundamental role of a clinical laboratory is to provide medical care to the patient population it serves. The ability to respond to a specific crisis also provides critical support to civic agencies. Although our laboratory did not perform surge testing for the DOHs, nor was testing specifically delineated between our laboratory and the DOHs, our ability to provide comprehensive virus testing, including influenza A subtyping, for such a large patient base (≈6.5 million persons) indirectly assisted the DOHs by providing key diagnostic information with which the DOHs could make management and testing decisions.

The steps taken by our laboratory were strongly supported by system leadership and enabled NSLIJHS to successfully meet this crisis. It is our hope that delineation of

these steps will be valuable to other health systems and their laboratories because there undoubtedly will be future public health crises that will demand an immediate increase in reference laboratory testing capacity. The toll of this crisis was on NSLIJHS laboratory personnel. The long hours required to meet testing demands were keenly felt in the first days, when the emergency response had not been activated. However, initial implementation of the emergency response only enabled us to keep up with the crisis, not go beyond it. Routine personnel worked extended hours, despite the support of cross-covering personnel. One key reason was the high level of expertise required to perform the virology and molecular testing; substitute personnel could not be deployed on short notice. The other key reason was the staff's dedication and their reluctance to go off-duty. Management created obligatory off-duty rotations to ensure our work force was as rested as possible.

The major surge response stratagems, established at the outset, guided NSLIJHS management actions throughout the crisis. Workforce management was top priority. Coordinating the System's general laboratories with CVL, daily reporting of test volumes and results, and providing support to Client Services enabled the laboratories to remain in synchrony with emergency departments, hospital facilities, and physician practices. Also imperative was the need for all NSLIJHS laboratories to maintain normal operations. At no time during a crisis can normal laboratory services undergo degradation.

Preparedness for infectious outbreaks has increasingly been a point of concern, owing to the threats of bioterrorism and natural diseases. Attention is given primarily to the hypothetical preparedness of first responders and acute-care facilities, either in the form of surveys (*9–12*), workflow analysis (*13*), or reviews (*12,14,15*). The outbreak of severe acute respiratory syndrome in 2003 generated reports from an actual global outbreak. In Hong Kong and Toronto, note was made of the frustrations arising from limited access to laboratory testing, resulting in a decreased ability to provide timely screening of patients for severe acute respiratory syndrome (*16*). The ability of a laboratory to deploy molecular respiratory virus testing was felt to be key for a successful response to an infectious disease outbreak, as such testing is highly sensitive, specific, and capable of high-throughput (*12*).

We believe that there will be future infectious outbreaks that will strain the standing capacity of clinical laboratories, requiring effective implementation of surge capacity responses independent of public health laboratory support. We believe that the steps taken by NSLIJHS laboratories during the initial outbreak of pandemic (H1N1) 2009 and the lessons learned (Table) from that experience are of value. The exceptional 2-way interaction between the NSLIJHS Laboratories and the NY public health laboratories was an excellent example of how sentinel laboratories function as a key component of the Laboratory Response Network system and can serve as a major support for public health in the time of crisis.

Table. Lessons learned during clinical laboratory response to pandemic (H1N1) 2009, New York City metropolitan area, USA, April 24–May 15, 2009*

The following were critical to an effective laboratory response:
1. Early assessment and decisive and immediate response by management to laboratory needs
Includes staffing, supplies, the LIS, physical plant, client relations, and local and state reporting requirements
2. Management of staffing needs
Plans for immediate cross-coverage by trained technical and nontechnical staff
3. Coordination of system general laboratories
Standardization of testing algorithms and prioritization of courier delivery to central clinical virology and Molecular Diagnostics Laboratories
4. Enhanced reporting
Verification of LIS operations for patient-based reporting
Communication to treating physicians
Daily epidemiology reports for System leadership, Infection Control, and hospital administrations
Daily contact with local civic health officials
5. Enhanced client services
Increase number of staff to communicate results and respond to incoming calls, including scripted responses to frequently-asked questions
Maintenance by sales staff of specimen-collection supplies and communication of guidelines for specimen procurement and testing to outreach physician practices
6. Public relations oversight
Communications to news agencies were restricted to the System's public relations office

*LIS, Laboratory Information Systems; System, North Shore–Long Island Jewish Health System.

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Dr Crawford is senior vice president for laboratory services and chair of the Department of Pathology and Laboratory Medicine for the North Shore–Long Island Jewish Health System. His primary research interest is advancing the role that pathology plays in public health and national healthcare reform.

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Address for correspondence: Christine C. Ginocchio, North Shore–Long Island Jewish Health System Laboratories, 10 Nevada Dr, Lake Success, NY 11042-1114, USA; e-mail: cginocch@nshs.edu

Projecting Global Occurrence of *Cryptococcus gattii*

Deborah J. Springer and Vishnu Chaturvedi

Cryptococcus gattii and *C. neoformans* cause pulmonary and systemic cryptococcosis. Recently, *C. gattii* was recognized as a distinct pathogen of humans and animals. We analyzed information from 400 publications (1948–2008) to examine whether the fungus occurs globally. Known distribution of *C. gattii* is possibly limited because specialized reagents for differentiation from *C. neoformans* are not readily available and not always used, and environmental surveys are patchy. However, autochthonous reports of *C. gattii* cryptococcosis have now been recognized from tropical and temperate regions. An ongoing outbreak in western Canada strengthens the case that the range of the pathogen has expanded. A few studies have highlighted differences in cryptococcosis between *C. gattii* and *C. neoformans*. More than 50 tree species have yielded *C. gattii* especially from decayed hollows suggesting a possible ecologic niche. This pathogen merits more attention so its environmental occurrence and role in cryptococcosis can be accurately determined.

The yeast genus *Cryptococcus* has been recognized for >125 years, first from fruit juice, milk, humans, soil, and pigeon droppings and from roosting areas (*1*). Although *C. neoformans* human infections were reported early in the 1900s, the overall number of cryptococcosis cases was extremely low. Cryptococcosis cases increased in Africa during 1947–1968, presumedly in association with the emergence of AIDS in the Congo River basin (*2*); however, no independent confirmation or laboratory data are available for this hypothesis. A unique variant, *C. neoformans* var. *gattii*, manifested by the unusual presence of elongated and cigar-shaped yeast morphology in cerebrospinal fluid, was first described in a Congolese Bantu boy (*3*,*4*).

Evans described and differentiated *C. neoformans* into 3 serologic types (A, B, and C) by agglutination (*5*). Diagnosis of cryptococcosis progressed further with identification of *C. neoformans* antibodies in body fluids and development of a latex agglutination test (*1*). Staib (*6*) developed a *Guizotia abyssinica* (Nigerseed) creatinine agar medium to distinguish pigment-producing *C. neoformans* from other *Cryptococcus* spp., which facilitated rapid screening of clinical and environmental samples for pathogenic *C. neoformans* isolates. A major advance in the classification and taxonomy of *C. neoformans* occurred with the discovery of a heterothallic, bipolar mating involved in the production of the perfect state for *C. neoformans* var. *gattii* (serotypes B and C). It was termed *Filobasidiella bacillispora* and differentiated from *F. neoformans* by production of smooth, elongate cylinder- to rod-shaped basidiospores (*7*).

Currently, *C. neoformans* is recognized as a species complex comprising *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D), which have distinct clinical manifestations and biological characteristics (*1*,*8*). *C. gattii* (serotypes B and D) was recognized as a species distinct from *C. neoformans* because of differences in basidiospore morphology, environmental niches, morphologic features in vivo, limited molecular identity (55%–61% relatedness of DNA), multiple gene genealogies, unique random amplified polymorphic DNA typing patterns, and inefficient cross-species mating with the production of sterile progeny and no recombination (*9*). During the previous 2 decades, the increased pace of discovery produced a new appreciation of the 2 major pathogenic species, namely, *C. neoformans* and *C. gattii*. This study aimed to critically examine published information about associated tree species, ecology, and

Author affiliations: New York State Department of Health, Albany, New York, USA; and University at Albany School of Public Health, Albany

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geographic occurrence of *C. gattii* to infer its environmental distribution.

Methods

We comprehensively searched for published reports using the PubMed database (US National Library of Medicine, National Institutes of Health) for 1948–2008. The keywords used in the search were *Cryptococcus* alone or in combination with *Cryptococcus gattii*; *Cryptococcus neoformans*; *Cryptococcus neoformans* var. *neoformans*; *Cryptococcus neoformans* var. *grubii*; *Cryptococcus neoformans* serotype A, B, C, D, or AD; and cryptococcosis alone or in combination with human, pigeon, and animal. Additionally, we scrutinized reference lists in publications obtained from PubMed searches for citations that had not been captured with our choice of keywords in PubMed searches. These citations were easily obtained by repeating the search criteria in the Web of Science (Thompson Reuters) and Google Scholar.

One of us (D.J.S.) independently examined the title, abstract, methods, data tables and figures of publications identified in the literature search. Information about *Cryptococcus* isolates, serotype, mating type, molecular type, geographic location, and other relevant details were entered into a master spreadsheet. All publications with adequate documentation of *C. gattii* by ≥1 valid laboratory methods were regarded as acceptable for inclusion.

Results

From 400 potentially useful publications, we shortlisted ≈200 and identified 105 that provided information about primary isolations of *C. gattii* from clinical, veterinary, and environmental sources. Geographically, the reports originated from a total of 48 countries, although most reports concentrated on few areas (Table 1). Because a certain level of selection bias existed in this search process, we might have missed some relevant publications (*10*).

Table 1. Number of publications per geographic region reporting isolation of *Cryptococcus gattii*

Region	Environmental isolation	Clinical and veterinary isolation	Total no. reports
South America	18	12	30
Australia/New Zealand	8	16	24
North America	5	14	19
South-central Asia	9	9	18
Africa	2	12	14
Europe	3	12	15
Eastern Asia	0	6	6
Central America	1	5	6
Southeast Asia	0	3	3

Distinguishing Features of *C. gattii*

C. gattii was easily and reliably differentiated from *C. neoformans* on creatinine dextrose bromthymol blue (CDB) medium. This work built on the discovery that *C. neoformans* can assimilate creatinine as sole source of carbon and nitrogen. Further modification in CDB medium led to development of canavanine-glycine-bromthymol blue agar, which has since become the differential medium of choice (*1*,*11*,*12*). Unfortunately, the medium is still not widely used in diagnostic laboratories, most likely because of limited availability from commercial suppliers.

C. gattii populations can be distinguished by the pairing of unknown isolates with compatible tester strains to distinguish *MATα* from *MAT**a*** strains. *MATα* is most prevalent clinically and environmentally, and *MAT**a*** is recovered less frequently (*7*,*13*,*14*). Four distinct *C. gattii* molecular subtypes (VGI, VGII, VGIII, and VGIV) have been recognized by PCR amplification of genomic DNA by using bacteriophage M13 single-stranded primers. Genotypes VGI and VGII are prevalent worldwide, and VGIII and VGIV are less common (Figure). Serotypes B and C are randomly dispersed among the M13 molecular types. Further molecular subtypes are now known to exist within the 4 molecular types (*15*).

The VGI molecular genotype has been reported from many areas and from 2 *C. neoformans–C. gattii* hybrids reported from Canada and the Netherlands (*14*,*16*). VGII has been reported from the Western Hemisphere, Australasia, Asia, and Africa and is reportedly the most fertile and virulent of the strains responsible for infection in healthy and immunocompromised humans and animals. This strain also is associated with an ongoing outbreak of *C. gattii* cryptococcosis from Vancouver Island, British Columbia, Canada (*14*). VGIII, which contains both serotypes B and C, is most commonly reported from South America but also is reported from North America, Central America, Australasia, and Southern Asia. VGIV, frequently associated with serotype C, has been reported from Africa and South and Central America. Further experimental studies on VG genotypes are needed to explain possible connections between distribution of various genotypes and propensity to cause cryptococcosis among exposed hosts.

C. gattii in Clinical and Environmental Specimens

Initially, reports of *C. gattii* originated from human clinical samples in tropical and subtropical regions, including portions of Africa, Europe, Australia, the United States, and South America. This accounted for the long-held impression that *C. gattii* is a tropical or subtropical pathogen (*4*,*17*,*18*). More recent clinical isolations from temperate regions in the United States, Canada, Europe, and Asia have greatly expanded the incidence areas of *C. gattii* (*4*). Accordingly, *C. gattii* has been reported from such diverse countries as Argentina, Austria, Canada, China, Congo, India, Italy, Japan, South Korea, the Netherlands, Spain,

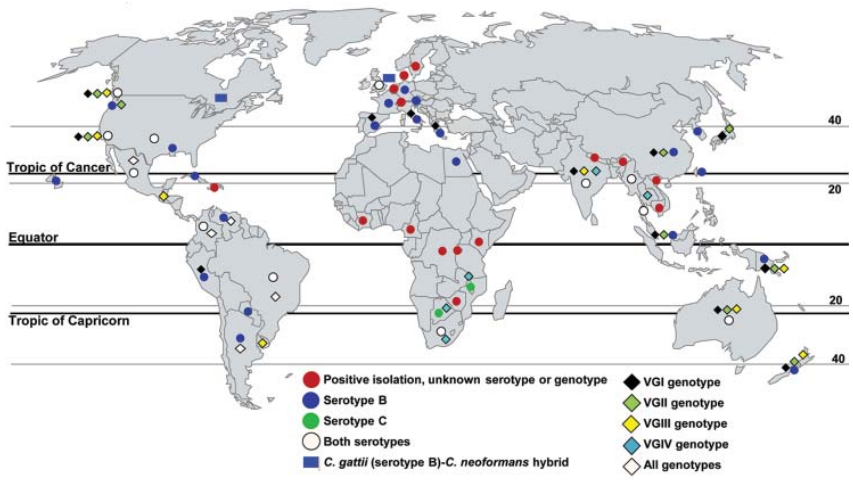

Figure. Worldwide isolations of *Cryptococcus gattii* from human clinical, veterinary, and environmental sources. Circles indicate serotype information, diamonds indicate genotype information, and rectangles indicate hybrids between *C. gattii* and *C. neoformans*. Existing reports and survey are patchy, and several areas between positive regions share tree species (Table 2) and climatic conditions and would most likely harbor the pathogen. Thus, *C. gattii* is likely to be more widely distributed than documented.

South Africa, United Kingdom, United States, and Democratic Republic of Congo, and this list expands every year. Infections in domestic animals, such as goats, dogs, cats, and horses, are common in Australia, New Zealand, Canada, and Brazil (*4*,*19*,*20*). Additionally, infections are reported in migratory, water-dwelling animals, including porpoises and dolphins (*21*). *C. gattii* also was associated with native animals in Australia (koala, echidna), New Zealand (kiwi), Africa (cheetah), and Canada (squirrel) (*14*,*19*,*22*). Nonnative zoo animals (koala, ferret, tapir, cheetah, llama) and exotic birds (cockatoo and parrots) have been affected with cryptococcosis caused by *C. gattii* in Australia, Canada, the United States, and Cuba (*23*). Thus, *C. gattii* affects a wide variety of native and domestic animals in regions of known clinical presence. Because, in comparison with human clinical samples, veterinary samples are less frequently analyzed for and diagnosed with *C. gattii* infection and subsequently reported, we suggest that the actual infection rates of *C. gattii* in animal populations are possibly much higher than presently known.

Published literature on the environmental isolation of *C. gattii* is patchy, sporadic, and centered in geographic regions reporting a high clinical incidence of *C. gattii* cryptococcosis. This is true for Canada, South America, and Australia. However, in India the environmental prevalence of *C. gattii* appears more pervasive than the reported prevalence of the fungus in clinical specimens (Figure). The first environmental isolation of *C. gattii* (serotype B) was reported by Ellis and Pfeiffer from *Eucalyptus* trees in 1990 (*24*) after unsuccessful attempts at environmental isolations from the same tree species in Oklahoma (*18*) and California (*25*). The first environmental isolation of serotype C was reported in 1998 from almond trees in Colombia (*26*). Environmental sampling is much more limited than clinical sampling because clinical isolates are a public health priority.

C. gattii serotype B is the most prevalent serotype in clinical and environmental samples (*17*,*18*). Curiously, *C. gattii* serotype C is a less common constituent of clinical and environmental isolations even though it is associated with AIDS patients and immunocompetent persons (*27*). *C. gattii* serotype C has been isolated from humans in clinical samples from India, the Western Hemisphere, and Africa (*18*,*27*). Although serotype C is rarely isolated from the environment, its most notable isolation occurred in association with detritus around nonnative almond trees in Colombia (*26*). More recent and extensive clinical surveys combining serotyping and molecular typing suggest that serotype C is less rare or restricted than previously thought (*27*). Future studies are unlikely to provide serotype information because the commercial typing reagents are no longer readily available, and thus genotypes will be the primary means to correlate strain characteristics with their environmental and clinical prevalence.

Existing reports and surveys of *C. gattii* from human clinical, veterinary, and environmental sources are patchy (Figure). Several areas between positive regions would most likely harbor the pathogen. Thus, *C. gattii* is likely to have a wider geographic distribution than documented. The environment has not been systematically explored to identify the source of *C. gattii* in the Congo River basin, where the first definitive report of *C. gattii* emerged. Such environmental surveys are imperative in view of reports of *C. gattii* cryptococcosis from a number of African countries (*3*,*17*,*27*,*28*).

C. gattii and Trees

Ellis and Pfeifer reported the first environmental isolation of *C. gattii* in 1990 in Australia from wood, bark, leaves, and plant debris of *Eucalyptus* trees (*24*). Although *Eucalyptus* is present in many of the areas known to have *C. gattii* cryptococcosis, the actual isolation of *C. gattii*

from *Eucalyptus* trees is rare outside Australia, despite extensive sampling. Imported *Eucalyptus* has not been associated with the environmental presence of *C. gattii* in Spain, central Africa, or Canada, and most *Eucalyptus* trees tested in Papua New Guinea, Egypt, and Italy were negative for *C. gattii*. Furthermore, early environmental surveys for *C. gattii* in imported *Eucalyptus* spp. rarely included other local tree species for testing (*4*). Although understandable, this was unfortunate because *C. gattii* is now known to have extensive associations with other tree species.

Evidently, *C. gattii* is established ecologically in trees other than *Eucalyptus* in many parts of the world, as supported by *C. gattii* association with native trees in Canada, Brazil, Colombia, India, and Argentina (Figure). *C. gattii* has been reported from 54 tree species; most (77%) are angiosperms; gymnosperms account for 23% of positive species (Table 2). Gymnosperms and angiosperms can develop decayed hollows, which differ in biochemical composition, available nutrients, presence of water, microbial communities, and fungal associations (*29*). *C. gattii* exhibits associations with the gymnosperms *Abies* spp., *Arbutus menziesii* var. *menziesii, Cedrus* spp., *Abies grandis*, *Picea* spp., *Pinus* spp., *Pseudotsuga menziesii* var. *menziesii*, and *Thuja plicata* in Canada; *Pinus radiate* (Monterey pine) and *Cupressus lusitanica* in Colombia; and *Cedrus deodara* and *Cupressus sempervirens* in Argentina. Angiosperms other than *Eucalyptus* spp. have been reported positive for *C. gattii* from North America, South America, Africa, and India. Like *Eucalyptus* spp., other angiosperm tree species reported as hosts for *C. gattii* have been extensively exported from their native areas (Table 2). Two prominent examples are *Ficus* spp. and *Terminalia* spp. (almond) trees. *Ficus* spp. are widely distributed in the tropics and subtropics, and many are exported as ornamentals. *Ficus* spp. have been recorded as *C. gattii* hosts in Brazil and Colombia but not in other regions (*30*).

C. gattii vis-à-vis *C. neoformans*

Cryptococcosis due to *C. gattii* is unlikely to be recognized in the laboratory without heightened awareness and sustained effort to differentiate these 2 closely related

Table 2. Tree species recorded as testing positive for *Cryptococcus gattii*

Location	Species (common name)	Native and exported tree ranges
Argentina	*Acacia visco* (arca), *Cedrus deodara** (deodar cedar), *Cupressus sempervirens** (Mediterranean cypress), *Eucalyptus microcorys* (tallowwood), *Tipuana tipu* (rosewood), *Ulmus campestris* (English elm)	Australia, Africa, Asia, Britain, Canada, Central America, England, Europe, Japan, South America, United States
Australia	*Angophora costata* (smooth bark apple), *E. blakelyi* (Blakely's red gum), *E. camaldulensis* (red river gum), *E. gomphocephala* (tuart tree), *E. grandis* (rose gum), *E. microcorys* (tallowwood), *E. rudis* (flood gum), *Eucalyptus* spp., *E. tereticornis* (forest red gum), *E. tetrodonta* (Darwin stringybark), *Syncarpia glomulifera* (turpentine)	Australia, Africa, Asia, Caribbean, Hawaii, Indonesia, New Zealand, Papua New Guinea, United States, South America, US Virgin Islands, British Virgin Islands
Brazil	*Adenanthera pavonina* (circassian seed), *Cassia grandis* (carao), *Erythrinia velutina* (coral tree), *E. camaldulensis* (red river gum), *E. microcorys* (tallowwood), *Eucalyptus* spp., *Ficus* spp., *Guettarda acrena, Moquilea tomentosa* (pottery tree)	Australia, Africa, Asia, Caribbean, Central America, Fiji, New Zealand, South America, United States, US Virgin Islands, British Virgin Islands
Canada	*Abies grandis** (grand fir), *Abies* spp.* (fir), *Acer* spp.(maple), *Alnus rubra* (red alder), *Aluns* spp. (alder), *Arbutus menziesii** (Pacific madrone), *Cedrus* spp.* (cedar), *Picea* spp.* (spruce), *Pinus* spp.* (pine), *Prunus emarginata* (bitter cherry), *Pseudotsuga menziesii** (coastal Douglas fir), *Quercus garryana* (Garry oak), *Thuja plicata** (western red cedar)	Australia, Europe, New Zealand, North America, South America
Colombia	*Acacia decurrens* (black wattle), *Coussapoa sp, Croton bogotanus, C. funckians (C. gossypiifolius), Cupressus lusitanica** (Mexican cypress), *E. camaldulensis* (red river gum), *E. globulus* (Tasmanian blue gum), *Ficus soatensis* (rubber Savanna), *Pinus radiata** (Monterey pine), *Terminalia catappa* (almond)	Africa, Asia, Australia, British Isles, Canada, Caribbean, Costa Rica, Europe, Costa Rica, Hawaii, Indonesia, Mediterranean region, Mexico, New Zealand, Pacific Islands, Papua New Guinea, Japan, United States, South America, US Virgin Islands, British Virgin Islands
India	*Acacia nilotica* (thorn tree), *Azadirachta indica* (neem tree), *Cassia fistula* (golden shower tree), *Cassia marginata, E. camaldulensis* (red river gum), *E. citriodora* (lemon-scented gum), *Eucalyptus* spp., *Mangifera indica* (mango), *Manilkara hexandra* (margosa), *Mimusops elengi* (bullet wood or Indian madlar tree), *Pithecolobium dulce* (Manila tamarind), *Polyalthia* longifolia (Indian mast tree), *Syzygium cumini* (java plum), *Tamarindus indica* (tamarind), *Terminalia arjuna* (arjuna)	Africa, Asia, Australia, Caribbean, Central America, Hawaii, Indonesia, Malaysia, Pacific Islands, Philippines, Portugal, South. America, New Zealand, United States, US Virgin Islands, British Virgin Islands
Egypt, Italy, Mexico, United States	*E. camaldulensis* (red river gum)	Africa, Australia, Caribbean, New Zealand, United States, South America, South Asia, US Virgin Islands, British Virgin Islands

*Gymnosperm tree species.

pathogens. Given the much more recent recognition of *C. gattii*, historical reports are likely to mention only *C. neoformans*; this is a major consideration in evaluating historical publications on cryptococcosis due to *C. gattii*. Pigeon droppings are a known ecologic niche for *C. neoformans* because the pathogen is predominantly isolated from avian environments or areas contaminated with avian feces (*1*). Thus, urban dwellings frequented by pigeons and containing accumulated pigeon droppings are an important reservoir for human and animal infections.

Pigeons are not known to acquire symptomatic disease but can carry yeast on feathers, skin, crops, or cloaca (*1*). Other animals reported positive for *C. neoformans* include macaw, swan, parakeet, Guenon monkey, fox, potoroo, and sheep (*1*). Thus, exotic and migratory birds and domestic and wild animals can be carriers or susceptible hosts for *C. neoformans*. The overwhelming association with avian droppings and environment, especially pigeons, sets *C. neoformans* apart from *C. gattii*. The ecologic niches for *C. neoformans* and *C. gattii* appear to be distinct.

Few reports exist of isolations of *C. neoformans* and *C. gattii* from the same habitats with the recognitions of natural hybrids between the 2 species. For instance, *C. neoformans* and *C. gattii* have been isolated from same sources, such as *Eucalyptus* spp. or *Syzygium cumini* trees or bird feces (*1*,*30*,*31*). *C. grubii* association with trees might represent fecal contamination by birds inhabiting these trees. Hybrid strains have been isolated from samples of bird feces in urban areas of South America and from patient samples obtained from the Netherlands and Canada (*16*). The existence of these hybrid strains suggests that at least in some parts of the world *C. neoformans* and *C. gattii* occupy either the same ecologic niche or closely overlapping areas.

Discussion

We suggest that *C. gattii* is an environmental pathogen with a specialized ecologic niche on the basis of accumulated reports of its widespread isolation from domestic and native animals, clinical presence in temperate climatic regions, increasing reports of isolations from native trees in temperate regions, and recapitulation of life cycle in association with plant material. The characteristics of such environmental pathogens include absence of any recognized animal host and maintenance of virulent traits by specific environmental associations. This concept has been well developed for a number of other environmental pathogens, such as *Mycobacterium ulcerans* (*32*) and *Burkholderia* spp. (*33*). The 54 tree species recorded positive for *C. gattii* are native to tropical, subtropical, and temperate regions of the world. Additionally, many of these trees are more widely distributed than their documented native range(s) indicate because of extensive exportation and cultivation, which suggest further expansion of the known range of *C. gattii*.

A corollary of this environmental distribution of the fungus is the diagnosis of autochthonous *C. gattii* cryptococcosis in native and domestic animals in Europe, Africa, Australia and New Zealand, and the Western Hemisphere, suggesting that habitats of many of these animals overlap ecologic niches with the fungus (*19*). A consistent feature of the association of *C. gattii* with trees is isolation of the fungus from decayed hollows of angiosperm and gymnosperm species (*24*,*30*). Decayed wood hollows develop slowly and are distinct ecologic niches inhabited by specialized microbial communities (*29*). Microbes that use wood or decayed hollows require specialized adaptations to inhabit this ecologic niche, which also offers a refuge from deleterious biotic and abiotic factors. Decayed hollows are characteristics of mature trees and thus occur most frequently in forested regions or rural to semirural areas with mature trees (*29*). This pattern is consistent with recognition of *C. gattii* cryptococcosis in Canada, Australia, Africa, Asia, and parts of South America. In some instances, especially in temperate areas, *C. gattii* has been isolated from trees in parks, on college campuses, and in zoos and animal refuges (*24*,*31*). Recent studies provide additional evidence for this specialized ecologic niche in trees and tree hollows by documenting long-term associations of *C. gattii* with trees, including seasonal variations in its isolation, and genetic recombination indicative of sexual and/or asexual mating in association with trees and tree hollows (*13*). An experimental study has recapitulated the sexual life cycle of the fungus in the laboratory on *Arabidopsis thaliana* and *Eucalyptus* spp. seedlings with production of easily airborne sexual spores (basidiospores) thus supporting the universal dispersal hypothesis, which suggests that most of the free-living microbial eukaryotes are likely to be globally distributed (*34*).

The association of *C. gattii* with woody materials distinguishes this species from *C. neoformans* niche in soil and pigeon droppings. Several publications provide additional evidence for this inference: 1) Escandon demonstrated that *C. gattii* can survive in live almond trees and can contaminate the soil in immediate surrounding (*35*); 2) mating has been associated with live plants and wood (*13*,*36*); and 3) positive soil sample are mostly isolated near positive trees and have been contaminated with woody plant debris.

C. gattii potentially can be dispersed through export of trees and woody products, air currents, water currents, and biotic sources, such as birds, animals, and insects. The ability of *C. gattii* to associate with vesicular elements in wood blocks, to survive in the vasculature of live almond trees, and to spread into soil (*35*,*37*) suggests that the pathogen can spread through the exportation of wood and trees (*24*).

Historically, *Eucalyptus* trees have been implicated in the spread of *C. gattii* to different areas in the world (*24*). Recently, *Pinus radiate*, *Cedrus deodara*, *Cupressus sempervirens*, *Cupressus lusitanica*, and *Terminalia catappa* (almond) have been recognized as *C. gattii* hosts; these trees have been widely exported from their native ranges as ornamental or commercially valuable trees. The evidence for *C. gattii* dispersal by wind and air currents is limited, but fungal isolations from air samples have been obtained around positive trees in Canada and India.

The following observations suggested dispersal of *C. gattii* in water or water currents: 1) naturally infected porpoises and dolphins have been identified, 2) the fungus has been isolated from natural freshwater and saltwater samples in British Columbia and from contaminated water in habitats of captive animals, and 3) *C. gattii* can survive in water in vitro for long periods (*21*). Multiple reports have suggested that birds and animals could play a role in dispersal of *C. gattii* to geographic areas presently uninhabited by *C. gattii*. Isolation of the fungus from psittacine bird excrement in South America is suggestive because many of these birds fly long distances and are migratory or are exported as exotic pets or exhibit items for zoos (*31*). Other native animals that could help in *C. gattii* spread include koalas in Australia (*22*), squirrels and porpoises in the Pacific Northwest (*19*), and dolphins (*21*) in North America.

C. gattii is likely to be acquired in areas where mature trees are abundant either in forested or rural to semiurban settings. We derived this conclusion from published clinical reports on *C. gattii* cryptococcosis in Aborigines in Australia, native Africans in the Congo River basin, Canadians who visited parks and forests on Vancouver Island, and a Spanish farmer and Italian farmer (*38*,*39*). A common theme among these clinical cases is presence of and human exposure to mature trees. Recovery of identical *C. gattii* strains from environmental sources from Canada and human clinical specimens from Italy strongly suggest that the point source of infection is the immediate vicinity of patients' residences (*14*,*39*). Association of *C. gattii* with decayed woody hollows, bark, and tree debris also suggests a role for mature trees (*19*,*24*).

Infections reported in domestic and wild animals in Australia, New Zealand, Africa, Spain, the United States, and Canada provide another important clue to risk areas for *C. gattii* acquisition (*19*). Overwhelmingly, these infections are reported from animals that either reside in or are exposed to areas with an abundance of mature trees. This situation is somewhat analogous to the fungus *Blastomyces dermatitidis*, another elusive primary pathogen, which causes blastomycosis. Some similarities in characteristics include clustered infection patterns in humans and mammals; increases in exposure risk from outdoor activities, and restricted and infrequent environmental isolations (*40*).

Conclusions

C. gattii is a globally established primary fungal pathogen with a specialized ecologic niche on trees and in hollows of trees. Future epidemiologic studies and environmental surveys are likely to reveal the extent of *C. gattii* prevalence in different environments especially in areas with known incidence of cryptococcosis but no reported isolations of *C. gattii*. Such information will be helpful in devising strategies to manage potential outbreaks of cryptococcosis. More clinical studies are also needed to follow up the course and outcome of *C. gattii* cryptococcosis, the salient point by which this fungus can be differentiated from the disease caused by *C. neoformans*, and any changes in patient management strategies.

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Ms Springer is a doctoral candidate in biomedical sciences at the University at Albany School of Public Health and a staff member of the Mycology Laboratory, Wadsworth Center, New York State Department of Health, Albany, New York, USA. Her research interests include the connections between pathogens and their environments using structural and cell biology approaches.

Dr Chaturvedi is director of the Mycology Laboratory, Wadsworth Center, New York State Department of Health, and an associate professor of biomedical sciences at the University at Albany School of Public Health. His research interests include fungal diagnostics, antifungal drugs, pathogenesis, and epidemiology.

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Address for correspondence: Vishnu Chaturvedi, Mycology Laboratory, Wadsworth Center, 120 New Scotland Ave, Albany, NY 12201-2002, USA; email: vishnu@wadsworth.org

Epidemiology of Travel-associated Pandemic (H1N1) 2009 Infection in 116 Patients, Singapore

Pratik Mukherjee, Poh Lian Lim, Angela Chow, Timothy Barkham, Eillyne Seow, Mar Kyaw Win, Arlene Chua, Yee Sin Leo, and Mark I-Cheng Chen

In June 2009, during Singapore's pandemic influenza plan containment phase, pandemic (H1N1) 2009 was introduced into the country through imported cases. To understand how travel patterns affected the initial outbreak, we examined epidemiologic and travel data for the first 116 case-patients admitted to Tan Tock Seng Hospital, Singapore, with travel-associated infection. Sixty-one percent and 54% of patients, respectively, met US Centers for Disease Control and Prevention and World Health Organization temperature criteria for influenza-like illness. One fourth of the case-patients traveled after illness onset, and 15% became ill while traveling. Regions of exposure for imported infections changed rapidly; case-patients initially arrived from North America, followed by Australasia and Southeast Asia. Case-patients on longer flights were more likely to become ill before arrival; those with shorter flights tended to become ill after arrival. Thermal scanners detected fevers in 12% of the arriving case-patients, resulting in a shorter time to isolation.

On April 24, 2009, international authorities reported cases of infection with a novel influenza A virus (H1N1) strain of swine origin, now known as pandemic (H1N1) 2009 virus; 7 cases in the United States and 3 clusters in Mexico were confirmed, and surveillance indicated influenza-like-illness (ILI) had been increasing in Mexico since March 18, 2009 (*1*). During the next 3 months, this virus spread rapidly across the globe, resulting in >254,206 cases and at least 2,837 deaths on 6 continents as of August 30, 2009 (*2*). For most countries, the initial introduction of this virus occurred through international travel and human-to-human transmission.

The role of air travel in the transmission and dissemination of respiratory infections has been examined for severe acute respiratory syndrome (SARS), pneumonic plague, and extensively drug-resistant tuberculosis (*3*–*6*). Grais et al. (*7*) explored the possible effect of airline travel on geographic spread of pandemic influenza in 2000 through simulation models of the pandemic influenza A virus (H3N2) of 1968–69, using air travel data for 53 global cities. The effect of air travel on facilitating the transmission and dissemination of influenza is borne out by other recent studies suggesting that decreased volumes of air travel in the 2–3 months after the terrorist attacks in the United States on September 11, 2001, delayed that winter's seasonal influenza peak and decreased transmission (*8*).

Human-to-human transmission of influenza during air travel has been reported to occur on flights of at least 8 hours and to affect passengers seated within 2 rows of the index case-patient (*6*). However, other reports show that respiratory infections can spread during shorter flights and over considerable distances within the cabin (*3*,*9*). For the purposes of influenza contact tracing, the World Health Organization (WHO) has defined considerable exposure on aircraft as being restricted to passengers sitting in the same row or within 2 rows of an infectious person for a flight time of >8 hours (*10*).

Singapore implemented the containment phase of its pandemic influenza plan on April 27, 2009, before pandemic (H1N1) 2009 was introduced into the country. Public health policy during this phase was to isolate infected case-patients and quarantine exposed persons to prevent local transmission for as long as possible. During this initial period, travel from a pandemic (H1N1) 2009–affected

Author affiliations: Tan Tock Seng Hospital, Singapore (P. Mukherjee, P.L. Lim, A. Chow, T. Barkham, E. Seow, M.K. Win, A. Chua, Y.S. Leo, M.I-C. Chen); National University of Singapore, Singapore (A. Chow, M.I-C. Chen); and Duke–National University of Singapore Graduate Medical School, Singapore (M.I-C. Chen)

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area was the major risk factor for infection; air travel was the main route of introduction. The containment phase was in effect for >2 months until epidemiologic surveillance indicated sustained community spread had begun during epidemiologic week 25 (week ending June 27, 2009). At that time, a gradual transition to mitigation measures was implemented.

During the containment phase, airport thermal scanners were used to detect fevers in arriving passengers at Singapore's Changi International Airport, and health advisories were used to encourage travelers in whom influenza-like symptoms developed after disembarkation to seek medical care. Ambulances, dedicated for this purpose only, were used to transport suspected case-patients from the airport to hospitals for screening. Adults with appropriate travel histories and ILI were referred to the designated screening center at Tan Tock Seng Hospital (TTSH) for treatment and isolation at the Communicable Disease Centre. During the mitigation phase, the focus of clinical and public health efforts shifted to caring for patients with severe illness and conditions that put them at risk for complications and to reducing transmission in the community through health education and voluntary self-isolation of persons with ILI.

We analyzed epidemiologic and travel data as well as data regarding source of referral for case-patients in relation to time of illness onset, time of arrival in Singapore, and time to isolation. Understanding how travel patterns affected propagation of the first pandemic wave of pandemic (H1N1) 2009 virus could yield insights into how the anticipated next wave might be disseminated and provide data on the effectiveness and limitations of different interventions used to slow dissemination.

Methods

We obtained demographic data and travel-related information (last port of embarkation and flight times) by direct interview and retrospective review of clinical notes. Duration of travel, which was calculated from flight times and transit time within airports, was categorized into 4 groups: short haul (<3 hours), medium haul (3–6 hours), long haul (6–15 hours), and extra-long haul (>15 hours). Clinical data were collected for symptoms and date and time of symptom onset (to the nearest hour in most instances and to the nearest 8-hour block [midnight to 7:59 AM, 8:00 AM to 3.59 PM, or 4:00 PM to 11:59 PM] if the patient was unsure). Symptom onset time was further categorized as before embarkation, during travel, or after disembarkation in Singapore. We also categorized hospitalized case-patients on the basis of whether their symptoms met US Centers for Disease Control and Prevention (CDC) ILI criteria (body temperature ≥37.8°C with either cough or sore throat in the absence of an alternative diagnosis) and WHO ILI criteria (body temperature >38°C with either cough or sore throat in the absence of an alternative diagnosis) (*11*,*12*).

A confirmed case of pandemic (H1N1) 2009 was defined as an ILI in a patient with a temperature >37.5°C, cough, rhinorrhea, sore throat, or myalgia and with laboratory confirmation of infection by real-time reverse transcription–PCR performed on respiratory samples (sputum or combined nasal and throat swab specimens). An imported, travel-associated case was defined as above but occurred in a person with recent travel outside Singapore who had arrived in Singapore during the containment period and had illness onset within 10 days of arrival.

We compared proportions by using the χ^2 test. Means were compared by use of *t* tests for dichotomous variables and 1-way analysis of variance for multichotomous variables. Stata 10.0 for Windows (StataCorp LP, College Station, TX, USA) was used for all statistical analyses.

Results

From April 27 through June 27, 2009 (epidemiologic week 25), 152 persons with confirmed pandemic (H1N1) 2009 were admitted to TTSH. Of the 152 patients, 116 met the definitions for having an imported, travel-associated case; the rest either did not have a history of travel or had onset of symptoms >10 days after their travel ended. These 116 cases span 5 weeks (epidemiologic weeks 21–25) from when the first confirmed case-patient arrived in Singapore on May 26, 2009.

Demographic and travel data for patients with cases of imported pandemic (H1N1) 2009 are shown in Table 1. Infections involved equal numbers of male and female travelers and occurred predominantly in young travelers (mean age 28.5 years; 70% were <30 and 7% were >50 years of age). Half of the travelers were Singaporeans returning from abroad, and the other half were travelers from other countries.

As shown in Figure 1, the regions where travelers were exposed to pandemic (H1N1) 2009 virus changed rapidly during the 5-week period of study. Most of the cases in epidemiologic weeks 21 and 22 (May 24 through June 6) were in patients who acquired their infection in the United States; however, within 2 weeks, this had changed dramatically, with a large proportion of cases coming from Australasia in epidemiologic week 23 (June 7–13). Within a week, this situation changed yet again; most infections originated from Southeast Asia by epidemiologic weeks 24–25 (June 14–27).

The first cases of pandemic (H1N1) 2009 detected among travelers arriving from the Philippines (week 22), Thailand (week 23), and Indonesia (week 25) indicated potential community transmission in those countries earlier than official announcements. Infections from Southeast Asian countries accounted for 29% of imported cases dur-

ing the containment phase; the Philippines were linked to 23% cases, mostly in weeks 24–25.

Travel duration reflects the distance between the regions of exposure and Singapore (Table 1). Of the 116 case-patients, 19% had a short travel duration (<3 hours), and 20% had a long travel duration (>15 hours). Although most case-patients became ill after arrival at their destination, 25% were ill before travel and boarded their flight despite symptomatic illness, and 15% became ill while traveling.

Table 1. Demographic and travel-related data for 116 persons with travel-associated pandemic (H1N1) 2009, Singapore, April 26–June 27, 2009*

Data	Case-patients, no. (%)
Sex	
M	59 (50.9)
F	57 (49.1)
Age group, y	
≤19	18 (15.5)
20–29	63 (54.3)
30–39	13 (11.2)
40–49	14 (12.1)
≥50	8 (6.9)
Nationality	
Singaporean	58 (50.0)
Others	58 (50.0)
Port of embarkation, by region	
Americas	22 (19.0)
Asia	56 (48.3)
Australasia	31 (26.7)
Europe	7 (6.1)
Duration of travel, h	
<3	22 (19.0)
3–5.9	34 (29.3)
6–14.9	36 (31.0)
≥15	24 (20.7)
Onset of symptoms	
Before embarkation	29 (25.0)
While travelling	17 (14.7)
After disembarkation	70 (60.3)
Source of referral to TTSH screening center	
Airport doctor	15 (12.9)
Community doctor	50 (43.1)
Self-referred	51 (44.0)
Screening criteria documented during clinical examination	
Temperature ≥37.5°C	84 (72.4)
Temperature ≥37.8°C	71 (61.2)
Temperature >38.0°C	61 (52.6)
US CDC ILI criteria†	60 (51.7)
WHO ILI criteria‡	51 (44.0)
Arrival at TTSH, by epidemiologic week§	
21	5 (4.3)
22	8 (6.9)
23	13 (11.2)
24	41 (35.3)
25	49 (42.2)

*TTSH, Tan Tock Seng Hospital, Singapore; US CDC, US Centers for Disease Control and Prevention; ILI, influenza-like illness; WHO, World Health Organization.
†Temperature ≥37.8°C plus cough or sore throat.
‡Temperature >38.0°C plus cough or sore throat.
§Week 21, May 24–30; week 22, May 30–June 6; week 23, June 7-13; week 24, June 14–20; week 25, June 21–27.

Doctors based at the airport referred 15 (12.9%) of the 116 patients to TTSH; thermal scanners used to screen arriving passengers had detected fever in 14 of these 15 patients. Of the remaining 101 patients, 51 (44%) self-reported to the screening center at TTSH and 50 (43%) were referred by doctors in the community.

At the time of examination, 72% of case-patients had temperatures >37.5°C. However, only 61.2% had temperatures ≥37.8°C and 54% had temperatures >38°C, the temperature criteria used in the US CDC and WHO ILI case definitions, respectively. Considering the entire symptom complex, 51% of patients would have fulfilled ILI criteria as defined by the US CDC, and 44% would have fulfilled the WHO criteria (*11*,*12*).

As the pandemic shifted toward Asian ports of embarkation, the number of case-patients with travel durations of <8 hours increased (Figure 2, panel A). The time of symptom onset relative to arrival in Singapore is shown in Figure 2, panel B; the figure does not include information for patients who were symptomatic before embarkation. Time of symptom onset was progressively closer to the time of arrival in Singapore for those arriving from longer distances ($p = 0.001$). Patients with longer travel durations were also more likely to have onset of symptoms before arrival ($p = 0.04$).

Port of embarkation, clinical symptoms, and duration of travel did not predict delay to isolation (Table 2). However, case-patients referred to TTSS by airport doctors had a shorter time to isolation (0.76 days) than self-referred patients or those referred by other sources (1.6–1.9 days). The number of case-patients referred by airport doctors increased over the 5-week period, but they represent only 12% of all travel-associated cases (Figure 3). Although the mean duration to isolation did not increase significantly over the study period, total numbers of case-patients with delays to isolation increased as the volume of travel-associated cases rose.

Discussion

Given Singapore's position as a major travel hub, with passenger traffic at Changi International Airport exceeding 37 million in 2008 (*13*), there is an ever-present risk of importation and subsequent community transmission of emerging respiratory infections. Such importation and transmission occurred during the SARS epidemic of 2003 (*14*) and remains a concern during the current pandemic of pandemic (H1N1) 2009 virus. In any epidemic, every imported case may start a cluster of locally transmitted cases, but the number of imported cases and delays to isolation

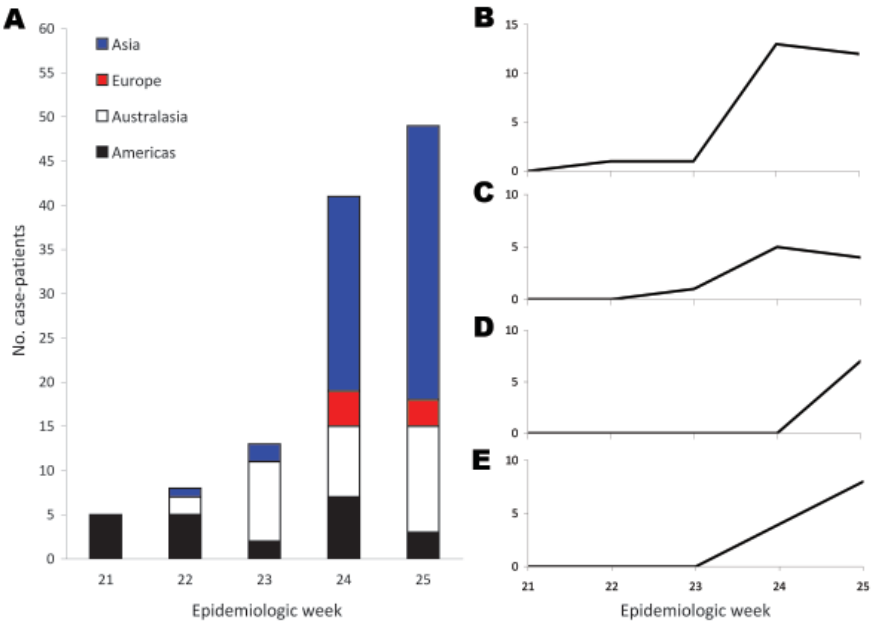

Figure 1. Sources of exposure, by region and country, among 116 patients in Singapore infected with pandemic (H1N1) 2009 virus identified during epidemiologic weeks 21–25, 2009. A) Asia compared with other regions; B) the Philippines; C) Thailand; D) Indonesia; E) other Asian countries. Week 21, May 24–30; week 22, May 30–June 6; week 23, June 7–13; week 24, June 14–20; week 25, June 21–27.

are particularly important at the start of the epidemic in determining speed of spread within the community. Improving detection and shortening time to isolation of infectious persons could modify the outbreak curve and allow more time for improving community preparedness.

Improving detection and shortening the time to isolation of sick persons is the rationale for using airport thermal scanners. Our data show that for the minority of cases detected by airport thermal scanners, detection does result in a hospital referral by an airport doctor and shorter time to isolation. However, intrinsic limitations of airport thermal scanners are that passengers have to become symptomatic before disembarking from a flight and have a fever high enough to be detected. Our data show that >30% of case-patients from all flights >3 hours had symptom onset before arrival, but overall, only 12% of all case-patients were detected by thermal scanners, suggesting that thermal scanners detected 40% of those symptomatic patients. This early detection and isolation may still have a valuable adjunctive role, especially in the initial phase of outbreaks. Situations favoring the use of airport thermal scanners include short-incubation diseases and geographically distant outbreak epicenters, such that arriving passengers have been on a long-haul flight. However, if the converse were true, with

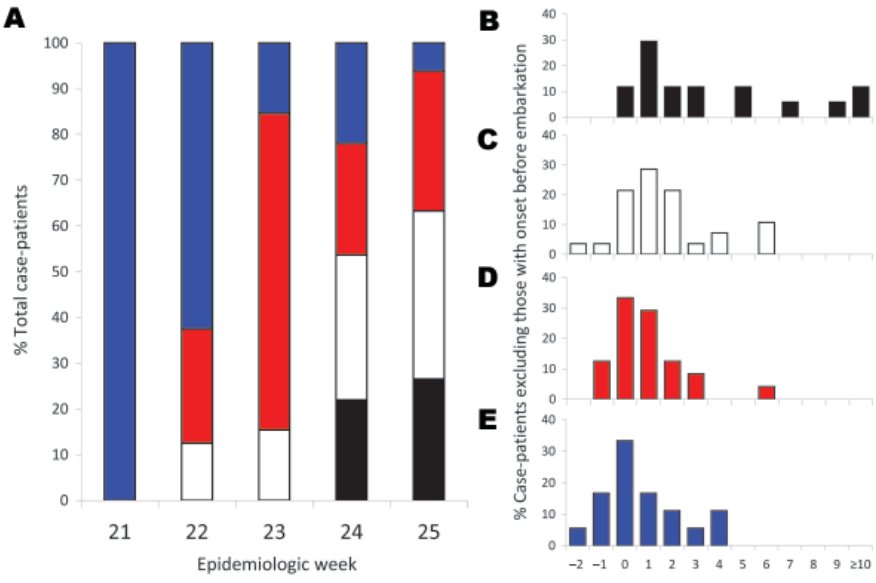

Figure 2. Travel duration and illness onset relative to arrival in Singapore for 116 patients infected with pandemic (H1N1) 2009 virus identified during epidemiologic weeks 21–25, 2009. A) Distribution of travel duration by epidemiologic week; B–E) timing of illness onset by travel duration in case-patients who did not have symptoms before embarkation (n = 87). Black, travel duration <3 h; white, 3–5.9 h; red, 6–14.9 h; blue, ≥15 h. Mean time from arrival to illness onset was 3.5 days (95% confidence interval [CI] 1.9–5.2), 1.7 days (95% CI 0.9–2.4), 1.0 days (95% CI 0.4–1.6), and 0.8 days (95% CI 0.0–1.5), respectively. The percentage of patients with symptom onset before arrival was 0%, 14%, 29%, and 33%, respectively. Week 21, May 24–30; week 22, May 30–June 6; week 23, June 7–13; week 24, June 14–20; week 25, June 21–27.

Table 2. Predictors of time to isolation for 116 patients with travel-associated pandemic (H1N1) 2009, Singapore, April 26–June 27, 2009*

Predictive factor	No. patients	Delay to isolation, mean no. days (95% CI)†	p value‡
Port of embarkation			0.718
Asian countries	56	1.64 (1.32–1.96)	
All other countries	60	1.55 (1.20–1.91)	
Source of referral to TTSH			0.015
Airport doctors	15	0.76 (0.33–1.19)	
Community doctors	50	1.58 (1.28–1.88)	
Self-referral	51	1.85 (1.44–2.27)	
Met US-CDC ILI criteria§			0.426
No	56	1.69 (1.31–2.08)	
Yes	60	1.50 (1.21–1.79)	
Met WHO ILI criteria¶			0.650
No	65	1.64 (1.30–1.99)	
Yes	51	1.53 (1.21–1.86)	
Time of onset of symptoms			0.664
Before embarkation	29	1.78 (1.14–2.42)	
While traveling	17	1.49 (0.87–2.12)	
After disembarkation	70	1.54 (1.29–1.80)	
Duration of travel, h			0.350
<3	22	1.96 (1.37–2.55)	
3–5.9	34	1.39 (1.03–1.74)	
6–14.9	36	1.69 (1.31–2.08)	
≥15	24	1.41 (0.74–2.08)	
Arrival at TTSH, by epidemiologic week#			0.868
21	5	1.83 (0.50–3.15)	
22	8	1.30 (0.44–2.16)	
23	13	1.87(1.13–2.61)	
24	41	1.61 (1.17–2.05)	
25	49	1.53 (1.19–1.87)	

*CI, confidence interval; TTSH, Tan Tock Seng Hospital, Singapore; US CDC, US Centers for Disease Control and Prevention; ILI, influenza-like illness; WHO, World Health Organization.
†No. days from arrival to hospital examination in patients with onset before arrival and no. days from onset to hospital examination in patients with onset after arrival.
‡p value by *t* test for dichotomous variables and 1-way analysis of variance for multichotomous variables.
§Temperature ≥37.8°C plus cough or sore throat.
¶Temperature >38.0°C plus cough or sore throat.
#Week 21, May 24–30; week 22, May 30–June 6; week 23, June 7-13; week 24, June 14–20; week 25, June 21–27.

transmission occurring in nearby countries and passengers arriving from short-haul flights, symptoms would develop in most passengers who become ill after entry and, thus, would be missed by airport thermal scanners.

The fact that one fourth of the case-patients in our study boarded a plane after becoming ill and traveled despite having symptoms illustrates the role of travelers in disseminating infection in a highly interconnected world. It raises the question of whether exit screening should be considered. However, the effectiveness of exit screening will depend on the role of asymptomatic persons in transmission, and such screening will still miss persons who are incubating the infection. Exit screening would severely hinder international travel, and because of its questionable efficacy, it may not be justified and may be contrary to the intent of the International Health Regulations 2005 (*15*)

Because 44% of the case-patients in this study were self-referred and 43% were referred by community physicians, prevention efforts should focus on other strategies. For example, health advisories should be distributed to arriving passengers, encouraging them to seek medical care if ill, and steps should be taken to improve detection by physicians who see patients in their clinics. The importance of clinical judgment, epidemiologic history, and access to diagnostic testing is emphasized by the substantial minority of case-patients who would not have met the WHO or US CDC ILI criteria but who did have laboratory-confirmed infection. However, our study did have limitations: clinical features were assessed at a single time point (on arrival at the hospital screening center) and there was potential recall bias.

Our data demonstrate how swiftly situations can change in a fast-moving pandemic; affected areas shifted from the Americas to Australasia to Asia within a matter of days. These rapid shifts pose a tremendous challenge to health authorities responsible for outbreak management, and they emphasize the narrow window of opportunity during which interventions can slow an epidemic. The monitor-

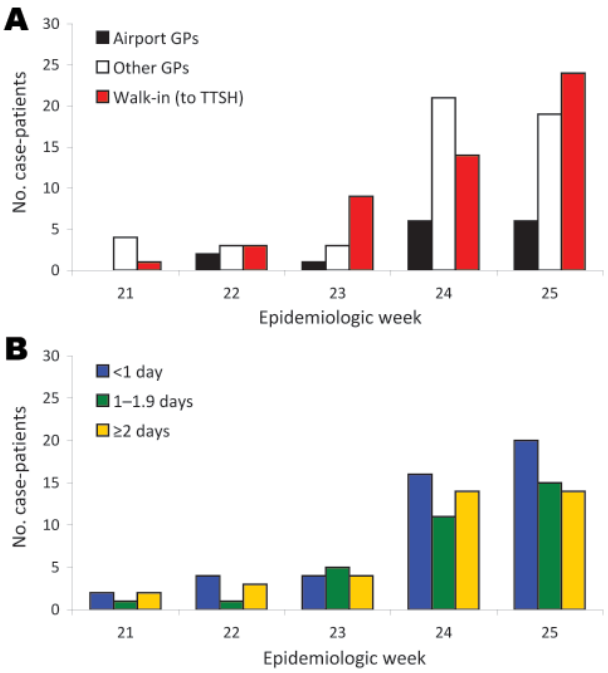

Figure 3. A) Source of referral to Tan Tock Seng Hospital (TTSH) and B) time to isolation for 116 patients infected with pandemic (H1N1) 2009 virus, Singapore. Week 21, May 24–30; week 22, May 30–June 6; week 23, June 7–13; week 24, June 14–20; week 25, June 21–27. GP, general practitioner.

ing of travelers fulfills a vital sentinel surveillance function, providing an early indicator of community transmission in countries even before transmission has been officially confirmed. Understanding how travel-associated infections propagated the first wave of this pandemic yields rich insights into how health authorities might respond to future outbreaks of emerging respiratory infections.

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Dr Mukherjee is a medical officer in communicable epidemiology at TTSH. His research interests include disease epidemiology, clinical research, and pandemic influenza.

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Address for correspondence: Poh Lian Lim, Department of Infectious Diseases, Tan Tock Seng Hospital, 11 Jalan Tan Tock Seng, Singapore 308433; email: pllim@post.harvard.edu

Severe Pneumonia Associated with Pandemic (H1N1) 2009 Outbreak, San Luis Potosí, Mexico

Alejandro Gómez-Gómez, Martin Magaña-Aquino, Christian A. García-Sepúlveda, Uciel R. Ochoa-Pérez, Reynaldo Falcón-Escobedo, Andreu Comas-García, Saray Aranda-Romo, Hugo I. Contreras-Treviño, Paulina V. Jiménez-Rico, Mario A. Banda-Barbosa, Félix Dominguez-Paulin, J. Mario Bernal-Blanco, Luis F. Pérez-González, and Daniel E. Noyola

We describe the clinical characteristics and outcomes of adults hospitalized with pneumonia during the pandemic (H1N1) 2009 outbreak. Patients admitted to a general hospital in San Luis Potosí, Mexico, from April 10 through May 11, 2009, suspected to have influenza virus–associated pneumonia were evaluated. We identified 50 patients with suspected influenza pneumonia; the presence of influenza virus was confirmed in 18: 11 with pandemic (H1N1) 2009 virus, 5 with unsubtypeable influenza A virus, 1 with seasonal influenza A virus (H3N2), and 1 in whom assay results for seasonal and pandemic (H1N1) viruses were positive. Eighteen patients were treated in the intensive care unit, and 10 died. During the pandemic (H1N1) 2009 outbreak, severe pneumonia developed in young adults who had no identifiable risk factors; early diagnosis and treatment of influenza virus infections may have a determinant role in outcome.

A novel influenza A virus, pandemic (H1N1) 2009 virus, has been identified as the cause of an epidemic outbreak of respiratory illness throughout the world (*1*). Current information indicates that the pandemic (H1N1) 2009 virus has been circulating in Mexico since at least March 2009 and that it has been the cause of an increase in the number of hospitalizations of young adults since April 2009 (*1*). The city of San Luis Potosí played a major role in raising awareness of the presence of an unusual and nonsubtypeable influenza A virus during the early days of the outbreak (*2*).

The Hospital Central "Dr. Ignacio Morones Prieto" is a public hospital located in San Luis Potosí that served as a reference center for evaluation and treatment of patients with suspected influenza virus pneumonia. In this article, we describe the results of investigations performed to determine the etiology of this outbreak and report the clinical findings, treatments, and patient outcomes.

Author affiliations: Hospital Central "Dr. Ignacio Morones Prieto," San Luis Potosí, Mexico (A. Gómez-Gómez, M. Magaña-Aquino, R. Falcón-Escobedo, J.M. Bernal-Blanco, L.F. Pérez-González); Universidad Autónoma de San Luis Potosí, San Luis Potosí (A. Gómez-Gómez, M. Magaña-Aquino, C.A. García-Sepúlveda, U.R. Ochoa-Pérez, R. Falcón-Escobedo, A. Comas-García, S. Aranda-Romo, H.I. Contreras-Treviño, P.V. Jiménez-Rico, M.A. Banda-Barbosa, F. Dominguez-Paulin, J.M. Bernal-Blanco, L.F. Pérez-González, D.E. Noyola); and Servicios de Salud del Estado de San Luis Potosí, San Luis Potosí (U.R. Ochoa-Pérez)

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Patients and Methods

Study Site

The city of San Luis Potosí is located in central Mexico and is the capital of the state of San Luis Potosí (*3*). The state population was 2,410,414 in 2005; the population of the San Luis Potosí metropolitan area in the same year was 957,753 (*4*). The Hospital Central provides medical services to mid- and low-income populations from all areas of the state; it has 269 beds in total, 250 in adult and pediatric wards and 19 in the intensive care unit (ICU).

Patients

Starting April 10, 2009, when the first case of pandemic (H1N1) 2009 was identified, all patients admitted to Hospital Central with lower respiratory tract infections (LRTIs) were screened for the presence of influenza virus. Patients who experienced acute onset of respiratory symp-

toms (cough, rhinorrhea, and/or dyspnea) and fever were assessed for possible influenza-related pneumonia, including radiologic evaluation. This report includes all adult patients with radiographic evidence of pneumonia admitted during the first month of the pandemic (H1N1) 2009 outbreak.

Clinical, Laboratory, and Bacteriologic Information

Patients admitted to medical wards and the ICU were subjected to the hospital's standard diagnostic protocol: blood cultures, HIV antibody testing, chest radiograph, laboratory tests, and spontaneous or induced sputum samples for Gram staining and culture. These tests were performed at admission and every 48–72 hours thereafter. Patients receiving mechanical ventilation had samples for minibronchoalveolar lavage (mini-BAL) (*5*), blood, and urine sent for bacterial cultures at admission and every 48–72 hours, or in the event of new or persistent fever. Bronchoscopy with BAL was performed as clinically indicated. A quantitative threshold of 10^5 CFU/mL for mini-BAL was used and of 10^4 CFU/mL for BAL (*6*). Patients were treated with at least the following standard antimicrobial drug regimens: those with criteria for acute lung injury (ALI, partial pressure of oxygen in arterial blood [paO_2]/fraction of inspired oxygen [FiO_2] = 201–300) or acute respiratory distress syndrome (ARDS, paO_2/FiO_2<200) received ceftriaxone and/or vancomycin plus levofloxacin or clarithromycin, and patients treated in medical wards received levofloxacin. Therapeutic decisions were individualized by the attending physician. Deceased patients for whom the family granted consent were subjected to postmortem examinations to recover lung specimens for bacteriologic culture, influenza virus detection, and histopathologic analysis.

Sample Collection and Processing

Respiratory samples obtained from patients with suspected influenza infection were placed in either normal saline or viral transport media and kept refrigerated at 4ºC until their arrival at the laboratory. A 200-μL aliquot of these samples was used for RNA extraction, and the remaining volume was stored at –70°C. Viral RNA was isolated using the High Pure Viral RNA Kit (Roche Diagnostics, Mannheim, Germany). Sample collection and processing were carried out according to national and international biosafety guidelines and recommendations (*7*,*8*).

Influenza Virus Detection

A multiplex reverse transcription–PCR (RT-PCR) capable of distinguishing type A from type B influenza virus was developed based on previously published primers (*9*). One-step RT-PCR was performed using the Access RT-PCR System (Promega Corporation, Madison, WI, USA) at 48ºC for 45 min, followed by 94°C for 2 min, 40 cycles of 30 s at 94°C, 30 s at 56°C, 30 s at 72°C, and a final step at 72ºC for 5 min. Influenza A samples were subsequently subtyped using a multiplex PCR with sequence specific primers approach adapted from previous publications (Table 1) (*11*). PCR products were subjected to electrophoresis on 1.5% agarose gels prestained with ethidium bromide at 6 volts/cm for 45 min and digitally documented. An RT-PCR for detection of pandemic (H1N1) 2009 virus was developed based on early sequencing data (EpiFlu Database, http://platform.gisaid.org). RT was carried out at 38ºC for 45 min by using UniFlu-RT oligonucleotide; PCR involved the newly developed Sw-NS-F 5′-ATG-GAC-TCC-AAC-ACC-3′ and Sw-NS-R 5′-TTA-AAT-AAG-CTG-AAA-CGA-G-3′ oligonucleotides at 1.6 μmol/L final concentration, 0.02 IU/μL of Taq (Vivantis Technologies Sdn Bhd Selangor D.E., Malaysia), 200 μmol/L deoxynucleoside triphosates, and 1.5 mmol/L $MgCl_2$. PCR program was 95ºC for 5 min, followed by 15 cycles of 95ºC for 20 s, 54ºC for 30 s, and 72ºC for 1.5 min, and a final step of 2 min at 72ºC. One microliter of a 1:1 dilution of the first PCR product was then used in a second PCR by using similar components except 1 mmol/L $MgCl_2$ and 800 nmol/L final concentration of internal oligonucleotides Sw1-F 5′-CTT-

Table 1. Oligonucleotide primers used for the subtyping of type A influenza viruses, Mexico

Oligonucleotide	Sequence (5′ → 3′)	Amplicon, bp	Reference
UniFlu-RT	AGC-AAA-AGC-AGG	Full genomic	(*10*)
HA1-F	GAA-ATT-TGC-TAT-GGC-TGA-CGG-GR	171	This study
HA1-R	GAC-ACT-ACA-GAG-ACA-TAA-GCA-TTT-TC		(*11*)
HA3-F	CAG-CAA-AGC-CTA-CAG-CAA-MTG-TT	236	This study
HA3-R	GGC-ATA-GTC-ACG-TTC-AAT-GCT-G		(*11*)
HA5-F	AAA-CTC-CAA-TRG-GGG-CGA-TAA-AC	344	This study
HA5-R1	CAA-CGG-CCT-CAA-ACT-GAG-TGT		(*11*)
HA5-R2	CCA-ACA-GCC-TCA-AAC-TGA-GTG-T		This study
NA1Nw-F	ACT-CAR-GAG-TCT-GAA-TGT-G	409	This study
NA1Nw-R1	GTC-CTT-CCT-ATC-CAA-ACA-CC		
NA1Nw-R2	GTT-CTC-CCG-AGC-CAG-ATA-CC		
NA2-F1	GGA-AAA-TCG-TTC-ATA-CTA-GCA-MAT-TG	176	This study
NA2-F2	GGG-AAA-ATC-GTT-CAT-ATT-AGC-ACA-TTG		(*11*)
N2-R1	AGC-ACA-CAT-AWC-TGG-AAA-CAA-TGC		This study

GAA-AGA-GGA-ATC-GAG-CG-3′ and Sw1-R 5′-GTC-TCC-CAT-TCT-CAT-CAC-AGT-3′ (429-bp amplicon). Influenza virus detection at the Instituto de Diagnóstico y Referencia Epidemiológicos (InDRE) and the State Public Health Laboratory used the real-time RT-PCR protocol developed by the US Centers for Disease Control and Prevention (CDC).

Virologic and Epidemiologic Data

To assess the presence of respiratory syncytial virus (RSV) and influenza in the community we reviewed the database at the Virology Laboratory Universidad Autónoma de San Luis Potosí (UASLP) and recorded the weekly number of viral detections from January 2008 through May 2009. In addition, the weekly number of acute respiratory infections (ARI) reported in the state of San Luis Potosí and the emergency department (ED) at the Hospital Central was recorded.

Statistical Analysis

We analyzed demographic and clinical characteristics using descriptive statistics. Means or medians were used for description of continuous variables according to data distribution; categorical variables were described with the use of percentages. Comparisons among patient groups were made to assess factors associated with severe infections. We compared categorical variables using the χ^2 or Fisher exact test; continuous variables were compared by using the Student *t* test or Mann-Whitney U test, according to data distribution. A p value <0.05 was considered significant. Statistical analyses were performed with SPSS for Windows (version 14.0, SPSS Inc., Chicago, IL, USA).

Results

An ARI outbreak was recorded in San Luis Potosí during April and May 2009. The most characteristic feature observed at the beginning of this outbreak was an increase in severe pneumonia cases requiring hospitalization of young adult patients. The Figure shows the epidemiologic curve of the weekly number of ARI cases reported to Servicios de Salud en el Estado de San Luis Potosí (state public health services) from January 2008 through May 2009 and the weekly number of ARI-related consultations provided in the ED at Hospital Central. In addition, the percentage of samples that were positive for influenza and RSV during each week at the Virology Laboratory UASLP is presented. Pneumonia patients included in this report were admitted during epidemiologic weeks 14 through 18 at a time that maximal pandemic (H1N1) 2009 virus circulation was documented.

From April 10 through May 11, 2009, a total of 70 patients >18 years of age were admitted to the Hospital Central with suspected LRTI. After review of clinical, radiologic, and laboratory data, suspected influenza virus–related pneumonia was diagnosed for 50 of those patients; other patients were determined to have cholangitis and ARDS (1 patient), congestive heart failure (2 patients), aspiration pneumonia (2 patients), asthma without evidence of pneumonia on chest radiograph (5 patients), and influenza-like syndrome without pneumonia (10 patients). Nine of the 20 excluded patients were tested for influenza infection, and results were negative for 8; samples from 1 of the patients found to have influenza-like illness without radiographic evidence of pneumonia were positive for pandemic (H1N1) 2009 virus.

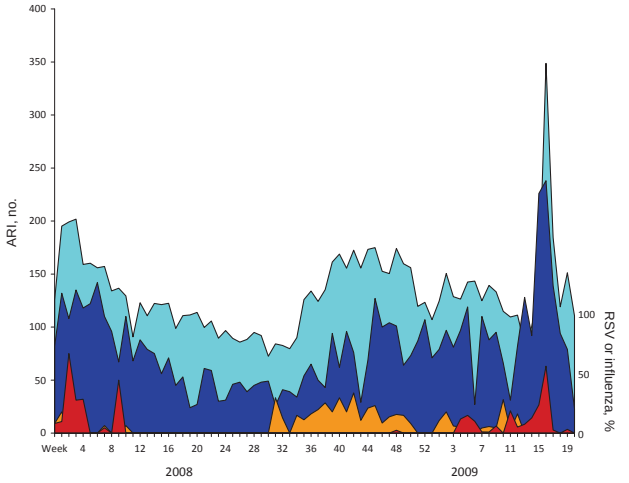

Figure. Weekly number of acute respiratory infections (ARI) reported in the state of San Luis Potosí, Mexico (no. of cases × 100, light blue area); weekly number of ARI visits at the emergency department of Hospital Central "Dr. Ignacio Morones Prieto" (dark blue area); and weekly percentage of samples positive for respiratory syncytial virus (RSV; orange area) or influenza (red area), Virology Laboratory, Universidad Autónoma de San Luis Potosí, during January 2008 through May 2009.

The main demographic features of our group of 50 patients are summarized in Table 2. Only 1 patient had an underlying pulmonary disorder. Obesity was the most frequent underlying disorder and was the only underlying disorder in 17 patients; 6 patients had morbid obesity (body mass index [BMI] >40). The most frequent symptoms patients had at hospital admission were fever (100%), headache (96%), cough (94%), and myalgias (94%). The predominant radiologic pattern on chest radiograph was consolidation, with multilobar and unilobar consolidation in 39 and 11 patients, respectively. Twenty-one patients had ill-defined interstitial opacities. Three patients had evidence of unilateral pleural effusion that affected ≈10% of the hemithorax. HIV testing was performed for 45 patients; all results were negative. Evidence of a systemic inflammatory response and muscle involvement was observed commonly, manifested by el-

Table 2. Demographic features of 50 adult patients admitted with acute pneumonia during the pandemic (H1N1) 2009 outbreak, San Luis Potosí, Mexico*

Characteristic	Value
Age, y, mean (SD, range)	38.4 (13.9, 21–69)
Gender	
M	29 (58)
F	21 (42)
Residence	
San Luis Potosí municipality	30 (60)
Other municipality	20 (40)
No. home contacts, median (range)	4 (1–16)
Presence of ≥1 underlying condition	30 (60)
Obesity (BMI > 30)	25 (55.6)†
Diabetes mellitus	8 (16)
Other conditions‡	9 (18)
Smoker	13 (26)
Arrival at the hospital	
Directly	40 (80)
Referred from other hospital or clinic	10 (20)
Influenza vaccination during previous winter season	5 (10)

*Values are no. (%) unless otherwise indicated. BMI, body mass index.
†BMI data available only for 45 patients.
‡Some patients had >1 additional underlying condition, including chronic renal failure (3), underlying heart disorder (3), systemic lupus erythematosus (1), chronic obstructive lung disease (1), hyperthyroidism (1), seizure disorder (1), and pregnancy (1).

evated levels of C-reactive protein, creatine phosphokinase (CPK), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST).

Thirty patients were treated in medical wards, 12 of whom met criteria for ALI, none had extrapulmonary involvement, and all were discharged to home. Two patients with ARDS were treated in the ER.

Eighteen patients were treated in the ICU (17 with ARDS and 1 with ALI). The median positive end expiratory pressure at admission was 16 cm H_2O (range 14–20 cm) and media FiO_2 was 80% (range 50%–100%); the mean Sepsis-related Organ Failure Assessment (SOFA) score and Acute Physiology and Chronic Health Evaluation (APACHE) II at admission were 9.44 (range 5–15) and 17 (range 8–32), respectively. Twelve patients had hemodynamic instability that required vasoconstrictors and 6 had arrhythmias, mainly supraventricular and ventricular extrasystoles. Acute renal failure developed in 7 patients (6 within 72 hours of admission); 6 of these 7 patients experienced hemodynamic instability, and 3 required hemodialysis. Nine patients with evidence of cardiac failure underwent transthoracic echocardiography. The mean left ventricular ejection fraction was 54.6%; in 2 patients, it was reduced at 40%. Nosocomial pneumonia developed in 8 patients in the ICU after a median of 9.5 days of mechanical ventilation.

Forty-five patients received antiviral treatment with oseltamivir. The time elapsed from onset of symptoms to the start of antiviral drug treatment ranged between 1 and 14 days (median 6 days). Four patients that did not receive antiviral drugs were discharged. The only patient who did not receive antiviral drugs and died was the first patient with pandemic (H1N1) 2009–associated pneumonia to be admitted to our hospital during this outbreak; he had severe pneumonia and ARDS and died in the ED.

All patients were treated with antimicrobial drugs. Levofloxacin or clarithromycin was included as part of the antimicrobial drug treatment of 48 (96%) patients; the 2 patients who did not receive 1 of these antimicrobial drugs recovered and were discharged to home. Sixteen patients received steroids as part of their treatment; steroid use was assessed by the attending physician. The median number of days from admission to start of steroid treatment was 2 days (range 0–20 days). The effect of steroid use on patient outcome is difficult to assess because this treatment was used more frequently in patients with more severe disease.

Postmortem open lung biopsies were performed on 5 patients and a complete necropsy was performed for 1. The pathologic findings were those of diffuse alveolar damage; additionally, some patients had hemophagocytosis and capillary and arteriolar thrombosis as well as enlargement of alveolar cells. One patient had acute bacterial pneumonia.

Respiratory samples from 45 patients were available for viral detection. Samples from 37 patients were tested at UASLP, while samples from 41 patients were tested at InDRE in Mexico City or the State Public Health Laboratory in San Luis Potosí using the real time RT-PCR protocol developed by CDC. Overall, influenza virus was detected in respiratory secretions of 15 (33.3%) of the 45 patients who had respiratory samples available for testing; pandemic (H1N1) 2009 virus was detected for 10 patients, seasonal influenza A virus was detected for 4, and results of assays for pandemic (H1N1) 2009 and seasonal influenza A viruses were positive for 1 patient. Subtyping of samples positive for seasonal influenza virus showed that 1 infection was caused by influenza A (H1N1) virus (this finding corresponded to the patient with positive results for both seasonal and pandemic [H1N1] 2009 virus; due to the potential for cross-reactivity, infection with pandemic [H1N1] 2009 virus only cannot be ruled out in this patient) and 1 by influenza A (H3N2); the other 3 samples were not subtypeable by our RT-PCR. In addition, RT-PCR was performed in lung tissue from 6 patients who died; the presence of influenza virus was detected in 3 of these patients (pandemic [H1N1] 2009 virus in 1 and unsubtypeable influenza virus in 2). Detection of influenza virus in respiratory samples from these deceased patients was either negative (in 2 patients) or not possible because no respiratory sample was collected before the patient's death (1 patient).

The duration of symptoms at the time of sample collection for samples that tested positive for influenza virus at

UASLP was significantly shorter (mean of 4.7 days) compared with those samples that tested negative (8.8 days; p = 0.02). Duration of symptoms at the time of sample collection was also demonstrated to be statistically significant for samples that tested positive with the use of CDC's real time RT-PCR protocol (mean 5.7 days) compared with those that were negative by this method (mean 9.5 days; p = 0.006).

We compared the demographic and clinical characteristics of patients with pandemic (H1N1) 2009 infections and those that were negative on viral testing (Table 3). The clinical presentation of both groups of patients was similar.

Sputum cultures obtained at time of admission were positive in 4 patients treated in medical wards; bacteria isolated included *Staphylococcus aureus* (2 patients) and

Table 3. Demographic, clinical, and laboratory features on admission of patients with pandemic (H1N1) 2009 and nonconfirmed influenza pneumonia cases during the pandemic (H1N1) 2009 outbreak, San Luis Potosí, Mexico*

Characteristic	Pandemic (H1N1) 2009 (n = 11)	Nonconfirmed influenza (n = 32)	p value
Demographic features			
Male sex	5 (45.5)	20 (62.5)	0.48
Age, y, mean (SD)	34 (10.97)	39.47 (14)	0.25
Resident of San Luis Potosí municipality	5 (45.5)	20 (62.5)	0.73
Clinical features			
Presence of underlying conditions			
Obesity (BMI >30)†	4 (40)	16 (57.1)	0.47
Diabetes	2 (18.2)	4 (12.5)	0.64
Other conditions‡	3 (27.3)	4 (12.5)	0.35
Days from symptom onset to admission, mean (SD)	5.36 (2.5)	6.72 (3.13)	0.2
Duration of symptoms at the onset of dyspnea, d, mean (SD)	2.73 (1.19)	3.91 (1.92)	0.06
Pneumonia severity index (PORT score)	2.82 (1.47)	2.81 (1.15)	0.99
Symptoms			
Fever	11 (100)	32 (100.0)	NA
Headache	10 (90.9)	31 (96.9)	0.45
Cough	11 (100)	31 (96.9)	1
Myalgias	9 (81.8)	32 (100.0)	0.06
Arthralgias	10 (90.9)	30 (93.8)	1
Dyspnea	11 (100)	25 (78.1)	0.16
Rhinorrea	8 (72.7)	25 (78.1)	0.69
Malaise	6 (54.5)	11 (34.4)	0.29
Blood-streaked sputum	3 (27.3)	13 (40.6)	0.49
Diarrhea	1 (9.1)	7 (21.9)	0.66
Pleuritic chest pain	1 (9.1)	5 (15.6)	1
Vomiting	2 (18.2)	2 (6.3)	0.27
Physical examination findings on admission			
BMI, mean (SD)	29.17 (6.48)	31.22 (5.4)	0.34
Respiratory rate, bpm, mean (SD)	28.44 (4.91)	28.31 (7.24)	0.92
Crackles			0.03
Unilateral	4 (36.4)	2 (6.3)	
Bilateral	7 (63.6)	30 (93.8)	
Wheezing	5 (45.5)	18 (56.3)	0.73
Laboratory features			
Total leukocyte count on admission (x 10^9/L), median (SD)	8.81 (6.1)	6.81 (4.13)	0.23
Platelet count (x 10^9/L), mean (SD)§	173.6 (61.48)	227 (216.24)	0.45
C-reactive protein, mg/dL, mean (SD)	14.51 (8.45)	18.74 (16.22)	0.41
Creatinine, mg/dL, median (SD)	1.24 (0.87)	1.11 (0.75)	0.13
Creatine phosphokinase, U/L, mean (SD)	376.78 (439.22)	462.2 (507.27)	0.65
Lactate dehydrogenase, U/L, median (SD)§	952 (674.72)	1089.31 (602.32)	0.54
AST, mg/dL, median (range)¶	43 (19–76)	69 (18–249)	0.07
ALT, mg/dL, median (range)#	29 (13–55)	43 (13–177)	0.05

*Values are no. (%) unless otherwise indicated. BMI, body mass index; bpm, breaths per minute; PORT, Pneumonia Patient Outcomes Research Team (www.ahrq.gov/clinic/pneuclin.htm); NA, not available; AST, aspartate aminotransferase; ALT; alanine transaminase.
†Data available for 38 patients.
‡Includes chronic renal failure, systemic lupus erythematosus, pregnancy, cardiac disorder, seizure disorder.
§Data available for 42 patients.
¶Data available for 41 patients.
#Data available for 40 patients.

Streptococcus pneumoniae (2 patients). For patients treated in the ICU all cultures performed on mini-BAL and BAL (1 patient) at admission were negative; however, nosocomial pneumonia developed in 8 patients; microorganisms isolated included *Acinetobacter baumanii* (5 patients), *S. aureus* (1 patient), *Escherichia coli* (1 patient) and *Candida* sp. (1 patient). BAL was performed for only 4 patients as part of evaluation of suspected nosocomial pneumonia. Six postmortem lung biopsy specimens were cultured; 1 showed growth of *E. coli*; the other 5 were negative.

Ten (20%) patients died; 8 were obese (BMI >30), and 4 of those were morbidly obese (BMI >40). Eight patients died in the ICU with a median length of stay of 8.5 days (range 2–14 days), and 2 patients with ARDS died in the ED within hours after arrival. Six patients died within the first 7 days following admission, and 4 died during the second week after admission. In 5 patients, the cause of death was multiorgan dysfunction (pulmonary, cardiovascular, and renal); 3 deaths were attributed to severe ARDS refractory to ventilator management protocols (*12*); 1 was caused by mixed ARDS and shock refractory to vasoconstrictors; and 1 was caused by septicemia. The median length of stay for patients treated in the ICU was 13 days (range 2–60 days); for those treated on the medical wards, this figure was 5 days (range 2–12 days).

The median duration of symptoms at time of admission for patients who died was longer (7.5 days) than for those who survived (5 days, $p = 0.025$); duration of symptoms before admission for patients who died in the ER was similar to duration of symptoms for patients who died in the ICU. The mean SOFA and APACHE II scores on admission for patients treated in the ICU who survived were 7.5 (range 5–11) and 13 (range 8–18), respectively; in comparison, for patients who died the mean values of these scores were 12 (range 10–15) and 21 (range 15–32), respectively. On the other hand, patients who received antiviral drug therapy during the first 5 days after initiation of symptoms had a better outcome than those who did not (survival of 95% of patients vs. 70%, respectively; $p = 0.037$). However, sample size was not sufficient to perform an appropriate multivariate analysis to define the independent contribution of duration of illness, antiviral drug therapy, and other variables to patient outcome.

Discussion

Since pandemic (H1N1) 2009 was first identified in spring 2009, there has been a worldwide dissemination of this virus (*1*). Although the precise origin of this strain has not yet been determined, Mexico was the first country to show the effects of the epidemic caused by this virus. In addition, more severe cases have been registered in our country, particularly in young adults, in comparison to the rest of the world.

Pneumonia is recognized as the most important complication of influenza infections (*13*). LRTI in patients with influenza may be caused directly by viral invasion or result from bacterial complications (*14*,*15*). The occurrence of progressive disease, with bilateral pulmonary consolidation or ARDS, associated with high death rates has been described for patients with influenza-associated pneumonia, particularly in those with infections caused by new subtypes of influenza virus. In contrast, bacterial pneumonia complicating influenza usually occurs after near resolution of influenza symptoms (*16*,*17*). Our study cohort was composed mostly of young adults without severe cardiovascular or pulmonary underlying disorders in whom the onset of dyspnea was, on average, 3.5 days after the onset of influenza symptoms. In only 4 (8%) of the 50 patients included in this report was there clear evidence of the participation of bacteria in the disease process; this number contrasts with previous reports of community-acquired pneumonia, for which bacteria are reported more frequently. Our results suggest that bacterial co-infection played a minor role as a cause of pneumonia in these patients. In addition, no histopathologic evidence of bacterial pneumonia was observed in 5 of the 6 deceased patients on whom lung biopsies were performed.

Although we were not able to confirm pandemic (H1N1) 2009 virus in all patients, several factors support the notion that a high proportion of their infections were caused by this virus. These factors include the clustering of cases of severe pneumonia in young adults (>85% of the patients became ill during a 2-week period) coincident with the identification of a novel influenza virus shown to be circulating in the community.

A striking feature observed in this cohort of patients was the high frequency of systemic involvement with laboratory evidence of myositis/rhabdomyolysis, elevated acute-phase reactants, ARDS, hemodynamic instability, and acute renal failure. Elevation of LDH levels has been reported for patients with seasonal influenza but has not been considered a specific marker of severity. Elevation of CPK levels was frequently observed in patients treated at our hospital, and elevation of CPK and AST levels suggest that muscle inflammation was present in these patients. Myositis (with or without rhabdomyolysis) has been associated previously to influenza infections (*18*–*21*). Of note, elevated CPK levels were observed in patients with and without confirmed influenza virus infections.

Why these severe manifestations of influenza infections occurred in young adult patients during this outbreak remains an unanswered question. Although almost two thirds of patients hospitalized with pneumonia at our institution had underlying health disorders, the presence of chronic respiratory or cardiovascular diseases were rare. Obesity was the most frequent underlying disorder and was present in

more than half of the patients; in contrast, the prevalence of obesity for adults >20 years of age in San Luis Potosí was 31.3% in 2006 (*22*). Eight of 10 patients who died were obese, including 4 who were morbidly obese. Obesity has been suggested to be a proinflammatory chronic condition and thus may have contributed to some of the manifestations observed in these patients (*23*,*24*). The presence of obesity as a possible risk factor for severe pandemic (H1N1) 2009 has been noted previously. In a report from Michigan, 9 of 10 patients with pandemic (H1N1) 2009 infection requiring intensive care were obese (*25*). However, larger studies are needed to determine the role of obesity as a risk factor for severe influenza pneumonia.

There are several limitations to our study. First, only pneumonia patients were included. Therefore, the effect of influenza as a cause of hospitalizations is probably underestimated because patients with exacerbations of chronic obstructive pulmonary disease and asthma, as well as with congestive heart failure decompensation triggered by influenza, may have been excluded. Second, we did not have appropriate respiratory samples from all patients admitted with pneumonia because some patients were admitted before the existence of an outbreak had been identified. In addition, patients with more severe infections tended to have a longer duration of illness at the time of admission, reducing the likelihood that influenza may have been detected. Third, influenza infection could not be confirmed in most patients, and other causes of acute pneumonia (such as other viruses) were not excluded. However, because the clinical manifestations were similar in all cases, patients became ill in a short period simultaneously with a sudden increase in ARI in San Luis Potosí, and pandemic (H1N1) 2009 infections were detected at a time when RSV and seasonal influenza circulation had declined, we consider it is likely that many cases may have been caused by influenza virus.

During the pandemic (H1N1) 2009 outbreak in San Luis Potosí, Mexico, young adults without identifiable risk factors became ill with severe pneumonia. Until predictors of severe pandemic (H1N1) 2009 virus infections are identified, early diagnosis and prompt antiviral treatment seem to be the best measures to avoid serious illness caused by this virus (*26*,*27*).

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Dr Gómez-Gómez is a pulmonologist at the Hospital Central "Dr. Ignacio Morones Prieto," San Luis Potosí, Mexico. His research interests include respiratory infections.

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Address for correspondence: Alejandro Gómez-Gómez, Facultad de Medicina, Universidad Autonoma de San Luis Potosi–Internal Medicine, Avenida Venustiano Carranza 2405 Colonia Los Filtros, San Luis Potosi 78210, Mexico; email: agomez.cer@prodigy.net.mx

Worldwide Dissemination of the *bla*$_{OXA-23}$ Carbapenemase Gene of *Acinetobacter baumannii*[1]

Pauline D. Mugnier, Laurent Poirel, Thierry Naas, and Patrice Nordmann

To assess dissemination of OXA-23–producing strains of *Acinetobacter baumannii*, we obtained 20 carbapenem-resistant, OXA-23–producing isolates from different regions. Their clonal relationship was assessed by pulsed-field gel electrophoresis and multilocus sequence typing. We identified 8 sequence types, including 4 novel types. All except 2 strains belonged to 2 main European clonal lineages. The *bla*$_{OXA-23}$ gene was either located on the chromosome or on plasmids and associated with 4 genetic structures.

Acinetobacter baumannii is a gram-negative organism that is increasingly recognized as a major pathogen causing nosocomial infections, including bacteremia and ventilator-associated pneumonia, particularly in patients admitted to intensive care units (*1*). Several studies have shown the geographically widespread occurrence of multidrug-resistant *A. baumannii* strains, which suggested a clonal relatedness of these strains. Three international *A. baumannii* clones associated with multidrug resistance (European clones I, II, and III) have been reported (*2*).

Increasing resistance to carbapenems has been observed worldwide in the past decade, frequently mediated by production of class D β-lactamases with carbapenemase activity. Three acquired class D β-lactamases with carbapenemase gene clusters have been described in *A. baumannii*, which correspond to *bla*$_{OXA-23}$-like, *bla*$_{OXA-40}$-like, and *bla*$_{OXA-58}$-like genes (*3*). The *bla*$_{OXA-23}$ gene, first characterized in Scotland (*4*), has been increasingly reported worldwide. *A. radioresistens* was recently identified as the progenitor of the *bla*$_{OXA-23}$-like genes (*5*). Clonal outbreaks of carbapenem-resistant and OXA-23–producing *A. baumannii* have been reported in many countries, such as Bulgaria (*6*), People's Republic of China (*7*), Brazil (*8*), Iraq (*9*), Afghanistan (*9*), and French Polynesia (*10*).

Genetic acquisition of the *bla*$_{OXA-23}$ gene was investigated and transposons Tn*2006*, Tn*2007*, and Tn*2008* were identified as genetic structures harboring this gene (*10*–*12*). In Tn*2006*, the *bla*$_{OXA-23}$ gene is flanked by 2 copies of the insertion sequence IS*Aba1*, which are located in opposite orientations (Figure 1). The functionality of Tn*2006* has been recently demonstrated (*13*). Tn*2008* is similar to Tn*2006* but lacks the second copy of IS*Aba1* and the *bla*$_{OXA-23}$ gene is associated with 1 copy of IS*Aba4* (which differs from IS*Aba1*) in Tn*2007* (Figure 1) (*11*). As reported for strains from United Arab Emirates and Bahrain, the *bla*$_{OXA-23}$ gene can be associated with only 1 copy of IS*Aba1* (*14*,*15*). We studied the clonal relationship and genomic environment of sequences surrounding the *bla*$_{OXA-23}$ gene among a collection of OXA-23–producing isolates from 15 countries.

Materials and Methods

Bacterial Strains and Susceptibility Testing

Twenty OXA-23–producing *A. baumannii* clinical isolates were obtained from 15 countries. These isolates had been obtained from patients hospitalized in intensive care units from December 2003 through March 2008. Isolates were obtained from tracheal aspirates (n = 3), bile (n = 1), urine (n = 4), wounds (n = 1), respiratory tract (n = 1), blood (n = 4), and sputum (n = 1). The isolates were initially chosen after preliminary pulsed-field gel electrophoresis (PFGE)–based typing had identified 13 pulsotypes. Isolates

Author affilations: Institut National de la Santé et de la Recherche Médicale Unité 914, Paris, France; and Université Paris Sud, Bicêtre, France

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were obtained from France (n = 4), Vietnam (n = 1), New Caledonia (n = 1), Thailand (n = 1), Australia (n = 1), Tahiti (n = 1), Reunion (n = 2), South Africa (n = 1), United Arab Emirates (n = 2), Libya, (n = 1), Bahrain (n = 1), Egypt (n = 1), Belgium (n = 1), Algeria (n = 1), and Brazil (n = 1).

Presence of the bla_{OXA-23} gene was screened by PCR by using specific primers (OXA-23-A 5′-GGA ATTCCATGAATAAATATTTTACTTGC-3′ and OXA-23-B5′-CGGGATCCCGTTAAATAATATTCAGGTC-3′) and additional sequencing (ABI 3100 sequencer; Applied Biosystems, Foster City, CA, USA). Susceptibility patterns to β-lactam antimicrobial drugs were determined by using a standard disk diffusion method according to published standards (*16*) and Etest strips (AB Biodisk, Solna, Sweden). Isolates were identified by using 16S rRNA gene sequencing (*17*).

Clonal Relationships

Isolates were typed by using *Apa*I macrorestriction analysis and PFGE according to the manufacturer's recommendations (Bio-Rad, Marnes-la-Coquette, France). Bacteria were grown in a medium appropriate for the strain until an optical density of 0.8 to 1 at 600 nm was reached. One milliliter of cells was centrifuged, washed, and resuspended in 10 mmol/L Tris, pH 7.2, 20 mmol/L NaCl, 50 mmol/L EDTA. Immediately after resuspension, an equal volume of 2% low melting point InCert agarose (Bio-Rad) was added. Solid agarose plugs were lysed at 37°C for 2 h in 1 mL of lysis buffer (10 mmol/L Tris, pH 7.2, 50 mmol/L NaCl, 0.5% sodium laurylsarcosine, 0.2% sodium deoxycholate) supplemented with 20 mg/L of lysozyme. The plugs were then incubated at 55°C for 16 h with proteinase K buffer (100 mmol/L EDTA, pH 8, 0.2% sodium deoxycholate, 1% sodium laurylsarcosine) supplemented with 20 mg/L of proteinase K. Plugs were washed with Tris-EDTA buffer containing 1 mmol/L phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA) and 3× with Tris-EDTA buffer at room temperature.

Whole-cell DNA of *A. baumannii* isolates was digested with *Apa*I overnight at room temperature (New England Biolabs, St. Quentin-en-Yvelines, France). Electrophoresis was performed on a 1% agarose gel with 0.5× Tris-borate-EDTA buffer by using a CHEF DRII apparatus (Bio-Rad). Samples were subjected to electrophoresis at 14°C, 6 volts/cm, and a switch angle with 1 linear switch ramp of 3–8 s for 10.5 h, and then for 12–20 s for 10.5 h.

Identification of PCR-based sequence groups was conducted by using 2 multiplex PCR assays designed to selectively amplify group 1 or group 2 alleles of the gene encoding outer-membrane protein A (*ompA*), the gene encoding part of a pilus assembly system required for biofilm formation (*csuE*), and the gene encoding the intrinsic carbapenemase gene of *A. baumannii*) (bla_{OXA-51}) (*18*). Clonal relationships were established by multilocus sequence typing (MLST) by using 7 standard housekeeping loci (citrate synthase [*gltA*], gyrase B [*gyrB*], glucose dehydrogenase B [*gdhB*], recombination A [*recA*], chaperone 60 [*cpn60*], glucose-6-phosphate isomerase [*gpi*], and RNA polymerase [*rpoD*]) as described (*18*). Sequencing of internal fragments was performed by using BigDye fluorescent terminators and primers described (*19*). Sequences were compared with the *A. baumannii* database at the MLST Website (http://mlst.zoo.ox.ac.uk). To supplement epidemiologic results, we performed a second MLST typing using the scheme developed by Nemec et al. (*20*). Sequences of the 7 housekeeping genes were analyzed by using an *A. baumannii* database (www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html).

Southern Blot Analysis and Location of bla_{OXA-23} Gene

Southern blot analysis was performed by using total genomic DNA digested with *Eco*RI, separated by electro-

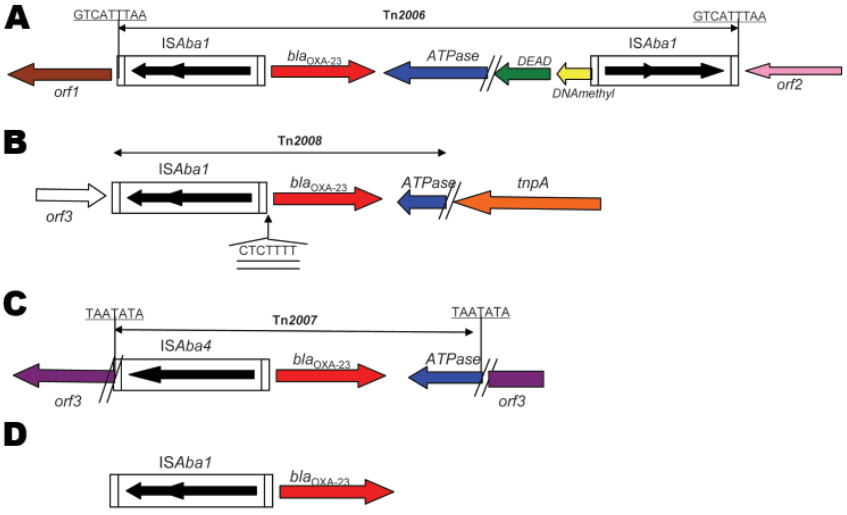


Figure 1. Genetic structures associated with the bla_{OXA-23} gene of *Acinetobacter baumannii*. A) Tn*2006* from isolates 240, 512, 810, 859, 883 and AUS (ST22/ST2). B) Tn*2008* from isolate 614. C) Tn*2007* from isolates Ab14, BEL, and DOS. D) IS*Aba1* from isolates AS3, 1190, 861, and 877. Boundaries of Tn*2006*, Tn*2007*, and Tn*2008* are indicated with the target site duplication likely generated by transposition events underlined. The 7-bp difference in the site of insertion of IS*Aba1* for isolate 614 is double-underlined. The open reading frame 1 (*orf1*), *orf2*, and *orf3* genes of unknown function is indicated. *tnpA*, gene encoding a putative transposase; *ATPase*, gene encoding the putative AAA ATPase; *DEAD*, gene encoding the putative DEAD (Asp-Glu-Ala-Asp) helicase; DNAmethyl, DNA methylase.

phoresis on 0.8% agarose gels, transferred onto Hybond N+ membranes, and hybridized with enhanced chemiluminescence labeled probes overnight at 42°C. The membranes were developed according to the manufacturer's instructions (GE Healthcare, Saclay, France). Chromosomal or plasmid locations of the β-lactamase gene were assessed by hybridization of I-*Ceu*I–digested genomic DNA with bla_{OXA-23} and 16S rDNA probes and electrophoresis (20–120 s for 9 h and 60–100 s for 11 h at 14°C and 5 V/cm^2) (*21*). DNA was transferred from an agarose gel to a nylon membrane by capillary transfer. Hybridization, labeling, and detection were conducted as described above. Mating-out assays were performed by using isolates that had plasmid-borne bla_{OXA-23} as donors and rifampin-resistant *A. baumannii* BM4547 as recipients as described (*22*). Transconjugants were selected on trypticase soy agar plates containing ticarcillin (50 mg/L) and rifampin (50 mg/L).

Cloning Experiments

To identify entire transposon structures containing the bla_{OXA-23} gene in different isolates and determine their location in the target DNA, a cloning procedure was used. Some data had been reported for 6 of 20 isolates (*11*). Total DNA was digested with either *Sac*I or *Sal*I, ligated into the *Sac*I or *Sal*I sites of plasmid pBK-CMV (kanamycin-resistant cloning vector), and the recombinant plasmids were transformed into *Escherichia coli* TOP10, as described (*14*). Recombinant plasmids were selected on trypticase soy agar plates containing amoxicillin (50 mg/L) and kanamycin (30 mg/L). Cloned DNA fragments of several recombinants plasmids were sequenced on both strands by primer walking as described (*11*).

Results

Clonal Relatedness of the Isolates

Twenty carbapenem-resistant *A. baumannii* isolates were obtained from 15 countries (Table). All isolates were highly resistant to ticarcillin (MIC >256 mg/L) and showed a high level of resistance to ceftazidime (MIC >256 mg/L), except isolates Ab14 (MIC 4 mg/L) 861 and DOS (MIC 8 mg/L). All isolates were resistant to imipenem and meropenem (MIC ≥16 mg/L) (Table).

Multiplex PCR for identification of sequence groups showed 10 isolates that belonged to group 1 according to Turton et al. (*18*), eight that belonged to group 2, and 2 isolates that did not belong to groups 1 or 2. The 10 isolates that belonged to group 1 and corresponded to European clone II (*18*) were classified into 2 sequence types (STs), ST22 and ST53, according to MLST analysis (*18*). ST22 (1–3-3–2-2–7-3) was the most frequent type identified. Nine isolates were identified: 2 from France and 1 each from Vietnam, New Caledonia, Thailand, Australia, Tahiti, Reunion, and South Africa. A single European clone II isolate was classified as ST53 (1–3-3–2-2,3-3), a single-locus variant of ST22. Among 10 other isolates, 8 belonged to group 2 (corresponding to European clone I). Four STs were identified: ST25 (10–12–4–11–1–9–5) (Libya, United Arab Emirates, and Bahrain), ST44 (10–12–4–11–4–9–5) (United Arab Emirates and Algeria), and 2 new STs, 1 for isolates from Reunion and Egypt (10–12–4–11–4–16–5) and another related ST identified in the single isolate from Belgium (10–12–4–11–4,4–5). These 4 STs differ by 1 locus. The 2 most recent isolates from France and Brazil did not belong to European clones I or II and corresponded to 2 STs (1–22–3-11–1-9–7 and 12–18–12–1-15–9-19, respectively) (Table). Although 8 STs were identified in this collection, 9 pulsotypes were characterized by PFGE according to the criteria of Tenover et al. (23) (Figure 2).

According to MLST analysis developed by Nemec et al. (*20*), all isolates that belonged to European clone II had the same sequence type (ST2) (2,2-2,2-2,2-2), including isolate 585, which had a distinct but related ST in the first analysis. Among isolates that belonged to European clone I, two sequence types were determined: ST20 (3–1-1,1-5–1-1) (Libya, United Arab Emirates, Bahrain) and ST1 (1,1-1,1-5–1-1) (United Arab Emirates, Reunion, Egypt, Belgium, Algeria). Isolates 910 (Reunion), 861 (Egypt), and BEL (Belgium) were included in ST1. These isolates had a distinct ST according to methods of Bartual et al. (*19*). The 2 most recent isolates were classified into 2 STs, a new ST (3–2-2,2-5–4-8) for isolate DOS (France) and ST15 (6,6-8–2-3–5-4) for isolate 877 (Brazil) (Table).

Location and Transferability of the bla_{OXA-23} Gene

Location of the bla_{OXA-23} gene was evaluated by using the I-*Ceu*I method. Eleven isolates had the bla_{OXA-23} gene on the chromosome, with a hybridization signal for an ≈40-kb band for isolate AS1 and an ≈200-kb band for 10 isolates (Table). Nine isolates carried the bla_{OXA-23} gene on a plasmid and 1 isolate had 2 copies of the bla_{OXA-23} gene, 1 on the chromosome and 1 on a 7–kb plasmid (Table).

To examine the copy number of the bla_{OXA-23} gene in different *A. baumannii* genomes, we performed Southern blot hybridization on *Eco*RI-digested DNA fragments using a 589-bp DNA probe specific for the bla_{OXA-23} gene. Sixteen isolates showed only 1 copy of the bla_{OXA-23} gene. Isolates BEL, Ab14, and DOS had 2 copies of the bla_{OXA-23} gene on different plasmids, and Ab13 had 1 copy on the chromosome and 1 copy on a plasmid according to results of the I-*Ceu1* technique.

Mating-out assays were performed by using the 10 plasmid-positive strains as donor strains and rifampin-resistant *A. baumannii* BM4547 as the recipient strain. Five transconjugants were obtained; all had a 130-kb plasmid that did not provide additional antimicrobial drug resistance

Table. Characteristics of 20 *bla*$_{OXA-23}$-positive *Acinetobacter baumannii* clinical isolates*

Isolate	Origin	Date of isolation	Specimen	EC	ST†	Copy no. of *bla*$_{OXA-23}$	Genetic location and size, kb	Genetic structure	MIC, μg/mL		
									CAZ	IPM	MEM
240	France	2003 Dec	Tracheal aspirate	II	22/2	1	Chromosome, ≈200‡	Tn*2006*	128	>32	>32
512	Tahiti	2004 Mar	Tracheal aspirate	II	22/2	1	Chromosome, ≈200‡	Tn*2006*	64	>32	>32
761	Vietnam	2005 May	Bile	II	22/2	1	Chromosome, ≈200‡	Tn*2006*	64	>32	>32
810	New Caledonia	2004 Jun	Blood	II	22/2	1	Chromosome, ≈200‡	Tn*2006*	96	>32	>32
863	Thailand	2006 Jun	Urine	II	22/2	1	Chromosome, ≈200‡	Tn*2006*	256	>32	>32
883	Reunion	2006 Jun	Unknown	II	22/2	1	Chromosome, ≈200‡	Tn*2006*	128	>32	>32
Ab13	France	2004 Jun	Urine	II	22/2	2	Chromosome, ≈200,‡ and plasmid, 70	Tn*2006*	128	>32	>32
AUS	Australia	2004 Oct	Urine	II	22/2	1	Chromosome, ≈200‡	Tn*2006*	96	>32	>32
859	South Africa	2006 Jan	Urine	II	22/2	1	Chromosome, ≈200‡	Tn*2006*	128	>32	>32
585	France	2004 Jul	Tracheal aspirate	II	53/2	1	Chromosome, ≈200‡	Tn*2006*	128	>32	>32
614	Libya	2004 Oct	Unknown	I	25/20	1	Plasmid, 130	Tn*2008*	256	>32	16
AS3	UAE†	2006 Oct	Blood	I	25/20	1	Plasmid, 130	IS*Aba1*	256	>32	>32
1190	Bahrain	2008 Mar	Blood	I	25/20	1	Plasmid, 130	IS*Aba1*	256	>32	>32
AS1	UAE	2006 Jul	Blood	I	44/1	1	Chromosome, ≈40‡	Tn*2006*	256	>32	>32
Ab14	Algeria	2004 Dec	Unknown	I	44/1	2	Plasmid, 25, and plasmid, >150	Tn*2007*	4	16	>32
910	Reunion	2006 Oct	Unknown	I	New1/1	1	Plasmid, 130	Tn*2006*	256	16	16
861	Egypt	2005 Nov	Sputum	I	New1/ 1	1	Plasmid, 130	IS*Aba1*	8	32	32
BEL	Belgium	2007 Jul	Respiratory tract	I	New2/ 1	2	Plasmid, 25, and plasmid, >150	Tn*2007*	256	>32	>32
DOS	France	2004 May	Unknown	–	New3/ New	2	Plasmid, 25, and plasmid, >150	Tn*2007*	8	>32	>32
877	Brazil	2006 Jul	Wound	–	New4/15	1	Plasmid, 130	IS*Aba1*	96	>32	>32

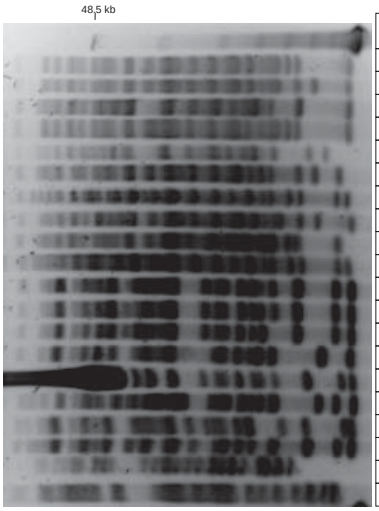
*EC, European clone; ST, sequence type; UAE, United Arab Emirates; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem. The MIC for ticarcillin was >256 μg/mL for all 20 isolates.
†ST determined by Bartual et al. (*19*) compared with ST determined by Nemec et al. (*20*).
‡Size of chromosome band carrying the *bla*$_{OXA-23}$ gene, as determined by using the I-*Ceu*I technique.

to the *A. baumannii* recipient strain, except in 1 case (co-resistance to kanamycin and amikacin on a *bla*$_{OXA-23}$–carrying plasmid that originated from isolate 1190). Plasmids carrying the *bla*$_{OXA-23}$ gene in isolates Ab14, DOS, BEL, and 877 were not self-transferable (Table) (*24*).

Variability of Genetic Structures Flanking the *bla*$_{OXA-23}$ Gene

The 10 isolates that belonged to European clone II had a *bla*$_{OXA-23}$ gene that was part of Tn*2006*. The 9-bp direct repeat (DR) that corresponded to duplication of the Tn*2006* target site, which was consistent with a transposition event, was identified in the 9 ST22/ST2 isolates. Tn*2006* was inserted in different locations on the chromosomes of those isolates (Table). For isolates 240, 512, 810, 859, 883, and Aus, the insertion occurred between 2 genes encoding hypothetical proteins (DR: GTCATTTAA) (Figure 1). In isolate 761, transposon Tn*2006* was located between a gene encoding a hypothetical protein and a gene encoding an isoleucyl tRNA synthase (DR: ATTCGCGGG). In isolate 863, Tn*2006* was identified between a gene encoding a cytochrome D terminale oxidase and a putative transposase (DR: ATAATTATT). In isolate 585, Tn*2006* was located between a gene encoding a hypothetical protein and a *sul1* gene (DR: ATTCGCGGG). The plasmid-borne *bla*$_{OXA-23}$ gene identified in isolate Ab13 was also part of Tn*2006* but was inserted into the *sul* gene that encoded a putative sulfonamide resistance determinant (DR: ATTCGCGGG).

Isolates that belonged to European clone I had diverse genetic structures at the origin of *bla*$_{OXA-23}$ acquisition. Two isolates had transposon Tn*2006*: one on the chromosome (AS1) and 1 on a plasmid (910). Transposon Tn*2007* was identified in 3 isolates; it was specific for the same open reading frame in 2 isolates (BEL and Ab14) (Figure 2).



Isolates M	PFGE	European clone	MLST*
240	A	2	22/2
512	A	2	22/2
761	A	2	22/2
810	A	2	22/2
863	B	2	22/2
883	A	2	22/2
Ab13	A	2	22/2
AUS	A	2	22/2
859	A	2	22/2
585	A	2	53/2
614	C	1	25/20
AS3	C	1	25/20
1190	C	1	25/20
AS1	D	1	44/1
Ab14	D	1	44/1
910	E	1	New1/1
861	F	1	New1/1
BEL	G	1	New2/1
DOS	H	-	New3/New
877	J	-	New4/15

Figure 2. Pulsed-field electrophoresis (PFGE) profiles of *Apa*I-digested genomic DNA from strains of *Acinetobacter baumannii*. PFGE types, European clone types, and multilocus sequence typing (MLST) results are shown. *ST, sequence type determined by Bartual et al. (*19*) compared with ST determined by Nemec et al. (*20*). Lane M, molecular size markers (48.5 kb).

Only 1 copy of IS*Aba1* was identified upstream of the bla_{OXA-23} gene in isolates AS3, 1190, 861, and 877. Transposon Tn*2008* was identified only in isolate 614 (Figure 1). Sequences of these specific genetic structures have been deposited in Genbank (accession nos. EF127491, EF059914, GQ861438, and GQ861439).

Discussion

This study was conducted to define which features may explain the worldwide dissemination of the bla_{OXA-23} gene in *A. baumannii*. Isolates were from the Middle East, Europe, and Asia; there were no isolates from North America. Except for 2 isolates, the isolates investigated in this study belonged to European clones I or II. Clustering of *A. baumannii* isolates was determined by MLST and PFGE; our collection was composed of 13 PFGE types corresponding to 9 STs. Eight STs were identified among the OXA-23–producing *A. baumannii*; the most common STs were ST22/ST2 found in France (n = 2), Vietnam, New Caledonia, Thailand, Australia, Reunion, South Africa, and Tahiti. Spread of bla_{OXA-23}–positive *A. baumannii* isolates that belong to clone ST22 has been demonstrated in South Korea (*25*). Analysis of the target site of bla_{OXA-23} acquisition showed that in the same clone, such as ST22, acquisition of the Tn*2006* composite transposon had occurred at different positions in the *A. baumannii* genome, which suggested that Tn*2006*-mediated acquisition of bla_{OXA-23} may occur as independent events, or that Tn*2006* is a structure that is mobile in a given genome. A single clone could have different genetic structures at the origin of the bla_{OXA-23} acquisition.

We showed that the bla_{OXA-23} gene associated with Tn*2006* could be located on the chromosome or a plasmid. This result agrees with our recent findings, which showed that Tn*2006* is capable of transposition (*13*). We have also observed that 5 isolates with different sequence types (ST-New1, ST25) harbored a similar 130-kb plasmid. The same strains with the same genetic structure were identified in 8 countries in different parts of the world.

In conclusion, the current worldwide dissemination of the bla_{OXA-23} gene is driven by ≥7 MLST types associated with different genetic structures and plasmids. We have identified complex and dynamic spreading of bla_{OXA-23} that will be difficult to control because this spread is not associated with a single entity.

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Ms Mugnier is a doctoral student at Hôpital de Bicêtre, Institut National et de la Santé et de la Recherche Médicale, Unité, South-Paris Medical School, University Paris-XI in Le Kremlin-Bicêtre, France. Her research interests are genetic plasticity and drug-resistance mechanisms in *A. baumannii*.

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Address for correspondence: Patrice Nordmann, Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 Rue du Général Leclerc, 94275 Le Kremlin-Bicêtre, France; email: nordmann.patrice@bct.ap-hop-paris.fr

Recombinant Canine Coronaviruses in Dogs, Europe

Nicola Decaro, Viviana Mari, Gabriella Elia, Diane D. Addie, Michele Camero, Maria Stella Lucente, Vito Martella, and Canio Buonavoglia

Coronaviruses of potential recombinant origin with porcine transmissible gastroenteritis virus (TGEV), referred to as a new subtype (IIb) of canine coronavirus (CCoV), were recently identified in dogs in Europe. To assess the distribution of the TGEV-like CCoV subtype, during 2001–2008 we tested fecal samples from dogs with gastroenteritis. Of 1,172 samples, 493 (42.06%) were positive for CCoV. CCoV-II was found in 218 samples, and CCoV-I and CCoV-II genotypes were found in 182. Approximately 20% of the samples with CCoV-II had the TGEV-like subtype; detection rates varied according to geographic origin. The highest and lowest rates of prevalence for CCoV-II infection were found in samples from Hungary and Greece (96.87% and 3.45%, respectively). Sequence and phylogenetic analyses showed that the CCoV-IIb strains were related to prototype TGEV-like strains in the 5′ and the 3′ ends of the spike protein gene.

Coronaviruses (CoVs) (order Nidovirales, family *Coronaviridae*) are exceptionally prone to genetic evolution through accumulation of point mutations in genes encoding for structural and nonstructural proteins and homologous recombination among members of the same antigenic group (*1*). CoVs are organized by antigenic group. The first group is subdivided into subgroups 1a and 1b. Subgroup 1a includes highly related viruses (i.e., porcine transmissible gastroenteritis virus [TGEV] and its derivative porcine respiratory coronavirus [PRCoV], feline coronaviruses [FCoVs], and canine coronaviruses [CCoVs]) (*2*). According to a proposal by the Coronavirus Study Group of the International Committee of Taxonomy of Viruses, and given the virus' close genetic relatedness (i.e., >96% aa identity in the key replicase 1ab domains), TGEV, FCoV, and CCoV should not be considered separate viruses. Instead, they should be considered host range variants of the same species (*3*).

CCoVs exemplify the genetic evolution and complexity of CoVs. To date, 2 CCoV genotypes are known, CCoV-I and CCoV-II (*4*); they share up to 96% of nucleotide identity in the viral genome (Lorusso et al., unpub. data) but are highly divergent in the spike protein gene (*5*). In addition, CCoV-I displays a novel open reading frame (ORF3) that encodes for a putative glycosylated protein, which is likely secreted from the infected cells (*6*). The 2 CCoV genotypes are commonly detected in the feces of dogs with diarrhea and often simultaneously infect the same dog (*7*). Both CCoV genotypes have been associated with mild clinical signs in pups, although hypervirulent strains have been reported to cause severe, fatal enteritis (*8–10*); a pantropic variant was responsible for systemic disease in natural and experimental conditions (*11–13*).

It has been postulated that TGEV originated from CCoV-II through cross-species transmission, which is supported by the high genetic relatedness between the 2 viruses and by the presence of ORF3 remnants in CCoV-II and TGEV genomes (*6*). More recently, novel CCoV-II strains, which likely originated from a double recombination event with TGEV, occurring in the 5′ end of the spike protein gene, have been isolated (*14*,*15*). Accordingly, genotype II has been further divided into 2 subtypes, CCoV-IIa and CCoV-IIb, including extant and TGEV-like CCoVs, respectively (*14*).

To assess the distribution of the TGEV-like CCoVs in canine populations of different geographic areas of Europe, we used a subtype IIb-specific reverse transcription–PCR

Author affiliations: Faculty of Veterinary Medicine of Bari, Valenzano, Italy (N. Decaro, V. Mari, G. Elia, M. Camero, M.S. Lucente, V. Martella, C. Buonavoglia); and Feline Institute Pyrenees, Etchebar, France (D.D. Addie)

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(RT-PCR) assay. In addition, we evaluated the genetic relationship among the identified strains by using sequence and phylogenetic analyses.

Methods

Sample Origin

During 2001–2008, a total of 1,172 fecal samples were collected from dogs with acute enteritis in 14 European countries: Italy (n = 760), United Kingdom (n = 199), Greece (n = 81), Hungary (n = 41), Portugal (n = 33), Spain (n = 32), Belgium (n = 7), Romania (n = 6), Bulgaria (n = 3), Germany (n = 3), Sweden (n = 3), Slovakia (n = 2), Poland (n = 1), and Slovenia (n = 1). Some samples were retrieved from previous studies (*7*,*16*–*18*), whereas the remaining samples were sent to our laboratory (from veterinarians, breeders, or other researchers) for routine diagnostic investigations.

RNA Extraction

Specimens were homogenized (10% wt/vol) in Dulbecco modified Eagle medium and subsequently clarified by centrifuging at 2,500 × *g* for 10 min. For RNA extraction, 140 μL of the supernatants were then used by means of QIAamp Viral RNA Mini Kit (QIAGEN S.p.A., Milan, Italy); according to the manufacturer's protocol, RNA templates were stored at –70°C until use.

CCoV RNA Detection, Quantification, and Genotyping

For rapid detection and quantification of CCoV RNA, all RNA extracts were subjected to a previously established TaqMan-based real-time RT-PCR (*16*) with minor modifications. Briefly, a 1-step method was adopted by using the Platinum Quantitative PCR ThermoScript One-Step System (Invitrogen S.R.L., Milan, Italy) and the following 50-μL mixture: 25 μL of master mix, 300 nM of primers CCoV-forward (5′-TTGATCGTTTTTATAACGGTTCTACAA-3′) and CCoV-reverse (5′-AATGGGCCATAATAGCCACATAAT-3′), 200 nM of probe CCoV-Pb (5′-FAM-ACCTCAATTTAGCTGGTTCGTGTATGGCATT-BHQ1-3′), and 10 μL of template RNA. To obtain a standard curve for absolute quantification, we simultaneously analyzed duplicates of $\log_{10}$ dilutions of standard RNA (*16*). The thermal profile consisted of reverse transcription at 50°C for 20 min, activation of Platinum Taq DNA polymerase at 95°C for 2 min, 45 cycles of denaturation at 95°C for 15 s, annealing at 48°C for 30 s, and extension at 60°C for 30 s.

The positive samples were characterized by 2 distinct genotype-specific assays (*17*) performed by using the Platinum Quantitative PCR ThermoScript One-Step System (Invitrogen S.R.L.) and the following oligonucleotide sets (final concentrations were 600 and 200 nM for primers and probes, respectively): primer pair CCoVI-F (5′-CGTTAGTGCACTTGGAAGAAGCT-3′)/CCoVI-R (5′-ACCAGCCATTTTAAATCCTTCA-3′) and probe CCoVI-Pb (5′-FAM-CCTCTTGAAGGTACACCAA-TAMRA-3′) for CCoV-I; primer pair CCoVII-F (5′-TAGTGCATTAGGAAGAAGCT-3′)/CCoVII-R (5′-AGCAATTTTGAACCCTTC-3′) and probe CCoVII-Pb (5′-FAM-CCTCTTGAAGGTGTGCC-TAMRA-3′) for CCoV-II. The thermal protocol was as described for CCoV detection except for different annealing temperatures (i.e., 53°C and 48°C for CCoV-I and CCoV-II, respectively).

Development of RT-PCRs Specific for Classical and TGEV-like CCoVs

Considering the high divergence observed in the 5′ end of the spike gene between classical (subtype IIa) and TGEV-like (subtype IIb) CCoVs, specific CCoV-IIa and CCoV-IIb gel-based RT-PCRs were developed. Primer 20179 (sense, 5′-GGCTCTATCACATAACTCAGTCCTAG-3′) binds a conserved region at the 3′ end of ORF1b and was recruited from a previous study (*13*), whereas antisense primers INS-R-dg (5′-GCTGTAACATAKTCRTCATTCCAC-3′) and 174-268 (5′-CAACATGTAACCTTTGTCTGTGATCTGC-3′) target regions at the 5′ end of the spike protein gene of feline CoV-II (FCoV-II)/classical CCoV-II and TGEV/TGEV-like CCoV, respectively. Separate RT-PCRs with primers 20179/INS-R (CCoV-IIa) or 20179/174-268 (CCoV-IIb) were conducted by using SuperScript One-Step RT-PCR for Long Templates (Invitrogen S.R.L.), according to the manufacturer's instructions. The following thermal protocol was used: reverse transcription at 50°C for 30 min, inactivation of Superscript II RT at 94°C for 2 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, 68°C for 30 s, and final extension at 68°C for 10 min. The PCR products were detected by using electrophoresis through a 1.5% agarose gel and examination under UV light after ethidium bromide staining.

RT-PCR for Amplification of the 3′ End of the Spike Protein Gene of CCoV-II

To rule out any potential infection by true TGEV strains and to confirm the recombinant origin of the TGEV-like CCoVs, we submitted 20 samples that were positive for CCoV-IIb and negative for CCoV-I to RT-PCR amplification of the 3′ end of the spike protein gene of CCoV-II (*7*). Primers S5 (5′-TGCATTTGTGTCTCAGACTT-3′) and S6 (5′-CCAAGGCCATTTTACATAAG-3′) were used in the RT-PCR, performed according to the protocol described for CCoV subtyping.

RT-PCR of the ORF7a/7b Region

To rule out the presence of true TGEV strains in the dog feces that were positive by CCoV-IIb–specif-

ic assay, we used an RT-PCR that had been proven to discriminate between TGEV and CCoV on the basis of amplicon size (*19*). In fact, primers N3SN (5′-GTGTTTGATGACACACAGGTTGAG-3′) and R3AS (5′-GCTTACCATTCTGTACAAGAGGTAG-3′) target the 3′ end of the viral genome, where CCoV/FCoV and TGEV display 2 (ORFs 7a and 7b) and 1 (ORF7) accessory genes, respectively. As controls, the following reference group-1a CoVs were used: TGEV-Purdue (kindly provided by P. Cordioli, Istituto Zooprofilattico Sperimentale di Lombardia ed Emilia Romagna, Brescia, Italy), FCoV-I-249/04 (*20*), FCoV-II-29/92 (*21*), CCoV-I-Elmo/02 (*5*), CCoV-IIa-CB/05 (*11*), CCoV-IIb-341/05, CCoV-IIb-174/06, CCoV-IIb-430/07, and CCoV-IIb-119/08 (*14*).

Sequence and Phylogenic Analyses

The RT-PCR products obtained with primer pairs 20179/174-268 and S5/S6 from 26 samples having positive CCoV-IIb–specific assay results and representative of the different geographic areas were subjected to direct sequencing at the BaseClear B.V. (Leiden, the Netherlands). The sequences were manually edited and analyzed by using BioEdit software (*22*) and National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and European Molecular Biology Laboratory (www.ebi.ac.uk) analysis tools. Phylogenetic and molecular evolutionary analyses were conducted by using Mega 4.1, beta (*23*). Phylogenetic trees on the basis of partial 5′ (339-nt) and 3′ (520-nt) ends of the spike protein gene were elaborated by using parsimony and neighbor-joining methods, which supplied statistical support with bootstrapping >1,000 replicates. Group-2 CoV canine respiratory CoV-240/05 (*24*) was used as an outgroup. The nucleotide sequences of the analyzed CCoV-IIb strains were deposited in GenBank under accession nos. GQ130243–GQ130268 and GQ148749–GQ148774 for 5′ and 3′ ends of the spike gene, respectively.

Results

CCoV Detection and Quantification

CCoV RNA was detected in 493 (42.06%) of 1,172 fecal samples from dogs with diarrhea. Viral RNA titers ranged from 1.25×10^1 to 7.56×10^7 copies/μL of template. For each geographic region of origin, detection rates of the CCoV real-time RT-PCR were Italy 330/760 (43.42%), United Kingdom 54/199 (27.13%), Greece 45/81 (55.5%), Hungary 32/42 (78.05%), Portugal 12/33 (36.36%), Spain 2/32 (6.25%), Belgium 4/7 (57.14), Romania 4/6 (66.66%), Bulgaria 1/3 (33.33%), Germany 3/3 (100%), Sweden 3/3 (100%), Slovakia 1/2 (50%), Poland 1/1 (100%), and Slovenia 1/1 (100%) (Table 1).

CCoV Genotype and Subtype Distribution

The geographic distribution of the CCoV types and subtypes is reported in Table 1. Genotype-specific amplification assays showed that 93 (18.86%) of 493 CCoV-positive samples were positive for CCoV-I, and 218 (44.22%) were positive for CCoV-II. In addition, 182 samples (36.92%) were positive for both genotypes. Both genotypes were found to circulate in most European countries that had been sampled, and an overall prevalence of CCoV-II was found in all countries except Greece, where the 2 genotypes were detected approximately to the same extent.

By using the developed TGEV-like RT-PCR, we found that 78 (19.50%) of 400 samples containing CCoV-II strains, alone or in combination with CCoV-I, were positive for CCoV-IIb. The remaining 322 CCoV-II strains were positive for subtype IIa, whereas mixed infections

Table 1. Distribution of CCoV genotypes and TGEV-like strains (CCoV-IIb) in dogs in European countries, 2001–2008*

Country	No. samples	No. or % positive					
		CCoV, no.	CCoV-I no.†	CCoV-II, no.†	CCoV-I+II, no.‡	CCoV-IIb, no.	CCoV-IIb/II, %
Italy	760	330	54	138	138	34	12.32
United Kingdom	199	54	13	24	17	10	24.39
Greece	81	45	16	15	14	1	3.45
Hungary	41	32	0	30	2	31	96.87
Portugal	33	12	6	3	3	0	0
Spain	32	2	0	1	1	0	0
Belgium	7	4	1	3	0	0	0
Romania	6	4	1	1	2	1	33.33
Bulgaria	3	1	0	1	0	0	0
Germany	3	3	0	0	3	0	0
Sweden	3	3	1	1	1	1	50
Slovakia	2	1	0	0	1	0	0
Poland	1	1	0	1	0	0	0
Slovenia	1	1	1	0	0	0	0
Total	1,172	493	93	218	182	78	19.50

*CCoV, canine coronavirus; TGEV, porcine transmissible gastroenteritis virus.
†Infections with a single CCoV genotype.
‡Infections with both CCoV genotypes.

caused by both CCoV-II subtypes were not detected in any samples. Almost all CCoV–IIb-positive samples were from Italy (34/276; 12.32%), the United Kingdom (10/41; 24.39%), and Hungary (31/32; 96.87%). Single CCoV-IIb strains were detected in samples from Greece (1/29; 3.45%), Romania (1/3; 33.33%), and Sweden (1/2; 50%), whereas no samples from the other countries had TGEV-like CCoVs.

RT-PCR of ORF7a/7b Region

After RT-PCR with primer pair N3SN/R3AS, CCoV and FCoV reference strains yielded an amplicon >1,000 bp, with the exception of TGEV-like CCoV 341/05, which gave a 929-bp product as a consequence of a 154-nt deletion in ORF7b (*14*). In contrast, a 367-bp product was obtained from TGEV-Purdue, as previously described (*19*). This pattern of amplification agreed with the absence of ORF7b in TGEV (*25*). All 20 samples that were positive according to the CCoV-IIb–specific assay and negative according to the CCoV-I TaqMan assay were confirmed to contain true CCoV strains because they yielded RT-PCR products considerably larger than the 367-bp amplicon obtained from TGEV.

Sequence and Phylogenetic Analyses

We selected the following TGEV-like strains for sequence analysis of the 5′ and 3′ ends of the spike protein gene: 12 strains from Italy, 8 from the United Kingdom, and 5 from Hungary, plus the single strains from Greece, Romania, and Sweden. All RT-PCR products were sequenced except those obtained from the samples from Greece and Sweden, which yielded weak bands despite the considerable viral RNA titers in the original fecal samples (5.02×10^4 and 5.76×10^6 RNA copies/μL of template, respectively).

The obtained sequences were compared with each other (Table 2) and with 3 CCoV-IIa, 4 CCoV-IIb, 2 FCoV-II, 2 CCoV-I, 3 FCoV-I, and 3 TGEV reference sequences (Table 3). Sequence comparison of the TGEV-like strains showed overall nucleotide identity of 83.6%–99.6% and 92.7%–100% in the 5′ and 3′ ends of the spike gene, respectively. By analyzing the TGEV-like strains by country of origin, we found the highest genetic variability among the viruses from Italy (86.8%–99.3% and 94.5%–99.8% of nucleotide identity in the gene 5′ and 3′ ends, respectively), whereas the strains from Hungary showed the highest relatedness (92.7%–99.3% and 98.4%–99.6% of nucleotide identity in the gene 5′ and 3′ ends, respectively) (Table 2). The TGEV-like strains exhibited the best identity to prototype strains (*14*) in both the 5′ (85.9%–99.3%) and the 3′ end (92.7%–99.8%) of the spike gene, whereas a slightly lower identity was found to the old strain UCD1 (*19*) in the 5′ end, which is the only sequence available in the GenBank database. When the 5′ end was analyzed, the identified TGEV-like CCoVs were more related to classical TGEVs

Table 3. Percent nucleotide identity of representative TGEV-like CCoVs to group-1a CoV reference strains in the 5′ and 3′ ends of the spike gene from dogs from Europe, 2001–2008*

CoV strain	GenBank accession no.	% Identity	
		5′ end†	3′ end‡
CCoV-IIb-341/05	EU856361	88.1–99.3	94.7–99.4
CCoV-IIb-174/06	EU856362	86.8–99.0	93.3–97.0
CCoV-IIb-430/07	EU924790	85.9–97.3	92.7–97.0
CCoV-IIb-119/08	EU924791	89.1–99.0	93.7–99.8
CCoV-IIb-UCD1	AF116248	76.6–85.7	NA
TGEV-Purdue	NC_002306	76.6–84.4	91.4–93.1
TGEV-TS	DQ201447	76.2–84.4	90.8–92.5
TGEV-96-1933	AF104420	74.0–81.8	87.1–88.8
PRCoV-RM4	Z24675	NA	90.2–91.6
PRCoV-86-137004	X60089	NA	90.2–91.6
CCoV-IIa-Insavc-1	D13096	35.4–38.3	92.1–95.3
CCoV-IIa-CB/05	DQ112226	35.1–37.7	93.7–99.0
CCoV-IIa-BGF10	AY342160	34.2–37.5	90.8–94.3
FCoV-II-79-1146	NC_007025	34.0–36.6	90.2–91.6
FCoV-II-79-1683	X80799	34.6–37.5	90.4–91.9
CCoV-I-Elmo/02	AY307020	37.6–40.4	61.8–62.8
CCoV-I-23/03	AY307021	31.8–34.8	60.3–61.2
FCoV-I-KU-2	D32044	37.4–40.8	59.1–60.3
FCoV-I-Black	EU186072	36.2–39.3	58.9–61.0
FCoV-I-UCD1	AB088222	38.3–42.7	57.9–58.9

*TGEV, porcine transmissible gastroenteritis virus; CCoV, canine coronavirus; NA, sequence not available.
†Nucleotides 1–305 of the spike gene of reference strain CCoV-IIb-341/05.
‡Nucleotides 3574–4085 of the spike gene of reference strain CCoV-IIb-341/05.

Table 2. Percent nucleotide identities in the 5′ (top right) and 3′ (bottom left) ends of the spike gene of CCoV-IIb (TGEV-like) strains from dogs in Europe, 2001–2008*

Country	Italy	United Kingdom	Hungary	Romania	Overall
Italy	86.8–99.3 94.5–99.8	83.6–92.7	86.5–99.0	89.1–99.3	83.6–99.3
United Kingdom	92.7–97.0	88.5–99.6 97.6–99.8	87.8–97.0	87.8–93.1	83.6–99.6
Hungary	94.7–100	92.9–94.9	92.7–99.3 98.4–99.6	94.7–99.3	86.5–99.3
Romania	95.3–99.6	93.5–94.5	98.6–99.6	ND	87.8–99.3
Overall	92.7–100	92.7–99.8	92.9–100	93.5–99.6	83.6–99.6 92.7–100

*CCoV, canine coronavirus; TGEV, porcine transmissible gastroenteritis virus; ND, not done because only a single strain from Romania was analyzed.

(76.6%–84.4%) than to type IIa CCoVs (34.2%–38.3%). In contrast, analysis of the 3′ end of the spike gene showed nucleotide identities of 87.1%–93.1% to TGEV and of 90.8%–99.0% to CCoV-IIa. The best identities among CCoV-IIa isolates were to strain Insavc-1, which has been proposed as intermediate virus between CCoV and TGEV (*26*) and to the more recent pantropic strain CB/05 (*11*) in the 5′ and 3′ ends, respectively.

At the phylogenetic level, the sequenced strains were grouped in the same cluster with TGEV and prototype CCoV-IIb strains at the 5′ end of the spike protein gene, displaying an obvious distance to both CCoV-IIa/FCoV-II and CCoV-I/FCoV-I (Figure, panel A). The TGEV-like CCoVs from the United Kingdom formed a unique clade, whereas the strains detected in eastern Europe were mixed with CCoV-IIb viruses from Italy. The old TGEV-like

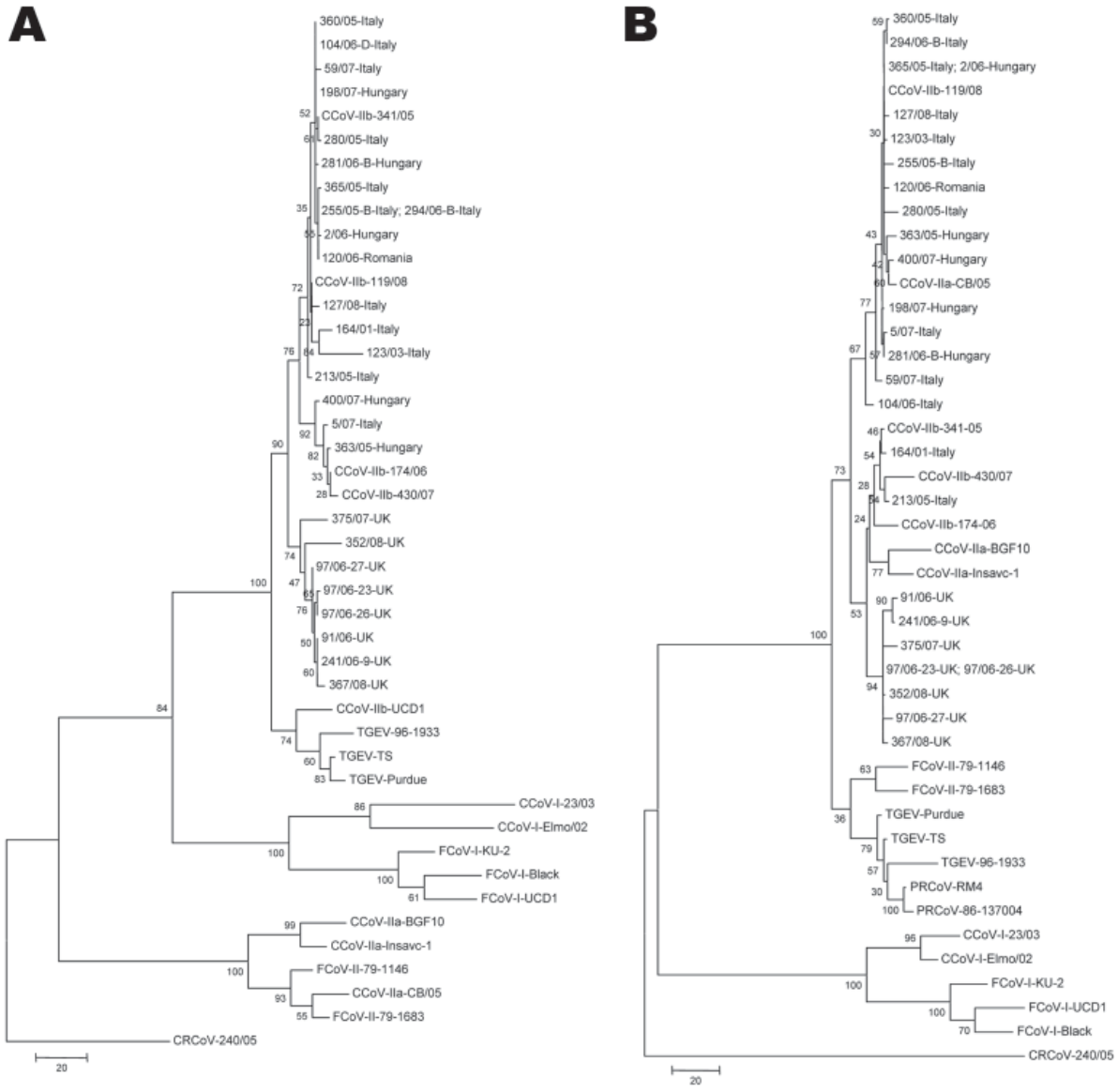

Figure. Phylogenetic analysis of canine coronavirus (CCoV) type IIb. Maximum parsimony trees based on partial 5′ (A) and 3′ (B) ends of the spike protein gene of group-1a coronaviruses (CoVs). For phylogenetic tree construction, the following reference strains were used (GenBank accession numbers are in parentheses): porcine transmissible gastroenteritis virus (TGEV) Purdue (NC_002306), TS (DQ201447), 96-1933 (AF104420), porcine respiratory coronavirus (PRCoV, only 3′ end) RM-4 (Z24675), 86-137004 (X60089), CCoV-IIa CB/05 (DQ112226), Insavc-1 (D13096), BGF10 (AY342160), CCoV-IIb 341/05 (EU856361), 174/06 (EU856362), 430/07 (EU924790), 118/08 (EU924791), UCD-1 (AF116248, only 5′ end), CCoV-I Elmo/02 (AY307020), 23/03 (AY307021), feline coronavirus (FCoV) type I Black (EU186072), KU-1 (D32044), UCD-1 (AB088222), FCoV-II 79-1146 (NC_007025), 79-1183 (X80799). The tree is rooted on the group-2 CoV canine respiratory coronavirus (CRCoV) 240/05 (EU999954). Statistical support was provided by bootstrapping >1,000 replicates. Scale bars indicate estimated numbers of nucleotide substitutions per site.

strain, UCD1, clustered with TGEV isolates. At the 3′ end of the same gene, subtype IIa and IIb strains segregated together and were separated from the FCoVs and the porcine CoVs TGEV and PRCoV (Figure, panel B). In addition, the strains from the United Kingdom were again grouped in a separate subcluster.

Discussion

CoVs are exceptionally prone to variability through accumulation of point mutations and recombination events. A CCoV strain displaying close relatedness to porcine CoVs in the N-terminus of the spike protein, which is related to porcine CoVs, was isolated ≈20 years ago, but the subsequent molecular characterization was restricted to the 5′ end of the spike gene (*19*). Additional TGEV-like CCoVs were reported more recently in Italy (*14*) and the United Kingdom (*15*). However, full biological and molecular characterization was carried out only for the isolates from Italy, showing that the TGEV-like strains are likely recombinant with TGEV at the level of the 5′ end of the spike gene (*14*). Experimental infection of CCoV-seronegative beagle pups showed that TGEV-like (i.e., subtype IIb) CCoV induces clinical signs resembling those of classical (i.e., subtype IIa) CCoVs, that is, mild diarrhea for a few days. (*14*) Unlike pantropic CCoV (*11–13*), TGEV-like CCoV was not able to spread systemically. Of the 4 recombinant strains detected in Italy, 2 had originated from eastern Europe, but at the phylogenetic level they were mixed with strains from Italy. The prevalence of this CCoV subtype in the canine population has not been determined in previous studies.

Our epidemiologic investigation assessed the distribution of CCoV-IIb in the dog population of Europe. Approximately 50% of the analyzed samples were positive for CCoV, showing the presence of CCoV-I or CCoV-II. Mixed infections caused by both genotypes were detected in <40% of the CCoV-positive samples, considerably lower than previously reported percentages (*7*,*17*). Approximately 20% of the CCoV–II-positive samples contained TGEV-like strains (Table 1). However, the prevalence of this CCoV subtype differed by geographic origin of the samples; the highest detection rates (96.87%) were observed in Hungary. The recombinant origin of all strains characterized by RT-PCR was confirmed by sequence analysis of 5′ and 3′ ends of the spike gene and by RT-PCR of the ORF7a/7b region. The selected 26 TGEV-like strains were related to prototype strains from Italy (*14*) in both the 5′ and the 3′ ends of the spike gene. A comparison with the prototype UK strains reported by Erles and Brownlie (*15*) was not possible, however, because the unique spike sequence deposited in the GenBank is located more downstream of the gene with respect to the sequences that we obtained.

On the basis of the spike gene sequences, the strains from Italy and eastern Europe were closely related, whereas the strains from the United Kingdom were more genetically distant (Table 3). This pattern of segregation was confirmed by phylogenic analysis, which showed that viruses detected in the United Kingdom formed a separate cluster with respect to the samples from Italy, Hungary, and Romania (Figure). The genetic relatedness between the TGEV-like strains from Italy and those from eastern Europe may be accounted for by extensive dog importation to Italy. In addition, dog exchange between eastern Europe and Italy has been associated with the reemergence of canine infectious hepatitis (*27*) and the spread of the arctic lineage of canine distemper virus (*28*).

In the 5′ end of the spike gene, the old TGEV-like strain, UCD1, was found to be genetically more related to true TGEV isolates than to recent CCoV-IIb strains, thereby accounting for recombination events occurring at different times. With the exception of strain UCD1, analysis of archival samples found the oldest TGEV-like strain in 2001, about 4 years before this CCoV subtype was reported in Italy (*14*).

Our study confirms that recombinant CCoVs are effectively circulating in dogs in different European countries. Considering the genetic distance in the spike protein, this circulation questions the efficacy of vaccines, which are based on classical (CCoV-IIa) strains, against the emerging TGEV-like (CCoV-IIb) viruses. Only vaccination trials and subsequent challenges by TGEV-like strains might assess whether the poor cross-reactivity between CCoV-IIa and CCoV-IIb observed in a previous study (*14*) might affect the immune response of dogs against the recombinant viruses.

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Dr Decaro is an associate professor at the Faculty of Veterinary Medicine of Bari, Italy. His research interests include viral pathogens in dogs, with particular emphasis on canine coronavirus and parvovirus.

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Address for correspondence: Nicola Decaro, Department of Veterinary Public Health, Faculty of Veterinary Medicine of Bari, Strada per Casamassima km 3, 70010 Valenzano, Bari, Italy; e-mail: n.decaro@veterinaria.uniba.it

Ceftiofur Resistance in *Salmonella enterica* Serovar Heidelberg from Chicken Meat and Humans, Canada

Lucie Dutil, Rebecca Irwin, Rita Finley, Lai King Ng, Brent Avery, Patrick Boerlin, Anne-Marie Bourgault, Linda Cole, Danielle Daignault, Andrea Desruisseau, Walter Demczuk, Linda Hoang, Greg B. Horsman, Johanne Ismail, Frances Jamieson, Anne Maki, Ana Pacagnella, and Dylan R. Pillai

The Canadian Integrated Program for Antimicrobial Resistance Surveillance describes a strong correlation (r = 0.9, p<0.0001) between ceftiofur-resistant *Salmonella enterica* serovar Heidelberg isolated from retail chicken and incidence of ceftiofur-resistant *Salmonella* serovar Heidelberg infections in humans across Canada. In Québec, changes of ceftiofur resistance in chicken *Salmonella* Heidelberg and *Escherichia coli* isolates appear related to changing levels of ceftiofur use in hatcheries during the study period, from highest to lowest levels before and after a voluntary withdrawal, to increasing levels after reintroduction of use (62% to 7% to 20%, and 34% to 6% to 19%, respectively). These events provide evidence that ceftiofur use in chickens results in extended-spectrum cephalosporin resistance in bacteria from chicken and humans. To ensure the continued effectiveness of extended-spectrum cephalosporins for treating serious infections in humans, multidisciplinary efforts are needed to scrutinize and, where appropriate, limit use of ceftiofur in chicken production in Canada.

Author affiliations: Public Health Agency of Canada, Saint-Hyacinthe, Québec, Canada (L. Dutil, D. Daignault); Public Health Agency of Canada, Guelph, Ontario, Canada (R. Irwin, R. Finley, B. Avery, L. Cole, A. Desruisseau); Public Health Agency of Canada, Winnipeg, Manitoba, Canada (L.K. Ng, W. Demczuk); Ontario Veterinary College, Guelph (P. Boerlin); Institut National de Santé Publique du Québec, Sainte-Anne-de-Bellevue, Québec, Canada (A.-M. Bourgault, J. Ismail); British Columbia Centre for Disease Control, Vancouver, British Columbia, Canada (L. Hoang, A. Pacagnella); Saskatchewan Disease Control Laboratory, Regina, Saskatchewan, Canada (G. Horsman); and Ontario Agency for Health Protection and Promotion, Toronto, Ontario, Canada (F. Jamieson, A. Maki, D.R. Pillai)

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Salmonella enterica serovar Heidelberg ranks among the top 3 serovars isolated from persons infected with *Salmonella* in Canada (*1*). It is more frequently reported in North America than in other regions of the world (*2*). Although many *Salmonella* Heidelberg infections result in mild to moderate illness, the bacterium also causes severe illness with complications such as septicemia, myocarditis, extraintestinal infections, and death (3,*4*). *Salmonella* Heidelberg appears more invasive than other gastroenteritis-causing serovars; ≈9% of isolates of this serovar received through the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) during 2003–2005 were recovered from blood samples (*5*). Treatment with antimicrobial agents may be life-saving in the case of invasive infections.

Sources of human *Salmonella* Heidelberg infection include consumption of poultry or eggs and egg-containing products (*6–10*). In Canada, *Salmonella* Heidelberg is commonly isolated from healthy chickens from farm, abattoir, and retail sources (*11*,*12*). It also has been isolated, although less frequently, from ground beef, pork, and turkey meat (*13–15*) and from clinical samples from various animal species (*12*).

Ceftiofur is an extended-spectrum cephalosporin drug approved in Canada for use with numerous label indications in cattle, swine, horses, sheep, turkeys, dogs, and cats. Ceftiofur is also injected in ovo to control *Escherichia coli* omphalitis in broiler chickens; this use is not an approved label indication.

A major public health concern is that use of third-generation cephalosporins, such as ceftiofur, in food animals is leading to resistance to other extended-spectrum cepha-

losporins, such as ceftriaxone and cephamycins (*16*–*20*), a group of antimicrobial agents used to treat a wide variety of human infections. Among other indications, ceftriaxone is the drug of choice for treating severe or invasive salmonellosis in children and pregnant women (*16*,*17*) where fluoroquinolones are not approved and treatment options are limited. Accordingly, third-generation cephalosporins have been classified as Critically Important Antimicrobials in Human Medicine by the World Health Organization (*21*) and as Class 1 Very High Importance in Human Medicine by the Canadian Veterinary Drugs Directorate, Health Canada (*22*).

In Canada, ceftiofur resistance in bacteria from healthy animals or food is mainly reported in chicken *Salmonella* Heidelberg isolates originating from farm, abattoir, and retail samples and in chicken abattoir and retail generic *E. coli* isolates (*11*,*12*). It also is occasionally reported in *Salmonella* isolates from sick animals or in bovine and porcine abattoir or retail *E. coli* isolates but at much lower frequency (*12*).

The objective of this study is to highlight the correlation between ceftiofur-resistant *Salmonella* Heidelberg isolated from retail chicken and the incidence of ceftiofur-resistant *Salmonella* Heidelberg infections in humans across Canada. Public health concerns raised by publication of the CIPARS 2003 annual report, specifically the higher rates of ceftiofur resistance in *Salmonella* Heidelberg isolates from chicken meat than from humans, prompted Québec broiler chicken hatcheries to voluntarily interrupt the extralabel in ovo use of ceftiofur during 2005–2006 (*23*). This study therefore also describes variations in ceftiofur resistance among chicken and human *Salmonella* Heidelberg and chicken *E. coli* strains in that province before, during, and after the voluntary withdrawal.

Materials and Methods

CIPARS is a national program led by the Public Health Agency of Canada (PHAC) dedicated to the preservation of effective antimicrobial drugs for humans and animals through the collection, integration, analysis, and communication of trends in antimicrobial resistance in selected bacterial organisms. Data presented here were collected during 2003–2008 from CIPARS' surveillance of human clinical *Salmonella* isolates and *E. coli* and *Salmonella* isolates from retail chicken. Detailed methods for sample collection, bacterial isolation, antimicrobial resistance testing, and data analysis are described in CIPARS's reports (*12*).

Sample Collection

Human *Salmonella* Isolates

Hospital-based and private clinical laboratories isolated and forwarded human *Salmonella* isolates to their Provincial Public Health Laboratory (PPHL). PPHLs forwarded *Salmonella* isolates to the Enteric Diseases Program, National Microbiology Laboratory (NML), PHAC, for phage type characterization and antimicrobial resistance testing. All isolates (outbreak and nonoutbreak) received passively by the Saskatchewan PPHL were forwarded; the more populated provinces (British Columbia, Ontario, and Québec) forwarded isolates received from days 1–15 of each month. Only 1 isolate per patient was kept for the analysis.

Retail Meat Samples

To use a similar geographic scale as CIPARS surveillance of human clinical *Salmonella* isolates and because we expected a certain level of provincial clustering in food distribution, we designed the study of CIPARS retail surveillance to provide a representative measurement of what consumers from each province were exposed to through ingestion of improperly cooked raw meat or cross-contamination. Randomization and weighted allocation of samples according to demography of the human population ensured that the data generated through retail sampling were representative and reliable at the provincial level. Retail raw chicken samples (most often chicken thigh with skin on) were collected as part of CIPARS retail program that purchases samples weekly (Ontario and Québec) or biweekly (Saskatchewan, British Columbia) from chain, independent, and butcher stores in 15–18 randomly selected census divisions in each participating province. Retail surveillance was initiated in Ontario and Québec in mid-2003 and at the beginning of 2005 in Saskatchewan. Surveillance also was conducted during part of 2007 and all of 2008 in British Columbia.

Microbiologic Analysis

Recovery of Isolates from Retail Chicken Meat

Primary isolations of *E. coli* and *Salmonella* spp. were conducted at the Laboratory for Foodborne Zoonoses, PHAC. Every retail chicken meat sample received was cultivated for *Salmonella*, but only 1 of every 2 samples was systematically selected to be tested for generic *E. coli* isolation. Incubated peptone rinses of chicken meat samples were streaked on eosin-methylene blue agar (Becton Dickinson, Sparks, MD, USA). Presumptive *E. coli* colonies were identified by using the Simmons' citrate and indole tests. Colonies showing negative indole results were identified by using the API 20E (bioMérieux Clinical Diagnostics, Marcy l'Étoile, France). All chicken samples were tested for *Salmonella* with a modified MFLP-75 method of the Compendium of Analytical Methods (*24*). Incubated peptone rinses were injected into modified semisolid Rappaport-Vassiliadis media. Presumptive *E. coli* colonies were injected into triple sugar iron and urea agar slants

and subjected to the indole test. Presumptive *Salmonella* isolates were verified by slide agglutination using PolyA-I and Vi *Salmonella* antiserum (Difco, Becton Dickinson). *Salmonella* isolates were shipped between laboratories on a tryptic soy agar slant by priority courier. No selective media were used to isolate ceftiofur-resistant bacteria.

Serotyping, Phage Typing, and Susceptibility Testing

Human and chicken *Salmonella* isolates were serotyped and phage typed by using published methods (*25*–*28*). MICs were determined by the NML (human isolates) and the Laboratory for Foodborne Zoonoses, PHAC (chicken isolates) by the broth microdilution method (Sensititre Automated Microbiology System; Trek Diagnostic Systems Ltd., Westlake, OH, USA). *Salmonella* and *E. coli* isolates were tested by using the National Antimicrobial Resistance Monitoring System custom susceptibility plate for gram-negative bacteria. The breakpoint used to determine ceftiofur resistance was >4 μg/mL (*29*).

Data Analysis

We analyzed data using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). The yearly proportion of retail chicken samples contaminated with ceftiofur-resistant *Salmonella* Heidelberg (or *E. coli*) and the incidence rate of human infection with ceftiofur-resistant *Salmonella* Heidelberg was calculated as described in CIPARS 2006 annual report (*12*). The Pearson product-moment correlation was used to verify the correlation between ceftiofur-resistant *Salmonella* Heidelberg isolated from retail chicken and human incidence estimates by using the Pearson option in the PROC CORR procedure in SAS. We computed the overall correlation coefficient using data across all provinces under study and computed a specific coefficient for provinces with >5 observations (*30*)

To describe ceftiofur resistance changes by quarter and reduce the noise around the estimate caused by the small number of observations per quarter, we computed a nonweighted rolling average of the prevalence of ceftiofur resistance using data from the current quarter and the previous 2 quarters for chicken *E. coli*, chicken *Salmonella* Heidelberg, and human *Salmonella* Heidelberg isolates from the province of Québec. We tested differences in ceftiofur resistance between years with SAS using χ^2 or Fisher exact tests when appropriate.

Results

Ceftiofur-Resistant *Salmonella* Heidelberg Isolated from Retail Chickens and from Humans

Across Canada, the annual percentage of chicken samples contaminated with ceftiofur-resistant *Salmonella* Heidelberg correlated strongly with the annual incidence of human cases related to this type of isolate (r = 0.91, p<0.0001) (Figure 1). This strongly significant correlation held across time and within different Canadian provinces (Ontario, r = 0.93, p<0.01; Québec, r = 0.89, p = 0.01).

Changes in ceftiofur resistance alone did not explain a number of the temporal changes in exposure (*12*). For example, in Ontario, the decrease in the prevalence of retail chicken contaminated with ceftiofur-resistant *Salmonella* Heidelberg isolates during 2004–2008 (Figure 1) was linked to a decrease in ceftiofur resistance from 58% to 14% (Table) and a decrease in the prevalence of *Salmonella* Heidelberg in chicken from 61% to 15% of all *Salmonella* isolates. In Québec, the decrease in contamination of chicken with ceftiofur-resistant *Salmonella* Heidelberg strains from 2003 to 2004 (Figure 1) was related mainly to a decrease in the prevalence of *Salmonella* Heidelberg (from 71% to 48%) in chicken, whereas the decrease from 2004 to 2006 was attributable mainly to a drop in ceftiofur resistance from 62% to 7% (Table). In British Columbia, the low level of chicken contamination with ceftiofur-resistant *Salmonella* Heidelberg strains resulted mainly from the rarity of *Salmonella* Heidelberg (only 11% of all *Salmonella* in 2007–2008), and low exposure levels in Saskatchewan were related mainly to low ceftiofur resistance among *Salmonella* Heidelberg (Table).

Ceftiofur-Resistant *E. coli* Isolated from Retail Chicken

Retail chicken generally was more frequently contaminated with ceftiofur-resistant commensal *E. coli* than with ceftiofur-resistant *Salmonella* Heidelberg isolates (Figure 1). The proportion of chicken contaminated with ceftiofur-resistant *E. coli* (Figure 1) closely followed changes in ceftiofur resistance (Table) because commensal *E. coli* was recovered from almost all (89%–100%) chicken samples

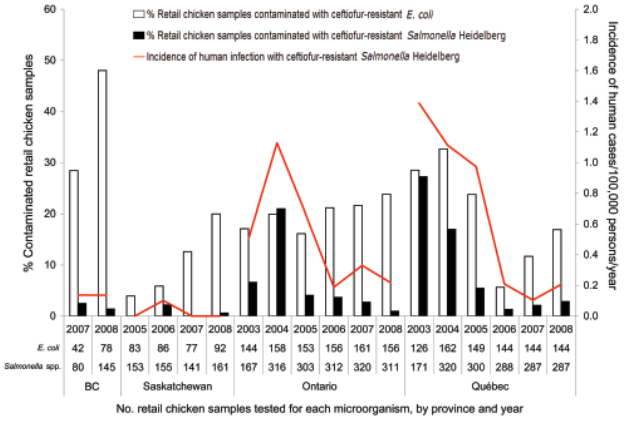

Figure 1. Prevalence of retail chicken contaminated with ceftiofur-resistant *Escherichia coli* and *Salmonella enterica* serovar Heidelberg and incidence of human infections from ceftiofur-resistant *Salmonella* Heidelberg in Canada.

Table. Prevalence of ceftiofur resistance among human and retail chicken *Salmonella* serovar Heidelberg isolates and retail chicken *Escherichia coli* isolates from Canadian provinces surveyed during 2003–2008

Isolate/province	Prevalence of ceftiofur resistance, % (no. resistant isolates/total no. isolates tested)					
	2003	2004	2005	2006	2007	2008
Human clinical *Salmonella* Heidelberg						
Québec	31 (52/167)	36 (42/116)	35 (37/106)	8 (8/96)	6 (4/63)	12 (8/65)
Ontario	18 (31/172)	38 (70/185)	30 (42/140)	10 (12/122)	22 (21/94)	32 (7/22)
Saskatchewan			0 (0/15)	7 (1/14)	0 (0/11)	0 (0/7)
British Columbia					23 (3/13)	19 (3/16)
Chicken retail *Salmonella* Heidelberg						
Québec	65 (13/20)	62 (18/29)	33 (4/12)	7 (1/14)	19 (6/32)	18 (7/38)
Ontario	16 (3/19)	58 (19/33)	27 (3/11)	21 (3/14)	21 (9/42)	14 (3/21)
Saskatchewan			0 (0/5)	13 (1/8)	0 (0/9)	8 (1/12)
British Columbia					50 (2/4)	67 (2/3)
Chicken retail *E. coli*						
Québec	32 (36/111)	34 (54/158)	25 (35/142)	6 (8/135)	13 (17/128)	18 (24/131)
Ontario	18 (24/136)	21 (32/150)	17 (25/145)	22 (34/152)	22 (35/157)	24 (36/150)
Saskatchewan			4 (3/82)	6 (5/85)	13 (10/75)	20 (18/92)
British Columbia					29 (12/42)	49 (34/70)

collected. Exposure to ceftiofur-resistant *E. coli* strains appeared to have increased in recent years in Canada (Figure 1). In 2008, exposure to ceftiofur-resistant *E. coli* strains was highest in British Columbia and lowest in Québec.

Temporal Changes in Ceftiofur Resistance in the Province of Québec, 2003–2008

In 2003–2004, >60% of the chicken *Salmonella* Heidelberg isolates were ceftiofur resistant, and ceftiofur resistance among chicken *E. coli* and human *Salmonella* Heidelberg isolates varied from 30% to 40% (Figure 2). Ceftiofur resistance declined sharply immediately after the first quarter of 2005 among chicken *E. coli* and *Salmonella* Heidelberg isolates, and a similar decline began in the next quarter among human *Salmonella* Heidelberg isolates (Figure 2). This decline steadily continued until the end of 2006. As a result, the prevalence of ceftiofur resistance significantly decreased from 2004 to 2006 among chicken (62% to 7%; p<0.001) and human (36% to 8%; p<0.0001) *Salmonella* Heidelberg isolates and chicken *E. coli* isolates (34% to 6%; p<0.0001 [Table]). Then, from 2006 to 2008, the prevalence of ceftiofur resistance significantly increased among chicken *E. coli* isolates (6% to 18%; p = 0.002), and prevalence of ceftiofur resistance increased, but not significantly, among *Salmonella* Heidelberg from chicken (7% to 18%; p = 0.32) and human (8% to 12%; p = 0.41) isolates (Table).

Discussion

CIPARS data clearly indicate a temporal association between changing levels of contamination of retail chicken with ceftiofur-resistant *Salmonella* Heidelberg strains and incidence of ceftiofur-resistant *Salmonella* Heidelberg infection in humans. This correlation is strong and applies to different regions of Canada. Our observation is consistent with published results from outbreak investigations and case-control studies suggesting that chicken products are a source of human infection with *Salmonella* Heidelberg in Canada (*7*,*8*).

Although humans potentially can become infected with ceftiofur-resistant *Salmonella* Heidelberg from sources other than chicken, chicken appears the most likely source in Canada. Ceftiofur-resistant *Salmonella* Heidelberg has never been reported among CIPARS porcine *Salmonella* of abattoir origin, and it has not been detected among retail pork, abattoir beef, or retail beef, in which *Salmonella* prevalence remains <2% (*12*). Data generated by National Antimicrobial Resistance Monitoring System retail surveillance in the United States indicated that 17% of *Salmonella* Heidelberg

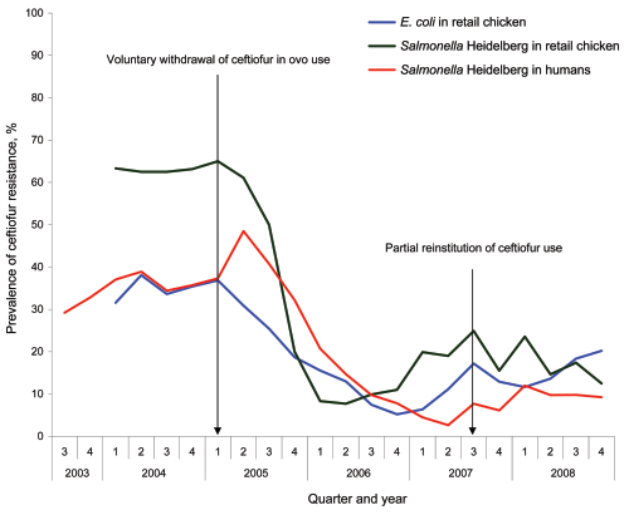

Figure 2. Prevalence of ceftiofur resistance (moving average of the current quarter and the previous 2 quarters) among retail chicken *Escherichia coli*, and retail chicken and human clinical *Salmonella enterica* serovar Heidelberg isolates during 2003–2008 in Québec, Canada.

isolates recovered from ground turkey in 2006 were resistant to ceftiofur (*13*). CIPARS does not conduct ongoing surveillance of retail turkey, and we cannot ignore the possibility that retail turkey could be a source of ceftiofur-resistant *Salmonella* Heidelberg for humans as well. However, turkey consumption in Canada (4.7 kg per capita) was much lower than chicken consumption (33.2 kg per capita) in 2007 (*31*). Lastly, *Salmonella* Heidelberg has been reported in clinical samples from various other animal species in Canada (*12*), and exposure to sick animals could potentially be another source of infection. However, ceftiofur resistance in clinical *Salmonella* Heidelberg isolates remains anecdotal in species other than chicken and turkey (*12*).

Drug use monitoring in chicken is nonexistent in Canada. However, research data indicate a high level of ceftiofur use in Québec hatcheries in 2003–2004, where at least 78% of the lots surveyed in Québec abattoirs (M. Boulianne et al., unpub. data) had received ceftiofur in ovo. During that same period, ceftiofur resistance among retail chicken *Salmonella* Heidelberg isolates were >60%. The rapid and important 82% (*E. coli*) and 89% (*Salmonella* Heidelberg) declines in ceftiofur resistance in Québec retail chicken meat that followed in 2005–2006, as well as in Québec chicken *E. coli* and *Salmonella* isolates collected from passive surveillance of animal clinical isolates conducted by the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ) (*32*), is consistent with an effective voluntary withdrawal in 2005 and 2006. In 2007, the Québec broiler industry announced a potential partial reinstitution of ceftiofur use to control omphalitis in young chicks, with the intention of using the drug on a rotational basis and limiting its use to no more than 6 months per year (*32*). Again, CIPARS data from Québec retail chicken sampling in 2007–2008 demonstrating a reemergence of ceftiofur resistance among *E. coli* but at lower levels than in 2003–2004 are consistent with a partial return to ceftiofur use. The simultaneous reduction (and reemergence) in ceftiofur resistance in both retail chicken *E. coli* and *Salmonella* Heidelberg isolates and in clinical chicken *E. coli* and *Salmonella* isolates from MAPAQ surveillance support the hypothesis that the fluctuations in ceftiofur resistance most likely were driven by a common exposure (or reduction of exposure) to ceftiofur in chicken hatcheries, rather than simply being secondary to the natural spread and disappearance of a ceftiofur-resistant clone unrelated to ceftiofur use.

Although Ontario hatcheries had never announced an official withdrawal of ceftiofur use, a drop in ceftiofur resistance also was observed among chicken *Salmonella* Heidelberg isolates in Ontario in 2005. Although some argue that this proves the absence of an association between ceftiofur use and ceftiofur resistance in broiler chicken, movement of hatching eggs, broiler chicks (mostly from Québec to Ontario), and retail chicken meat between these 2 provinces could explain some of the similarities among *Salmonella* Heidelberg isolates in Ontario and Québec (*33*). The withdrawal in Québec might also have led Ontario broiler chicken hatcheries to temporarily decrease their use of ceftiofur in 2005.

In the absence of reliable comprehensive drug use information in the broiler chicken industry, we use resistance in commensal *E. coli* as a surrogate measure of the level of drug use (*34*). The high prevalence of ceftiofur resistance among *E. coli* isolates from British Columbia (almost half of the isolates in 2008 in that province), the increasing prevalence of resistance measured in Saskatchewan, and the 22% ceftiofur resistance among chicken *E. coli* isolates from Ontario when ceftiofur resistance prevalence was at its lowest level in Québec in 2006, indicates that ceftiofur use is unlikely to be restricted to the province of Québec. Lastly, in ovo ceftiofur use has also been reported in US chicken hatcheries (*35*).

Coselection of resistance to cephalosporins by exposure to other antimicrobials or to chemicals in the agricultural environment has been suggested as a confounding factor for the increase in observed resistance. Giles et al. (*36*) report the presence of the *sug*E gene on the same element as the bla_{CMY-2} gene in *Salmonella,* but the capacity of this gene to effectively confer resistance to quaternary ammonium compounds and provide coselection remains uncertain.

The levels of contamination of retail chicken with ceftiofur-resistant *E. coli* represent an additional concern. No selective media for ceftiofur-resistant strains was used, and the level of contamination of retail chicken with ceftiofur-resistant *E. coli* (and *Salmonella* Heidelberg) strains was most likely underestimated. Although this study describes exposure to commensal *E. coli*, such strains occasionally may cause infections in predisposed humans. In addition, the species *E. coli* includes a variety of strains commonly pathogenic for humans, and some strains from the normal flora of animals may carry a variety of virulence determinants that increase their potential for causing disease in humans (*37*). Poppe et al. (*38*) also demonstrated experimentally the acquisition of resistance to extended-spectrum cephalosporins by *Salmonella* serovar Newport from *E. coli* strains by conjugation in poultry intestinal tracts. In addition, molecular characterization of plasmids from field isolates demonstrates that identical bla_{CMY-2} plasmids can be found in both *Salmonella* and *E. coli* from the same chicken (P. Boerlin et al., unpub. data). Because the bla_{CMY-2} gene is horizontally transferable and is frequently observed in ceftiofur-resistant isolates of chicken origin, chicken could potentially be a reservoir of this gene for human pathogens, including *Salmonella* and others.

Except for anecdotal information, little information is available about drugs used by broiler chicken hatcheries and growers in Canada. The absence of on-farm drug use monitoring data prevents us from fully determining the effect of subtle changes in the level of use of ceftiofur (or other drugs) on resistance among bacteria recovered from chickens in Canada. Surveillance data from turkey or other nonsurveyed commodities would be useful to adequately quantify the contribution of each commodity to the overall number of cases related to ceftiofur-resistant *Salmonella* Heidelberg in humans. The impact of disinfectants used by the broiler industry at the farm or processing level on the selection of ceftiofur-resistant strains also needs to be assessed. Lastly, CIPARS is planning a burden-of-illness study to measure the impact of extended-spectrum cephalosporin resistance in *Salmonella* Heidelberg on human health.

Efforts undertaken by Québec chicken hatcheries to voluntarily withdraw use of ceftiofur in 2005–2006 coincided with a markedly reduced prevalence of ceftiofur-resistant *Salmonella* Heidelberg in retail chicken. This drop also effectively reduced the number of ceftiofur-resistant *Salmonella* Heidelberg infections in humans in this province during the same period. This reduction suggests that control of resistance to extended-spectrum cephalosporins is possible by managing ceftiofur use at the hatchery level. The partial reintroduction of ceftiofur use in Québec chicken hatcheries in 2007 with increasing rates of ceftiofur resistance after reintroduction, and indications that ceftiofur is used for the same purpose in other Canadian provinces, is of high concern. An increasing level of exposure to *E. coli* strains carrying horizontally transferable genes conferring resistance to extended-cephalosporins complicates the situation. To ensure the continued effectiveness of extended-spectrum cephalosporins to treat serious human infections, multidisciplinary efforts are needed to scrutinize, and where appropriate, limit use of ceftiofur in Canadian food animal production, particularly in chicken.

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Dr Dutil is a veterinary epidemiologist at the Laboratory for Foodborne Zoonosis of the Public Health Agency of Canada. She is in charge of the coordination of the data analysis of the Canadian Integrated Program for Antimicrobial Resistance Surveillance.

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Address for correspondence: Lucie Dutil, Agence de la Santé Publique du Canada, 3200 Sicotte, CP 5000, Saint-Hyacinthe, Quebec J2S 7C6, Canada; email: lucie_dutil@phac-aspc.gc.ca

Healthcare-associated Viral Gastroenteritis among Children in a Large Pediatric Hospital, United Kingdom

Nigel A. Cunliffe, J. Angela Booth, Claire Elliot, Sharon J. Lowe, Will Sopwith, Nick Kitchin, Osamu Nakagomi, Toyoko Nakagomi, C. Anthony Hart,[1] and Martyn Regan

Viruses are the major pathogens of community-acquired (CA) acute gastroenteritis (AGE) in children, but their role in healthcare-associated (HA) AGE is poorly understood. Children with AGE hospitalized at Alder Hey Children's Hospital, Liverpool, UK, were enrolled over a 2-year period. AGE was classified as HA if diarrhea developed ≥48 hours after admission. Rotavirus, norovirus, adenovirus 40/41, astrovirus, and sapovirus were detected by PCR. A total of 225 children with HA-AGE and 351 with CA-AGE were enrolled in the study. HA viral gastroenteritis constituted one fifth of the diarrheal diseases among hospitalized children and commonly occurred in critical care areas. We detected ≥1 virus in 120 (53%) of HA-AGE cases; rotavirus (31%), norovirus (16%), and adenovirus 40/41 (15%) were the predominant viruses identified. Molecular evidence indicated rotaviruses and noroviruses were frequently introduced into the hospital from the community. Rotavirus vaccines could substantially reduce the incidence of HA-AGE in children.

Enteric viruses are major etiologic agents of acute gastroenteritis (AGE) among infants and young children worldwide (*1*). Rotavirus is the most common single cause of severe diarrhea leading to dehydration and death (*2*). Other recognized viral causes of pediatric gastroenteritis include norovirus, astrovirus, enteric adenovirus (serotypes 40 and 41), and sapovirus (*1*).

Author affiliations: University of Liverpool, Liverpool, UK (N.A. Cunliffe, J.A. Booth, O. Nakagomi, T. Nakagomi, C.A. Hart); Royal Liverpool Children's National Health Service Foundation Trust, Liverpool (N.A. Cunliffe, C. Elliot, S.J. Lowe, C.A. Hart); Health Protection Agency NW, Liverpool (C. Elliot, W. Sopwith, M. Regan); Sanofi Pasteur MSD, Maidenhead, UK (N. Kitchin); and Nagasaki University, Nagasaki, Japan (O. Nakagomi, T. Nakagomi)

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Although studies of viral gastroenteritis in children have mainly focused on community-acquired (CA) infection, the importance of healthcare-associated (HA) rotavirus infection and the potential for its prevention by vaccination has been highlighted in several recent publications (*3*–*8*). Despite improved understanding of the disease impact and epidemiology of HA rotavirus gastroenteritis, many studies have been limited by inadequate design and methodology, short duration, and small size.

The value of molecular methods in defining the contribution of multiple viruses to pediatric diarrheal disease is being increasingly recognized (*9*,*10*). However, no studies have examined each of the known viral gastroenteritis agents among children with HA-AGE. In a 2-year prospective study in a large pediatric hospital, we examined the contribution to HA-AGE and CA-AGE of rotavirus and 4 additional enteric viruses using the most sensitive molecular detection methods available.

Materials and Methods

Study Setting

The study was conducted at the Royal Liverpool Children's National Health Service Foundation Trust (Alder Hey Hospital). Alder Hey provides primary, secondary, and tertiary care facilities for >200,000 children each year and has ≈300 inpatient beds. General medicine, general surgery, and a range of specialist services including critical care, oncology, cardiac, and neurosurgery are provided.

Enrollment Procedures

Children <16 years of age who were admitted with AGE during January 1, 2006–December 31, 2007, or

[1]Deceased.

those in whom AGE developed after hospitalization, were eligible for inclusion in the study. AGE was defined as diarrhea (≥3 loose, or looser-than-normal, stools in a 24-hour period), with or without vomiting, of <7 days' duration. Gastroenteritis was considered HA if symptoms developed ≥48 hours after admission. Written informed consent was obtained from the child's parent or guardian before enrollment.

Collection of Clinical Data and Fecal Specimens

Study nurses identified case-patients by daily chart review. Clinical data were collected when patients were admitted and prospectively until hospital discharge. We used these data to calculate a severity score using a modified 20-point scoring system (Table 1). A stool sample was collected as soon as possible after onset of diarrhea. Fecal specimens were stored at −80°C until virus detection was undertaken. Rates of AGE were calculated by using total admission numbers obtained from hospital records.

Laboratory Analyses

Virus Detection

Nucleic acid was extracted from 10% fecal suspensions in phosphate buffered saline by using the RNeasy mini kit (QIAGEN, Crawley, UK). After reverse transcription–PCR (RT-PCR) was performed by using random hexamers, rotavirus, adenovirus 40/41, astrovirus, and sapovirus were each detected by conventional PCR using virus-specific primers, agarose gel electrophoresis, and ethidium bromide staining (*9*,*12*). Norovirus was detected by using a modification of the real-time PCR method of Kageyama et al. (*13*) as described by Amar et al. (*9*).

Table 1. Modified 20-point scoring system used to evaluate severity of gastroenteritis (*11*)

Clinical sign or symptom	Points
Duration of diarrhea, d	
<2	1
2–4	2
>4	3
Maximum no. diarrheal stools in 24 h	
3	1
4–5	2
>5	3
Duration of vomiting, d	
No vomiting	0
1–2	2
≥3	3
Maximum no. vomiting episodes in 24 h	
1	1
2	2
≥3	3
Rehydration	
None	0
Oral	1
Intravenous	2
Fever, ºC	
<37.6	0
37.6ºC–38.5	2
>38.5	3
Behavior	
Normal	0
Lethargic/irritable	1
Convulsion	3

Molecular Characterization of Rotaviruses and Noroviruses

Rotavirus viral protein (VP) 7 (G) and VP4 (P) were genotyped by using multiplex, heminested RT-PCR (*14*,*15*). The dsRNA genomes of genotype P[8],G1 rotavirus strains were examined by polyacrylamide gel electrophoresis (PAGE). The VP7 gene of P[8],G1 rotavirus strains was amplified by RT-PCR using primers 9con1 and 9con2 (*15*). The capsid region of Norovirus genogroup II strains was amplified by RT-PCR using primers G2SKF and G2SKR (*16*). All RT-PCR amplification products were purified by using Micro-spin columns (GE Healthcare, Buckinghamshire, UK), and sequenced by Cogenics (Hope End, Essex, UK). Phylogenetic trees were constructed according to the neighbor-joining method (*17*) in the ClustalW software package (*18*).

Results

Subject Enrollment and Case Classification

AGE in 669 children met the case definition for investigation during the study. We excluded 93 children from analysis because of failure to obtain fecal specimens (80 children), consent refusal/withdrawal (12), and age >16 years (1). Of the remaining 576 children with AGE, 351 cases (61%) were determined to be CA and 225 (39%) were HA.

Virus Detection Rates

During the study period, ≥1 viruses were detected in 339 (59%) of 576 specimens. Rotavirus was identified most frequently (38% of all AGE cases), followed by norovirus (16%), adenovirus 40/41 (14%), astrovirus (5%), and sapovirus (5%) (Table 2). The order of detection of each virus did not differ between CA- and HA-AGE cases. We detected ≥1 virus in 120 (53%) of 225 HA-AGE cases. Rotavirus was detected in 70 (58%) of 120 children with HA-AGE in whom a virus was identified.

In 98 (17%) of 576 children with AGE, >1 virus was detected; this proportion did not significantly differ between CA (65/351 cases, 19%) and HA (33/225, 15%) infection. Rotavirus was the agent least likely to be identified as a mixed infection; it occurred as the only virus in 144 (65%) of 220 AGE cases in which it was identified.

Table 2. Virus detection rates by case origin, Royal Liverpool Children's NHS Foundation Trust (Alder Hey Hospital), Liverpool, UK*

Virus	HA-AGE cases, no. (%), n = 225	CA-AGE cases, no. (%), n = 351
Rotavirus	70 (31)	150 (43)
Norovirus	36 (16)	54 (15)
Adenovirus 40/41	34 (15)	49 (14)
Astrovirus	12 (5)	16 (5)
Sapovirus	5 (2)	22 (6)
Any virus detected	120 (53)	219 (62)
No virus detected	105 (47)	132 (38)

*CA-AGE, community-acquired acute gastroenteritis; HA-AGE, healthcare-associated acute gastroenteritis.

This proportion did not differ between patients with CA (100/150, 66%) and HA (44/70, 62%) rotavirus infection (Table 3). Sapovirus was least likely to be identified as a sole infection, with only 4 (15%) of 27 cases involving no other virus.

Age Distribution of Case-patients

The median age of children with all-cause CA-AGE (10 mo, range 1–180 mo) did not differ from children with all-cause HA-AGE (12 mo, range 1–192 mo). Among case-patients, the median age of children with rotavirus infection (11 mo, range 1–192 mo) was similar to children excreting norovirus (10 mo, range 1–180 mo); adenovirus 40/41 (10 mo, 1–180 mo); astrovirus (9 mo, 1–180 mo); and sapovirus (12 mo, 1–96 mo). The median age of children excreting any virus did not differ according to exposure setting (data not shown).

Severity of Viral Gastroenteritis

The severity of AGE caused by infection only with rotavirus or norovirus was greater than with other viruses for CA and HA infections (Table 4). The proportion of children with severe gastroenteritis (defined as severity score ≥11) was greatest with rotavirus (42/144, 29%) and norovirus (12/46, 26%) and lowest for children in whom virus was not detected (39/237, 16%). Rotavirus was associated with severe illness in 36% of HA-AGE cases in which it was identified as the only infection, not significantly different to the proportion (24%) of HA-AGE judged to be severe as a consequence of single norovirus infection (p = 0.400 by Fisher exact test). Overall, 28% of HA-viral gastroenteritis patients were defined as having severe disease.

Molecular Epidemiology of Virus Infections

Rotavirus genotype could be determined for 93 (62%) of 150 CA-AGE cases and for 23 (33%) of 70 HA-AGE cases (rotaviruses that could not be genotyped were considered to contain insufficient virus load in stool [data not shown]). Distribution of rotavirus strains did not differ between CA and HA cases, with the P[8],G1 strain predominating in each. Thus, of rotaviruses genotyped from 93 patients with CA rotavirus, genotypes of 62 gastroenteritis patients were P[8], G1; 7 were P[8], G3; 4 were P[4], G2; 18 were P[8], G9; and 1 was P[8], G4; 1 strain could not be G or P typed. Of rotaviruses genotyped from 23 patients with HA rotavirus gastroenteritis, 19 were P[8], G1; 1 was P[8], G3; 2 were P[4], G2; and 1 was P[4], G1 + G2.

To explore whether HA-AGE cases were associated with similar or identical rotavirus strains causing CA-AGE, we examined P[8], G1 rotaviruses further by PAGE and by VP7 gene sequencing. Three distinct electropherotypes (L1, L2, and L3) were identified among 19 HA P[8], G1 strains, and each of these electropherotypes also was recognized among 7 electropherotypes assigned to 62 CA P[8], G1 strains (data not shown). Similarly, all except 2 VP7 nucleotide sequences from HA-AGE rotavirus infections clustered with corresponding sequences from CA strains with which they shared almost identical or identical nucleotide sequence (Figure 1). For noroviruses, all except 1 HA-AGE norovirus sequences were identical, or almost identical, to those derived from CA strains (Figure 2).

Hospital Distribution of Viral Gastroenteritis Cases

Although 274 (94%) of 291 patients with CA viral gastroenteritis were located in acute general medical and surgical wards, 88 (56%) of 157 with HA viral gastroenteritis were located elsewhere in the hospital. In particular, the highest rates of HA viral gastroenteritis infection were noted in critical care units (intensive care unit and high dependency unit), neurology, cardiology, and long stay wards where children with chronic conditions who have complex healthcare needs are patients (although this represents <5 case-patients) (Figure 3). Median duration of hospital stay before onset of

Table 3. Mixed virus infections by case origin, Royal Liverpool Children's NHS Foundation Trust (Alder Hey Hospital), Liverpool, UK*

Virus	Total	Rotavirus	Norovirus	Adenovirus 40/41	Astrovirus	Sapovirus	>2 viruses
Rotavirus	76		14	16	3	10	7
Norovirus	44	6		6	2	3	4
Adenovirus 40/41	48	10	2		1	2	5
Astrovirus	16	4	2	2		0	2
Sapovirus	23	1	1	0	0		4
>2 viruses	37	5	4	4	0	2	

*Enumerates persons in which >1 virus was identified. Numbers above empty cells represent community-acquired acute gastroenteritis (AGE) cases; numbers below the line represent healthcare-associated AGE cases. NHS, National Health Service.

Table 4. Median AGE severity scores among children hospitalized at Alder Hey Hospital for HA-AGE versus CA-AGE, by types of viruses detected, Liverpool, UK, 2006–2007*

Virus	HA-AGE, n = 225 (range)	CA-AGE, n = 351 (range)
Rotavirus	8 (3–17)	8 (2–16)
Norovirus	8 (3–14)	9 (2–15)
Adenovirus 40/41	6 (3–12)	6 (4–14)
Astrovirus	7.5 (6–9)	7.5 (5–11)
Sapovirus	11 (11)	5 (4–8)
Any virus detected	8 (3–17)	8 (2–16)
No virus detected	7 (3–15)	7 (2–15)

*CA-AGE, community-acquired acute gastroenteritis; HA-AGE, healthcare-associated acute gastroenteritis.

symptoms among children with HA viral gastroenteritis was 8 days (range 2–1,365 days); two thirds of infections occurred at least 1 week after hospital admission.

Discussion

In our study, one fifth of AGE among children within a large pediatric hospital was of viral origin and acquired within the healthcare setting. Furthermore, at least 1 virus was detected in >50% of HA-AGE patients, and in 28%, >1 virus was identified. HA viral gastroenteritis is a major infection control issue in hospital pediatric wards (*19*). Our study systematically detected each of the 5 established causes of viral gastroenteritis.

Rotavirus was the most common virus identified among children with CA- and HA-AGE (43% and 31%, respectively). It was also the virus most likely to occur alone and was the virus with which severe symptoms were most often associated. A recent review of European studies identified rotavirus in 31%–87% of all-cause HA-AGE, reflecting the differences in methods used in individual studies (*4*). HA rotavirus infections in our study accounted for 32% of all rotavirus infections identified. This finding is consistent with 2 recent retrospective studies showing that 21% (*20*) and 36% (*21*) of rotavirus gastroenteritis cases were HA and 2 reviews documenting that 14%–51% of rotavirus infections among hospitalized children were HA (*3*,*5*). Therefore, the impact of HA rotavirus gastroenteritis in major pediatric hospitals has not decreased since the first major study of this subject published nearly 20 years ago (*22*).

With the availability and application of molecular assays capable of detecting a broad range of norovirus genotypes, norovirus is now recognized as a major cause of sporadic CA-AGE in children (*23*–*25*). We document that norovirus was the second most common virus identified (after rotavirus) among children with HA-AGE (detected in 16% of HA-AGE patients and in 24% of HA-AGE patients in whom a single virus was identified). Furthermore, norovirus was associated with severe illness in a substantial percentage (24%) of HA-AGE patients in which it was identified as a sole infection, a percentage similar to that obtained for rotavirus (36%).

Previous studies have investigated adenovirus 40/41 in CA-AGE, and we systematically looked for it in children with HA-AGE. Adenovirus 40/41 was the third most commonly identified virus in our study among children with CA- and HA-AGE (detected in 14% and 15% of patients, respectively). Studies that have investigated for adenovirus 40/41 among children with CA-AGE by using antigen-based detection methods have generally reported detection rates of ≈5% (*1*). We used molecular methods, therefore suggesting a greater role for adenovirus 40/41 in CA-AGE than has been previously appreciated, and now indicating it also plays a prominent role in HA-AGE. Notably, adenovirus 40/41 was also the third most commonly identified enteric virus in a recent study in East Anglia, UK where it was detected by PCR in 9.6% of all children examined (*10*). Astroviruses and sapoviruses were much less frequently identified in our study, despite the application of molecular methods.

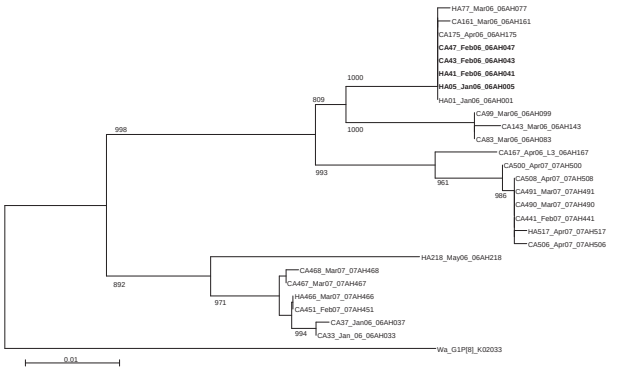

Figure 1. Phylogenetic tree based on viral protein (VP) 7 nucleotide sequences of serotype G1 rotavirus strains from Royal Liverpool Children's National Health Service Foundation Trust (Alder Hey Hospital), Liverpool, UK. For each strain the source (healthcare-associated [HA] or community-acquired [CA]), specimen number, month/year of detection, and name of the strain is indicated. Reference G1P[8] strain Wa is included. Horizontal lengths are proportional to the genetic distance calculated with Kimura's 2-parameter method. Scale bar shows genetic distance expressed as nucleotide substitutions per site. Bootstrap probabilities >80% (>800 of 1,000 pseudoreplicate trials) are indicated at each node. Hatched VP7 sequences are from strains whose electropherotypes shared an identical L1 pattern. The VP7 nucleotide sequences used in the tree have been deposited under the strain name and accession number (in parentheses) as follows; 06AH001 (FJ797814), 06AH005 (FJ797815), 06AH033 (FJ797816), 06AH037 (FJ797817), 06AH041 (FJ797818), 06AH043 (FJ797819), 06AH047 (FJ797820), 06AH077 (FJ797821), 06AH083 (FJ797822), 06AH099 (FJ797823), 06AH143 (FJ797824), 06AH161 (FJ797825), 06AH167 (FJ797826), 06AH175 (FJ797827), 06AH218 (FJ797828), 07AH441 (FJ797829), 07AH451 (797830), 07AH466 (FJ797831), 07AH467 (FJ797832), 07AH468 (FJ797833), 07AH490 (FJ797834), 07AH491 (FJ797835), 07AH500 (FJ797836), 07AH506 (FJ797837), 07AH508 (FJ797838), 07AH517 (FJ797839).

With the use of molecular methods to detect 5 established viral agents of gastroenteritis, we demonstrated that 15% of HA-AGE patients contained >1 virus, emphasizing the value of the simultaneous examination for multiple viruses. Thus, in a recent community-based study in the United Kingdom that examined for each of these 5 viruses using molecular assays, mixed virus infections were identified in 11.7% of case-patients (*24*). In almost 50% of the norovirus infections in our study, an additional virus was identified (most commonly rotavirus). Although we found no evidence of increased disease severity among rotavirus–norovirus co-infections (data not shown), our data clearly demonstrate the frequency with which both viruses cocirculate with the potential for nosocomial spread. Dual norovirus–rotavirus co-infections were also commonly recognized in a recent study of hospitalized children in Italy, in whom the severity of illness was higher than with norovirus infection alone (*26*). Similar to the observations made for norovirus, additional virus infections were commonly identified among case-patients excreting adenovirus 40/41, in whom more than half had additional viruses.

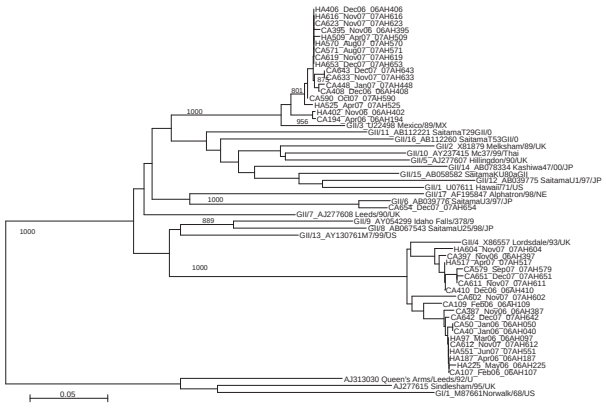

Figure 2. Phylogenetic tree based on 387 nucleotide sequences of the 5′ end of open reading frame 2 (encoding viral protein 1) of norovirus strains from Royal Liverpool Children's National Health Service Foundation Trust (Alder Hey Hospital), Liverpool, UK. For each strain the source (healthcare-associated [HA] or community-acquired [CA]), specimen number, month/year of detection, and the name of the strain is indicated. Reference strains included on the tree are GII/1 U07611 Hawaii/71/US, GII/2 X81879 Melksham/89/UK, GII/3 U22498 Mexico/89/MX, GII/4 X86557 Lordsdale/93/UK, GII/5 AJ277607 Hillingdon/90/UK, GII/6AB039776 SaitamaU3/97/JP, GII/7 AJ277608 Leeds/90/UK, GII/8 AB067543 SaitamaU25/98/JP, GII/9 AY054299 IdahoFalls/378/96/US, GII/10 AY237415 Mc37/99/Thai, GII/11 AB112221 SaitamaT29GII/01/JP, GII/12 AB039775 SaitamaU1/97/JP, GII/13 AY130761 M7/99/US, GII/14 AB078334 Kashiwa47/00/JP, GII/15 AB058582 SaitamaKu80aGII/99/JP, GII/16 AB112260 SaitamaT53GII/02/JP, GII/17 AF195847 Alphatron/98/NE, GI/1 M87661 Norwalk/68/US, AJ313030 Queen'sArms/Leeds/92/UK, AJ277615 Sindlesham/95/UK. Horizontal lengths are proportional to the genetic distance calculated with the Kimura 2-parameter method. Scale bar shows genetic distance expressed as nucleotide substitutions per site. Bootstrap probabilities >80% (>800 of 1,000 pseudoreplicate trials) are indicated at each node. The nucleotide sequences used in the tree have been deposited under the strain name and accession number (in parentheses) as follows; 06AH107 (FJ797840), 06AH109 (FJ797841), 06AH187 (FJ797842), 06AH194 (FJ797843), 06AH225 (FJ797844), 06AH387 (FJ797845), 06AH395 (FJ797846), 06AH397 (FJ797847), 06AH040 (FJ797848), 06AH402 (FJ797849), 06AH406 (FJ797850), 06AH408 (FJ797851), 06AH410 (FJ797852), 07AH448 (FJ797853), 06AH050 (FJ797854), 07AH509 (FJ797855), 07AH517 (FJ797856) 07AH525 (FJ797857), 07AH551 (FJ797858), 07AH570 (FJ797859), 07AH571 (FJ797860), 07AH579 (FJ797861), 07AH590 (FJ797862), 07AH602 (FJ797863), 07AH604 (FJ797864), 07AH611 (FJ797865), 07AH612 (FJ797866), 07AH616 (FJ797867), 07AH619 (FJ797868), 07AH623 (FJ797869), 07AH633 (FJ797870), 07AH642 (FJ797871), 07AH643 (FJ797872), 07AH651 (FJ797873), 07AH653 (FJ797874), 07AH654 (FJ797875), 06AH097 (FJ797876).

Although each of the enteric viruses detected in this study is firmly established as a gastroenteritis pathogen, the detection of a virus in a child with AGE does not necessarily imply causation. This is most clearly relevant in the context of co-infections including mixed virus infections, where ≥1 detected organisms may not be primarily responsible for diarrhea. Although we did not exhaustively and systematically search for other potential infective causes of diarrhea, we identified enteropathogenic bacteria and parasites in <2% of specimens examined, which suggests that they were not major causes of diarrhea in our study population (data not shown). Additionally, although detection of a virus by a sensitive molecular method may be more likely than a less sensitive antigen-based assay to indicate subclinical infection (*27*), prolonged rotavirus shedding demonstrated by RT-PCR was associated with symptoms of diarrhea in a longitudinal study of children recovering from severe rotavirus infection (*28*). Because molecular-based assays are widely accepted as the preferred detection method for norovirus, but not for other gastroenteritis viruses, application in this study of molecular methods that likely have similar (high) levels of sensitivity for detection of all 5 gastroenteritis viruses allowed more appropriate recognition of the extent of virus circulation within the hospital. Finally, although a stool sample obtained from a patient at hospital admission would be required to conclusively categorize a viral gastroenteritis episode as CA or HA, two thirds of these infections occurred ≥1 weeks after admission, and a 48-hour symptom-based definition has been used extensively in previous studies (*3–5*).

The entry and spread of enteric viruses into and within pediatric healthcare settings is not completely understood. Several studies have indicated that multiple rotavirus introductions from the community into the hospital and subsequent spread between patients accounts for most HA rotavirus infections because of the diversity among strains recovered from hospitalized children and similarity at the genotype level between viruses circulating within the community and hospital (*20*,*29*,*30*). Similarly, a recent study

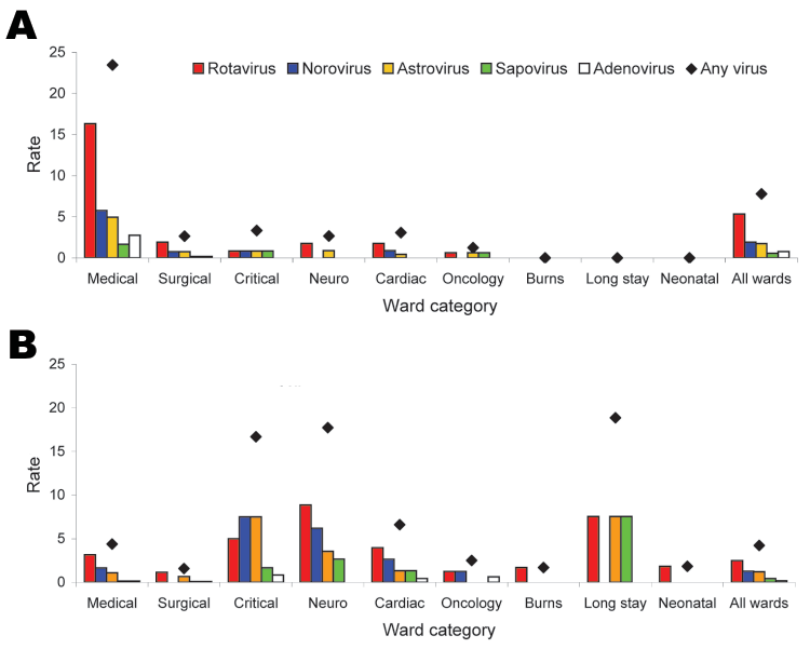

Figure 3. Distribution of case-patients with A) community-acquired versus B) healthcare-associated acute gastroenteritis (AGE) in whom a virus was detected, by ward category and virus detected, Alder Hey Hospital, Liverpool, UK, 2006–2007. Rates were calculated as numbers of cases per 1,000 admissions to each ward throughout the study and are shown for each virus tested, with a comparison of all cases where at least 1 virus was detected (diamonds).

that described frequent, brief clusters of norovirus gastroenteritis among pediatric inpatients attributed these findings to frequent introduction of noroviruses into the hospital from the community (*31*). For both rotaviruses and noroviruses, however, previous studies have not provided direct evidence that strains detected among hospitalized children originated in the community. In this respect, our study provides strong molecular epidemiologic support, at the nucleotide level, for the hypothesis that rotaviruses and noroviruses in the community are the sources of HA infection. Thus, rotaviruses (judged by VP7 sequence), and noroviruses (examined by sequence of the capsid region) detected among HA-AGE cases were similar or identical to corresponding viruses detected in children with CA-AGE.

In contrast to CA-AGE cases, the highest rates of HA-viral enteric infections did not occur on the acute medical and surgical wards but instead in the critical care areas, neurologic, cardiac, and long stay wards where chronically ill children are patients. We observed this trend for each of the 3 major viruses identified (rotavirus, norovirus, and adenovirus 40/41). Although we noticed differences in the hospital distribution of HA-AGE cases, the ages of children with HA-AGE did not differ significantly from children with CA-AGE. We did, however, find evidence for severe disease among 28% of children with HA viral gastroenteritis in our study; 22% of such children had an underlying medical condition (data not shown). Given that CA viruses are likely to be the source of HA viral gastroenteritis, standard hospital infection prevention measures (e.g., isolation of children with AGE) should be emphasized (*32*). Hand washing by staff before patient contact may be particularly important because asymptomatic staff members could be a source of HA rotavirus infection (*33*) and hand washing has been shown to reduce the rate for nosocomial rotavirus infection (*34*). However, because HA rotavirus infection remains a substantial clinical problem ≈20 years after publication of a seminal study on the subject (*22*), even stringent infection control precautions are likely to be insufficient to greatly reduce the problem. In the context of rotavirus, the major pathogen responsible for CA- and HA-AGE, rotavirus vaccines offer a further opportunity to reduce the impact of HA-AGE. Routine rotavirus vaccination of children in the community is expected to greatly reduce the number of CA, rotavirus-associated hospitalizations (*35,36*), thereby reducing the hospital rotavirus reservoir and HA rotavirus gastroenteritis. The prevention of HA rotavirus gastroenteritis should represent an important secondary goal of rotavirus vaccines (*3,6*).

In conclusion, we have demonstrated that viruses accounted for more than half the cases of HA-AGE in a large pediatric hospital and resulted from the frequent introduction of gastroenteritis viruses from the community. Because rotavirus is the single most common pathogen, introduction of a rotavirus vaccine into childhood immunization programs is expected to substantially reduce the incidence of CA and HA rotavirus gastroenteritis in hospitals.

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Dr Cunliffe is a reader in medical microbiology, University of Liverpool, and Honorary Consultant Microbiologist, Royal Liverpool Children's National Health Service Foundation Trust. His research focuses on the epidemiology and prevention of viral gastroenteritis.

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Address for correspondence: Nigel A. Cunliffe, Division of Medical Microbiology, University of Liverpool, Daulby St, Liverpool L69 3GA, UK; email: n.a.cunliffe@liv.ac.uk

Meningitis and a Febrile Vomiting Illness Caused by Echovirus Type 4, Northern Territory, Australia

Peter G. Markey, Joshua S. Davis, Gerry B. Harnett, Simon H. Williams, and David J. Speers

In July 2007, a cluster of meningitis cases caused by an echovirus 4 strain was detected in 1 indigenous community in the Top End of the Northern Territory of Australia. Illness was characterized by fever, vomiting, and headache. Over the next 4 months, additional cases of meningitis and the fever and vomiting syndrome emerged in other indigenous communities and subsequently in the major urban center of Darwin. We describe the epidemiology of 95 laboratory-confirmed meningitis cases and conclude that the epidemic fever and vomiting syndrome was caused by the same enterovirus. Nucleotide sequencing of the whole genome verified this enterovirus (AUS250G) as a strain of echovirus type 4. Viral protein 1 nucleotide sequencing demonstrated 96% homology with an echovirus 4 strain responsible for a large outbreak of meningitis in the Yanbian Prefecture of China in 1996.

Enteroviruses are among the most common human viral pathogens. Recent reports from the United States have documented >50 serotypes of enterovirus causing illness in humans (*1*). Illness syndromes with established causal links to enteroviruses include acute hemorrhagic conjunctivitis; viral meningitis; hand, foot, and mouth disease; and acute ascending paralysis (including poliomyelitis). In July 2007, a disease control unit in the Northern Territory of Australia reported a cluster of viral meningitis cases in a nearby community. Nucleic acid testing of the cerebrospinal fluid (CSF) of these patients detected an enterovirus.

The Northern Territory of Australia has a population of 210,000 living in an area of 1.35 million km^2; the climate varies from desert and semiarid in the south to subtropical in the north. The northern part, known as the Top End, is characterized by several small urban centers and many small scattered indigenous communities with populations of 300–2,000. Darwin (population 100,000) is the major urban center. Approximately 30% of the population of the Northern Territory consists of indigenous Australians.

This cluster of meningitis cases coincided with an outbreak of another viral illness in the same and nearby communities; the illness affected mainly children and was characterized by fever, vomiting, and headache. During the next 4 months this epidemic febrile vomiting syndrome (EFVS) was reported in multiple communities in the northern part of the Northern Territory and eventually in Darwin. During the same period, clusters of viral meningitis were also being reported in some of the communities experiencing the EFVS.

We hypothesize that the cluster of viral meningitis cases and the EFVS were different manifestations of the same infection, caused by a strain of echovirus type 4 (E4) virus. This enterovirus was closely related to 2 E4 strains that caused a large outbreak of viral meningitis in the Yanbian prefecture of China in 1996 (*2*); ≈5,000 cases from a population of 2.16 million were identified.

Methods

Patients

A case of acute E4 enteroviral illness was defined as the detection, in 2007 in a resident of the Northern Territory, either of E4 in a CSF specimen or E4 in samples from another site during an illness characterized by fever and severe headache. Infants were children aged <1 year; children (including infants), <15 years of age; and adults, ≥15 years. Cases were included in our study only if specimens

Author affiliations: Centre for Disease Control, Darwin, Northern Territory, Australia (P.G. Markey); Royal Darwin Hospital, Darwin (J.S. Davis); and Queen Elizabeth II Medical Centre, Nedlands, Western Australia, Australia (G.B. Harnett, S.H. Williams, D.J. Speers)

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were collected in 2007 and samples from patients had a positive PCR or culture for E4 at the reference laboratory. A questionnaire was developed and details of cases were collected by a review of hospital case notes and, where possible, by telephone interviews with case-patients. The questionnaire documented clinical symptoms and signs together with laboratory results and risk factors, such as potential occupational exposure, child care, institutional exposure, or illness in the immediate family. Duration of illness was identified by discussion with the case-patient or, if the case-patient could not be contacted, was defined as the difference between date of symptom onset according to the medical record and date of hospital discharge.

The spread of the EFVS was investigated by asking senior clinic staff at all the remote community health centers in the regions affected about the local presence of a recent epidemic of fever, vomiting, and headache. If staff recalled such an epidemic, details were recorded about its timing, number of case-patients seen in the community health center, and the proportion of case-patients who were children. These interviews were all conducted within 2 months after the outbreak. Attack rates were calculated by using these estimates and the population of each community according to 2006 census data from the Australian Bureau of Statistics (*3*).

We collated data in Microsoft Excel 2000 (Redmond, WA, USA) and performed statistical analysis using STATA version 9.0 (StataCorp LP, College Park, TX, USA). Logistic regression was used to examine the relationship between the outcome variables (duration of illness, hospital admission, and length of stay) with the independent variables discussed below. Multivariate logistic regression models were built using a backwards stepwise approach. We compared categorical variables using the χ^2 test and continuous variables using the Wilcoxon rank-sum test. A p value <0.05 was considered significant.

Virus Isolation, Detection, and Identification

Feces samples were cultured for enterovirus by using a human diploid fibroblast cell line. Enterovirus molecular testing was performed directly on CSF samples, dry throat swabs, feces samples, and fecal cell culture supernatants that demonstrated a typical cytopathic effect. In-house seminested reverse transcription–PCR (RT-PCR) was used, which was specific for 2 regions of the 5′ untranslated region (UTR) of the enterovirus genome (*4*,*5*). This 2-region RT-PCR method detects a wide range of enteroviruses. CSF samples were also routinely tested for bacterial pathogens by culture and for herpes simplex virus by PCR.

Enterovirus genotyping was performed by direct sequencing of the viral protein (VP) 1 capsid coding gene. Total RNA was extracted from cell culture supernatant followed by RT-PCR amplification by using primers previously described (*6*). The products were sequenced on both strands by using the ABI Prism BigDye Terminator v3.1 system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequencing reactions were interrogated on an ABI Prism 3130XL 16-channel Genetic Analyzer (Applied Biosystems). The deduced sequence was compared for identification by alignment with enterovirus sequences available in GenBank by using BLASTn (http://blast.ncbi.nlm.nih.gov). The whole viral genome was sequenced by use of primers designed from the obtained sequences and from aligned GenBank E4 sequences. We performed phylogenetic analysis for the VP1 gene using MEGA version 3.0 software (www.megasoftware.net) by the neighbor-joining method with the Kimura 2-parameter model and 1,000 bootstrap replicates.

Results

We identified 95 cases of acute E4 viral illness. Seventy-six of these cases had a positive PCR CSF result; 8 also had virus detected in throat or feces samples. In the remaining 19, E4 was detected by PCR from a throat swab, a fecal specimen, or both. Records were reviewed on all cases, and interviews were conducted with 48 case-patients or a parent if the case-patient was a child. Interviews were not possible for most indigenous case-patients who lived in remote communities because of a lack of home telephones.

Approximately equal numbers of male and female patients were affected (Table 1); most affected children were male (M:F ratio 1.4), and most affected adults were female (M:F ratio 0.8). Forty-six percent of case-patients were indigenous Australians. From the beginning of July through the first week of October, most cases occurred in indigenous infants from remote communities in the Top End; from October through December, most were nonindigenous adults from urban Darwin and nearby communities (Figure 1). The pooled incidence in the 3 main affected regions was 59.1/100,000. Ages of case-patients ranged from 3 days to 56 years (median 12 years), with indigenous case-patients being significantly younger (p<0.0001). Twenty-eight

Table 1. Incidence of meningitis caused by echovirus type 4 virus, by age group and sex, Northern Territory, Australia, 2007

Case-patient age group, y	Case-patient sex		Total no. case-patients	Incidence*
	M	F		
<1	16	12	28	1,025.0
1–4	3	3	6	54.9
5–14	11	7	18	71.4
15–24	12	11	23	94.0
25–34	3	10	13	47.3
35–44	2	1	3	11.4
45–64	2	2	4	11.3
≥65	0	0	0	0.0
Total	49	46	95	59.1

*Per 100,000 population.

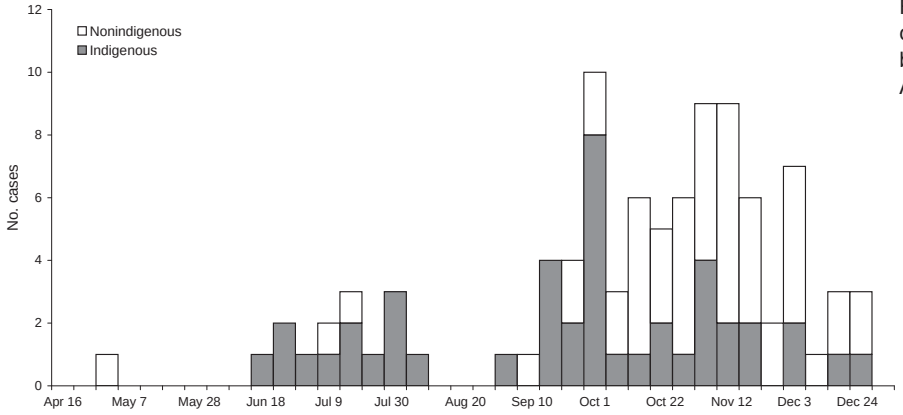

Figure 1. Indigenous and nonindigenous cases of echovirus type 4 virus illness, by week of onset, Northern Territory, Australia, 2007.

(30%) case-patients were <1 year of age, and 43 (45%) were >15 years of age. Incidence was highest in infants <1 year of age, followed by persons 15–24 years of age.

For adults, the most common symptom at onset was headache (70%); for infants and children, it was fever (48%). Common symptoms varied by age group (Table 2). For infants, symptoms were invariably fever and irritability, although for several, poor peripheral perfusion was noted and neonatal bacterial sepsis was clinically suspected. Children and infants were significantly more likely than adults to have fever ($p = 0.001$); infants were less likely than older children and adults to have vomiting ($p<0.0001$).

Duration of illness ranged from 3 to 28 days (median 7 days); length of hospital stay ranged from 0 to 10 days (median 3 days). Sequelae, reported for only 4 case-patients, included persistent headache and lethargy up to 4 weeks post onset, but all were mild. Recent similar but milder symptoms, such as fever and headache, were reported for immediate family members in 33 (56%) of 59 case-patients from whom information was available. We did not seek clinical details and investigations of these family members.

Lumbar puncture was performed on 82 case-patients. Fifty-nine (72%) CSF samples showed an increased leukocyte count, 45 (76%) of which had a predominance of mononuclear cells (55% of all samples). Of the 27 infants who had a lumbar puncture, 19 had normal microscopy without pleocytosis, despite all but 1 having the virus detected in the CSF. Initial peripheral leukocyte differential, available for 90 case-patients, was normal for 30 (33%); 50 (55%) had lymphopenia, 21 (23%) had neutrophilia, 2 (2%) had lymphocytosis, and 1 (1%) had neutropenia.

Multivariate analysis showed that of all the potential markers of severity (age, sex, indigenous status, high CSF protein, CSF pleocytosis, lymphopenia, and neutrophilia) only age was associated with admission to hospital and length of stay. The odds ratio of children being admitted to hospital compared with adults was 14.8 (95% confidence interval [CI] 3.2–69.3, $p<0.001$). When adjusted for

Table 2. Prevalence of clinical features and CSF abnormalities in persons affected by echovirus type 4 viral illness, by age group, Northern Territory, Australia, 2007*

Features	No. (%) case-patients by age group, y				Total no. (%) case-patients
	<1	1–4	5–14	≥15	
Clinical features	n = 28	n = 6	n = 18	n = 43	N = 95
Fever	28 (100)	6 (100)	17 (94)	32 (74)	83 (87)
Headache	0 (0)	4 (67)	17 (94)	43 (100)	64 (67)
Vomiting	5 (18)	6 (100)	17 (94)	35 (81)	63 (66)
Photophobia†	1 (4)	2 (40)	7 (39)	34 (79)	44 (47)
Diarrhea	16 (57)	0	2 (12)	18 (42)	36 (39)
Neck stiffness	0	1 (17)	9 (50)	21 (51)	31 (33)
Confusion/irritability	16 (57)	2 (33)	2 (11)	7 (17)	27 (29)
Rash	6 (22)	2 (33)	3 (19)	5 (12)	16 (17)
CSF abnormalities	n = 27	n = 5	n = 15	n = 35	n = 82
Glucose <2.7 mmol/L	7 (27)	0	0	3 (9)	10 (12)
Increased CSF protein‡	10 (39)	1 (20)	1 (7)	26 (74)	38 (47)
Leukocyte count >5 cells/mL	8 (30)	5 (100)	15 (100)	31 (90)	59 (72)
Leukocyte count >5 cells/mL and >50% monocytes	5 (19)	2 (40)	10 (67)	28 (80)	45 (55)

*CSF, cerebrospinal fluid.
†Presence or absence of clinical features was not discernible in all cases.
‡Age-specific normal values for protein were defined as follows: <1 month, <0.9 mg/L; 1–2 months, <0.77 mg/L; 3 months, 0.6 mg/L; and >3 months, 0.45

indigenous status and sex the odds ratio was 13.0 (95% CI 2.4–69.5, p = 0.003).

The EFVS affected 26 of the 28 communities contacted, and the median attack rate for children was 22% (range 2%–57%); the overall attack rate was 18.6%. Twenty-six cases of viral meningitis occurred in remote indigenous communities in the same area; 19 of these occurred within 3 weeks after the reported onset of the EFVS in their respective communities. Cases of proven viral meningitis in children from remote communities represented 2.0% of the estimated number of EFVS cases in children; however, >50% of these cases came from just 2 communities.

The epidemic began in the east of the northern part of the Northern Territory and progressed to the west over the next 6 months (Figure 2). Nevertheless, the earliest case of E4 meningitis was in April in a Darwin adult, who also reported similar illness in fellow workers.

Not all E4 case-patients exhibited the clinical features of meningitis. For example, 3 adults had neither meningeal symptoms nor CSF pleocytosis, but nevertheless had E4 detected in the CSF or feces. This finding suggests a spectrum of illness typical of enteroviruses, ranging from mild illness to meningitis, and supports the hypothesis that the EVFS was caused by the same virus.

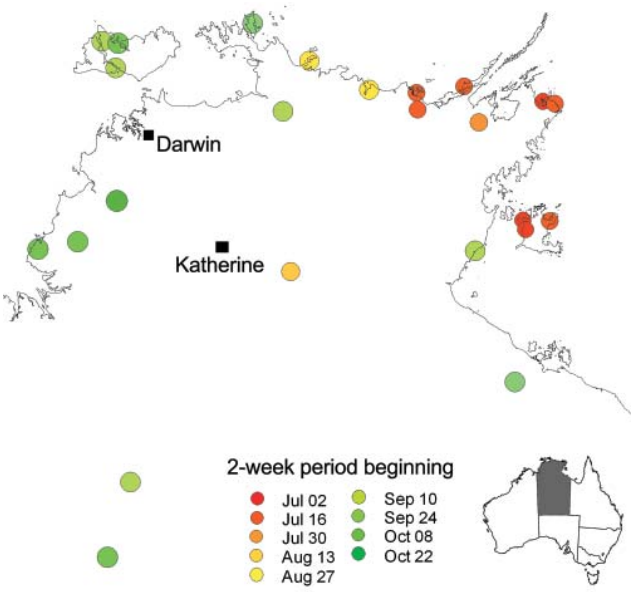

Figure 2. Northern part of the Northern Territory of Australia showing communities affected by the epidemic febrile vomiting syndrome, by week of epidemic peak.

Molecular Epidemiology

Product from the 5′ UTR PCRs was sequenced from all positive samples and by BLASTn search of the GenBank database; all had the closest homology to the Yanbian strains of echovirus type 4 (AF230973, AF233852). In addition, VP1 capsid coding gene sequences were obtained from 9 of the isolates, and all closely matched the AUS250G strain.

A GenBank BLASTn search showed that the whole genome nucleotide sequence of the E4 strain (AUS250G) had 84% homology with the Yanbian strains, and 81% homology with E4 Pesacek (AY302557), a strain isolated in the United States in 1951 (*8*). The AUS250G strain also had a 96% aa homology with the Pesacek strain, which is the only other complete E4 sequence in GenBank. Whole genome amino acid comparisons were not made with the Yanbian strains because amino acid translations of the Yanbian sequences were faulty, presumably due to nucleotide sequence errors. A phylogenetic tree of the complete VP1 nucleotide sequences (Figure 3) shows the relationship of AUS250G to other E4 strains, with the closest matches being a strain isolated in Shiga, Japan (AB166855) and the 2 Yanbian strains. The entire AUS250G genome sequence has been deposited in GenBank under accession no. FJ172447.

Discussion

This cluster of viral meningitis cases was discovered quickly and was able to be accurately mapped and described because of use of PCR testing of CSF samples. The overlapping symptomatology of both E4 meningitis and EFVS, together with the coincidence of cases of the former with outbreaks of the latter in remote communities and the high incidence of a similar illness in the relatives of meningitis cases, led us to conclude that the EFVS was a milder form of infection with the same virus. Furthermore, E4 was detected in specimens from several patients with fever and vomiting but with no clinical diagnosis of meningitis. The infection appears to have had a high attack rate, particularly in children in remote communities, and ≈2% of case-patients either developed meningitis or were systemically sick enough to be admitted to hospital.

Our estimate of the attack rate must be viewed with caution because we relied on clinic staff estimates of case numbers some weeks after the epidemic rather than through contemporaneous records. Given the time and resource constraints on staff in the remote setting, case numbers could have been overstated. Nevertheless, the EFVS and the high rates of viral meningitis in the community unquestionably stressed the healthcare system, particularly in remote communities. In addition, some schools in urban areas reported several cases of meningitis and absenteeism caused by viral illness in both staff and students, which led to anxiety in the school community. The news media was also interested and published reports of a “brain virus” infecting large numbers of people on the front page of the local newspaper (*9*).

An interesting feature of this study is the number of infants (19/27) whose CSF tested positive for E4 but

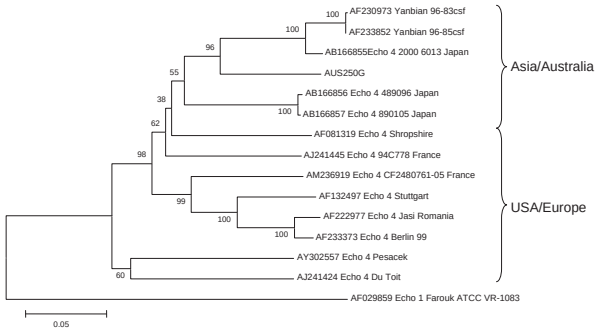

Figure 3. Phylogenetic tree of viral protein (VP) 1 gene sequences showing the relationship of the Australian echovirus type 4 virus (E4) isolate, AUS250G, to E4 strains, 2 Yanbian strains, and an echovirus type 1 sequence. The tree was constructed in MEGA version 3.0 software (www.megasoftware.net) using the neighbor-joining method with the Kimura 2-parameter model and 1,000 bootstrap replicates. Branch numbers represent bootstrap % values. Scale bar represents nucleotide substitutions per site.

lacked concomitant pleocytosis. This finding may have been due to our broad case definition and the diligence in investigating febrile neonates to exclude bacterial causes. The immaturity of the immune system in infants who were moderately ill with viremia could also have been a factor.

Previously reported outbreaks of enteroviral meningitis have demonstrated a predominance of males in childhood cases (*10–12*), and our study's findings were consistent with this. Also consistent with previous reports, a significant proportion (30%) of our case-patients were <1 year of age; however, ≈25% of the case-patients in our study were 15–24 years of age, an age group not previously recognized to be at risk.

Most of our cases occurred during July through December, which is late in the dry season of the local subtropical climate. Other reports from regions with temperate climates describe epidemics in the summer and autumn (*10–14*).

The spread of the viral illness across the Top End mirrors that reported in Spain in 1991 (*14*). Late in 2007 and early in 2008, spread of the syndrome and enteroviral meningitis into Western Australia and South Australia were reported. Additionally, 116 cases were subsequently laboratory confirmed from Western Australia with all 5′ UTR and 3 VP1 sequences matching the Northern Territory isolates, beginning in the eastern Kimberley region adjacent to the northern part of the Northern Territory and then spreading throughout most of the state. The E4 strain was detected by PCR from the CSF cases of meningitis and from throat swabs from EFVS patients in Western Australia.

From VP1 sequences the E4 strain was most closely related to a Japanese E4 strain and 2 Chinese E4 strains. No further clinical or sequence information is available for the Japanese strain, but the Chinese strains were responsible for a large outbreak of viral meningitis in the Yanbian prefecture of China in 1996 (*2*). Little epidemiologic information is available for this large outbreak, which affected thousands of persons. These authors reported that this outbreak was caused by a new enterovirus isolated from 23 CSF and feces samples. Our review of the whole genome sequence information deposited in GenBank demonstrates that the Yanbian strains and this strain are closely related and belong to the E4 serotype.

This E4 strain is therefore most closely related to other E4 strains from the Western Pacific Rim region. We could not conclude that this E4 enterovirus evolved from the Japanese or Yanbian viruses. However, this E4 strain could have either descended from 1 of these strains or shared a common ancestor virus due to their close VP1 genetic relatedness compared with other E4 strains. The large outbreaks of meningitis caused by both this E4 strain and the Yanbian E4 strains suggest a lack of previous immunity in the populations affected.

We have described an outbreak of human infection with E4 enterovirus in Australia. The high attack rate and the way in which it spread across the Northern Territory and into other states of Australia suggest it may be a newly introduced virus to which the local population has had no previous exposure. The virus likely has been introduced through the northern border where there is considerable human movement between the Northern Territory and the countries to the north. Our findings demonstrate the clinical spectrum of illness and geographic spread of E4 enterovirus infection and add to the knowledge of the molecular epidemiology of echoviruses.

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Dr Markey is a public health physician and Head of Disease Surveillance at the Centre for Disease Control in the Department of Health and Families, Northern Territory Government, Northern Territory, Australia. His background is in general practice, Aboriginal health, epidemiology, and communicable diseases.

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Address for correspondence: Peter G. Markey, Centre for Disease Control, PO Box 40596, Casuarina, 0811, Northern Territory, Australia; email: peter.markey@nt.gov.au

Methicillin-Resistant and -Susceptible *Staphylococcus aureus* Infections in Dogs

Meredith C. Faires, Michelle Traverse, Kathy C. Tater, David L. Pearl, and J. Scott Weese

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a pathogen of animals. To compare types of infections, clinical outcomes, and risk factors associated with MRSA in dogs with those associated with methicillin-susceptible *Staphylococcus aureus* (MSSA) infections, we conducted a case–control study at 3 veterinary referral hospitals in the United States and Canada during 2001–2007. Risk factors analyzed were signalment, medical and surgical history, and infection site. Among 40 dogs with MRSA and 80 with MSSA infections, highest prevalence of both infections was found in skin and ears. Although most (92.3%) dogs with MRSA infections were discharged from the hospital, we found that significant risk factors for MRSA infection were receipt of antimicrobial drugs (odds ratio [OR] 3.84, p = 0.02), β-lactams (OR 3.58, p = 0.04), or fluoroquinolones (OR 5.34, p = 0.01), and intravenous catheterization (OR 3.72, p = 0.02). Prudent use of antimicrobial drugs in veterinary hospitals is advised.

During the past 2 decades, methicillin-resistant *Staphylococcus aureus* (MRSA) has gained global attention as a human pathogen in hospitals and in communities. Recent reports of MRSA infection and colonization of dogs and cats (*1*–*5*) indicate that MRSA has apparently emerged as a pathogen of animals as well. Most reported MRSA infections in dogs have involved wound and postoperative infections (*2*), but evaluation is lacking regarding specific types of infections, clinical outcomes, and risk factors associated with such MRSA infections in dogs. No current evidence points to whether MRSA infections, in terms of location of infection, severity of disease, or clinical outcome, differ from methicillin-susceptible *S. aureus* (MSSA) infections.

The literature about human medicine has compared MRSA-infection risk factors (*6*,*7*), mortality rates (*6*,*8*–*10*), and clinical features (*6*) with those for MSSA infections. Results from a meta-analysis of 31 cohort studies showed that for patients with MRSA bacteremia, mortality rates were significantly higher than for patients with MSSA bacteremia (*11*). This mortality rate difference between MRSA and MSSA infections might result from treatment with inappropriate antimicrobial drugs or the restricted number of antimicrobial drugs available for treatment (*12*). With respect to animals, however, limited data are available; only 1 study has evaluated these MRSA and MSSA infections. Morris et al. (*13*) compared MRSA and MSSA infections in cats but were unable to detect significant differences in signalment, clinical presentations, or outcomes. Two other studies have reported potential risk factors associated with MRSA colonization in horses admitted to a veterinary referral hospital (*14*,*15*). Accurate data are needed to identify the differences between MRSA and MSSA infections in dogs as well as the clinical relevance of MRSA beyond concerns associated with antimicrobial drug resistance. To gain those data, epidemiologic research is required. Research is also required for proper medical treatment of dogs with MRSA infections, for counseling of clients of infected animals, and for elucidation of possible reasons for the emergence of MRSA in pets. Our study objectives were to compare the types of infections, clinical outcomes, and risk factors for MRSA and MSSA infections in dogs.

Author affiliations: University of Guelph, Guelph, Ontario, Canada (M.C. Faires, D.L. Pearl, J.S. Weese); University of Pennsylvania, Philadelphia, Pennsylvania, USA (M. Traverse); and Angell Animal Medical Center, Boston, Massachusetts, USA (K.C. Tater)

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Materials and Methods

Selection of Case-Patients and Controls

From 2001 through 2007, we conducted a retrospective, secondary-base, case–control study at 3 veterinary re-

ferral hospitals: the Ontario Veterinary College Veterinary Teaching Hospital (Guelph, Ontario, Canada), Matthew J. Ryan Veterinary Hospital of the University of Pennsylvania (Philadelphia, PA, USA), and Angell Animal Medical Center (Boston, MA, USA). These hospitals offer a variety of small animal medical specialties and services. Each year they receive ≈14,000, 30,000, and 50,000 patients, respectively. Each hospital used its microbiology database to identify MRSA and MSSA infections in dogs. Each dog with an identified MRSA infection (case-patient) was matched—by veterinary referral hospital and by date of admission—to 2 control dogs with MSSA (the dogs seen immediately before and after the dog with MRSA). Dogs merely colonized by MRSA or MSSA were excluded from analysis.

Data Collection

To collect information, we used medical records of all case-patient and control dogs to answer a pretested, standardized questionnaire. Data were collected on signalment, medical and surgical history, infection, hospital duration, and clinical outcome. Signalment data included breed, age, and sex. Medical and surgical history was limited to a 90-day period before admission to the referral hospital and included antimicrobial drug treatment, hospitalization, and surgical procedure. MRSA or MSSA infection data comprised site of infection and procedures performed before onset of infection, such as surgery, endoscopy, colonoscopy, intravenous catheterization, and urinary catheterization. Clinical outcome data covered whether surgery was required because of the infection and whether the animal was discharged, was euthanized, or died.

Because of a large number of categories, we recategorized several variables. Breed was categorized according to size based on weight: small (1–10 kg), medium (>10–25 kg), or large (>25 kg). Age was categorized as young (≤2 years), middle aged (>2–8 years), or old (>8 years). Site (where MRSA or MSSA was cultured) was categorized as skin, ear, urinary, skeletal, and other (abdominal fluid, thoracic fluid, blood, oral cavity, lymph node, vagina, transtracheal wash fluid, and milk). The number of days dogs were hospitalized was categorized as short (≤2 days), medium (3–7 days), or long (>7 days). Finally, specific antimicrobial drugs were grouped according to classes, i.e., aminoglycoside, β-lactam, chloramphenicol, fluoroquinolone, lincosamide, nitroimidazole, and tetracycline. For analytical purposes, dogs were subcategorized into 4 groups: did not receive any antimicrobial drug in the previous 90 days, received that specific class of antimicrobial drug, received another class of antimicrobial drug, and unknown status.

Statistical Analyses

All descriptive statistics, model building, and analyses were performed by using Stata 10.0 (StataCorp, College Station, TX, USA) and by using exact logistic regression. In Stata, the score method was used for calculating p values, and the group option was applied to account for matching (*16*). All tests were 2 sided, and significance was based on $p<0.05$. For predictor variables, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. For descriptive variables listed in the categories of signalment, medical and surgical history, and infection, the outcome was defined as having MRSA or MSSA infection. Variables in the clinical outcome category were modeled as dependent variables, and MRSA or MSSA infection was the independent variable. To avoid problems associated with colinearity, we performed a correlation analysis to identify pairs of predictor variables that had high colinearity ($|r|\geq 0.8$). We did not construct a multivariable model because of the relatively small sample size, resultant concerns of stability, and problems associated with overfitting. Consequently, we constructed only univariable models.

Results

A total of 40 MRSA case-patients and 80 MSSA controls were eligible for inclusion. From each hospital, the number of case-patients and controls were, respectively, 7 and 14 (Ontario Veterinary College), 20 and 40 (Matthew J. Ryan Veterinary Hospital), and 13 and 26 (Angell Animal Medical Center).

Breed distribution was categorized according to weight (Table 1). Ages of MRSA case-patients ranged from 1 to 13 years (mean 5.6 and median 5.0 years). Ages of MSSA control dogs ranged from 6 months to 16 years (mean 6.8 and median 7.0 years). No distinction was made between intact or sterilized dogs. Overall, no significant differences appeared between case-patients and controls with respect to breed ($p = 0.18$), age ($p = 0.50$), or sex ($p = 0.29$).

Regarding previous hospitalization or surgical procedures, no overall significant differences appeared between the MRSA and MSSA groups ($p = 0.62$ and 0.40, respectively). Results from the univariable analysis (Table 2) indicate that receipt of antimicrobial drugs (OR 3.84, 95% CI 1.21–14.74, $p = 0.02$), β-lactams (OR 3.58, 95% CI 1.04–14.79, $p = 0.04$), or fluoroquinolones (OR 4.61, 95% CI 1.08–27.37, $p = 0.02$), within 90 days before admission was significantly associated with a MRSA infection. Furthermore, when fluoroquinolones and β-lactams were included in the "other classes" category, the odds of a dog having MRSA versus MSSA infection increased over odds for dogs that had not received antimicrobial drugs (Table 2).

Most MRSA and MSSA infections were located on the skin. Overall, with regard to infection site, we found no significant difference between the MRSA and MSSA groups ($p = 0.50$) (Table 3). Before onset of the MRSA and MSSA infections, the most common procedure was intravenous catheterization—a significant risk factor for a MRSA in-

Table 1. Univariable analysis of demographic (signalment) risk factors for MRSA versus MSSA infections in dogs, United States and Canada, 2001–2007*

Variable	MRSA, no. (%) dogs, n = 40	MSSA, no. (%) dogs, n = 80†	Odds ratio (95% CI)	p value‡
Breed, kg				
Small, 1–10	10 (25)	11/79 (13.9)	Ref	
Medium, >10–25	16 (40)	28/79 (35.4)	0.63 (0.19–2.01)	0.43
Large, >25	14 (35)	40/79 (50.6)	0.39 (0.12–1.25)	0.10
Age group, y				
≤2	10 (25)	13 (16.3)	Ref	
3–8	20 (50)	43 (53.8)	0.63 (0.22–1.78)	0.34
>8	10 (25)	24 (30)	0.54 (0.15–1.91)	0.39
Sex				
F	14 (35)	36 (45)	Ref	
M	26 (65)	44 (55)	1.47 (0.65–3.45)	0.35

*MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; CI, confidence interval; Ref, referent category. Dogs with MRSA (case-patients) and MSSA (controls) infections were matched for veterinary referral hospital and date of admission.
†Except as indicated.
‡Score method for estimating p values does not assume a symmetrical distribution for discrete data. p<0.05 was considered significant.

fection (OR 3.27, 95% CI 1.14–10.65, p = 0.02). Neither colonoscopy nor endoscopy was performed on any animal. Dogs with MRSA infection were hospitalized 0–29 days (mean 3.4 and median 1.5 days), whereas dogs with MSSA infection were hospitalized 0–13 days (mean 2.0 and median 0 days). Overall, in terms of duration of hospitalization, we found no significant difference between case-patients and controls (p = 0.49).

Surgery was required for treatment of 16 (40.0%) of 40 dogs with MRSA infection and 34 (42.5%) of 80 dogs with MSSA infection. Most dogs with MRSA and MSSA infections were discharged from the hospital (Table 4). For all dogs in the MRSA and MSSA groups that were euthanized, the infection was reported as the attributed cause of death. Overall, no significant differences were noted between case-patients and controls with regard to surgery (p = 0.79) or outcome (p = 0.64).

Discussion

The identification of receipt of antimicrobial drugs—specifically β-lactams and fluoroquinolones—as risk factors for a MRSA infection was not unexpected. Data from human medicine and a logical hypothesis each indicate that antimicrobial drug use in animals would increase the likelihood of selection for multidrug-resistant bacteria such as MRSA. The case and control dogs included in this study were from veterinary referral hospitals; that is, tertiary care facilities that manage complicated medical and surgical cases referred from other veterinary facilities where treatment, surgery, or both might have been initiated. This study identified the highest prevalence of MRSA and MSSA infections from the skin (pyoderma) and ears (otitis), which in dogs are frequently treated with β-lactams and fluoroquinolones, respectively (*17*). Moreover, these conditions can become chronic and can result in repeated or prolonged antimicrobial drug treatments that might select for the development of antimicrobial drug resistance (*18*). Before admission to the referral hospitals, >50% of dogs with MRSA infection were given antimicrobial drugs from the β-lactam family. Methicillin resistance in staphylococci involves the *mecA* gene, which encodes for the penicillin-binding protein 2a and results in reduced affinity for all β-lactam antimicrobial drugs. Thus, medical management of MRSA cases can become complicated and can result in the administration of various classes of antimicrobial drugs (some of which can be ineffective), especially when culture and susceptibility testing have not been conducted.

In small animal medicine, fluoroquinolones are commonly used because of their activity against a wide range of bacteria and their ability to be given orally (*19*). In humans, administration of antimicrobial drugs, including macrolides (*7*), β-lactams (*20*), and fluoroquinolones (*7*,*21*), has been associated with increased risk for development of nosocomial MRSA infections compared with nosocomial MSSA infections. Specifically, use of fluoroquinolones has been positively correlated with the incidence of hospital-associated MRSA (*22*) infections. In addition to the direct effect of antimicrobial drugs on selection for antimicrobial drug–resistant organisms, other mechanisms could facilitate emergence of MRSA during fluoroquinolone treatment. Research performed by Bisognano et al. (*23*) demonstrated that fluoroquinolone-resistant MRSA and MSSA isolates exposed to subinhibitory levels of ciprofloxacin resulted in increased production of binding proteins, leading to higher levels of bacterial attachment. Thus, exposure to fluoroquinolones might promote the attachment of *S. aureus* while eradicating MSSA strains and might therefore promote acquisition of MRSA strains (*24*).

In our study, information pertaining to antimicrobial drug exposure in the 90 days before admission to the veterinary referral hospital was selected because that period was sufficient for adequate review of medical charts. In the literature, periods for antimicrobial drug exposure as a risk factor for MRSA acquisition have ranged from 1 to

Table 2. Univariable analysis of medical and surgical risk factors for MRSA versus MSSA infections in dogs, United States and Canada, 2001–2007*

Variable†	MRSA, no. (%) dogs, n = 40	MSSA, no. (%) dogs, n = 80	Odds ratio (95% CI)	p value‡
Received antimicrobial drugs				
No	8 (20.0)	30 (37.5)	Ref	
Yes	26 (65.0)	33 (41.3)	3.84 (1.21–14.74)	0.02
Don't know	6 (15.0)	17 (21.2)	1.29 (0.28–5.65)	0.75
Received ≥2 antimicrobial drugs				
No	25 (62.5)	56 (70.0)	Ref	
Yes	9 (22.5)	7 (8.8)	2.87 (0.81–11.49)	0.08
Don't know	6 (15.0)	17 (21.2)	0.79 (0.19–2.79)	0.78
Received an aminoglycoside				
No	8 (20.0)	30 (37.5)	Ref	
Yes	1 (2.5)	1 (1.3)	5.71 (0.06–517.45)	0.30
Other classes§	25 (62.5)	32 (40.0)	3.80 (1.19–14.62)	0.02
Don't know	6 (15.0)	17 (21.2)	1.29 (0.28–5.62)	0.75
Received a β-lactam				
No	8 (20.0)	30 (37.5)	Ref	
Yes	18 (45.0)	25 (31.3)	3.58 (1.04–14.79)	0.04
Other classes	8 (20.0)	8 (10.0)	4.18 (0.96–20.88)	0.04
Don't know	6 (15.0)	17 (21.2)	1.31 (0.28–5.69)	0.75
Received chloramphenicol				
No	8 (20.0)	30 (37.5)	Ref	
Yes	1 (2.5)	2 (2.5)	2.61 (0.04–65.5)	1.00
Other classes	25 (62.5)	31 (38.8)	3.84 (1.21–14.74)	0.02
Don't know	6 (15)	17 (21.2)	1.29 (0.28–5.65)	0.75
Received a fluoroquinolone				
No	8 (20.0)	30 (37.5)	Ref	
Yes	9 (22.5)	7 (8.8)	5.34 (1.24–27.38)	0.01
Other classes	17 (42.5)	26 (32.5)	3.24 (0.94–13.2)	0.06
Don't know	6 (15.0)	17 (21.2)	1.31 (0.29–5.74)	0.75
Received a lincosamide				
No	8 (20.0)	30 (37.5)	Ref	
Yes	2 (5.0)	2 (2.5)	4.43 (0.27–75.86)	0.19
Other classes	24 (60.0)	31 (38.8)	3.77 (1.18–14.53)	0.02
Don't know	6 (15.0)	17 (21.2)	1.32 (0.29–5.73)	0.75
Received a nitroimidazole				
No	8 (20.0)	30 (37.5)	Ref	
Yes	2 (5.0)	0	7.18 (0.53–∞)	0.07
Other classes	24 (60.0)	33 (41.3)	3.47 (1.08–13.33)	0.03
Don't know	6 (15.0)	17 (21.2)	1.38 (0.29–6.17)	0.74
Received a tetracycline				
No	8 (20.0)	30 (37.5)	Ref	
Yes	2 (5.0)	1 (1.3)	6.63 (0.29–463.75)	0.17
Other classes	24 (60.0)	32 (40.0)	3.54 (1.11–13.65)	0.03
Don't know	6 (15.0)	17 (21.2)	1.33 (0.29–5.88)	0.74
Hospitalized				
No	16 (40.0)	38 (47.5)	Ref	
Yes	16 (40.0)	25 (31.3)	1.54 (0.58–4.14)	0.37
Don't know	8 (20.0)	17 (21.3)	1.17 (0.32–4.06)	0.08
Underwent surgical procedure				
No	25 (62.5)	46 (57.5)	Ref	
Yes	10 (25.0)	16 (20.0)	1.07 (0.39–2.83)	1.00
Don't know	5 (12.5)	18 (22.5)	0.45 (0.09–1.68)	0.26

*MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; CI, confidence interval; Ref, referent category. Dogs with MRSA (case-patients) and MSSA (controls) infections were matched for veterinary referral hospital and date of admission.
†Information obtained refers to the 90 days before admission to the veterinary referral hospital.
‡Score method for estimating p values does not assume a symmetrical distribution for discrete data. $p<0.05$ was considered significant.
§Refers to dogs given antimicrobial drugs from other drug classes.

Table 3. Univariable analysis of infection site, duration of hospitalization, and medical and surgical risk factors for MRSA versus MSSA infections in dogs, United States and Canada, 2001–2007*

Variable	MRSA, no. (%) dogs, n = 40	MSSA, no. (%) dogs, n = 80†	Odds ratio (95% CI)	p value‡
Site of infection				
Skin	19 (47.5)	38/78 (48.7)	Ref	
Ear	5 (12.5)	11/78 (14.1)	0.89 (0.21–3.28)	1.00
Skeletal§	7 (17.5)	6/78 (7.7)	2.69 (0.53–17.96)	0.23
Urinary¶	3 (7.5)	11/78 (14.1)	0.37 (0.03–2.20)	0.29
Other#	6 (15.0)	12/78 (15.4)	1.06 (0.24–4.14)	1.00
Duration of hospitalization				
Short (≤2 d)	25 (62.5)	57 (71.3)	Ref	
Medium (3–7 d)	11 (27.5)	19 (23.8)	1.59 (0.52–4.94)	0.45
Long (>7 d)	4 (10.0)	4 (5.0)	2.70 (0.43–17.49)	0.23
Intravenous catheterization**				
No	21 (52.5)	58 (72.5)	Ref	
Yes	19 (47.5)	22 (27.5)	3.27 (1.14–10.65)	0.02
Surgery**				
No	25 (62.5)	58 (72.5)	Ref	
Yes	15 (37.5)	22 (27.5)	1.61 (0.65–4.09)	0.29
Urinary catheterization**				
No	36 (90.0)	77 (96.3)	Ref	
Yes	4 (10.0)	3 (3.8)	6.00 (0.48–314.98)	0.11

*MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; CI, confidence interval; Ref, referent category. Dogs with MRSA (case-patients) and MSSA (controls) infections were matched for veterinary referral hospital and date of admission.
†Except as indicated.
‡Score method for estimating p values does not assume a symmetrical distribution for discrete data. p<0.05 was considered significant.
§Specimens were from internal joint surface, joint fluid, intramedullary pin, and orthopedic implant.
¶Specimens were urine, urinary calculus, urinary catheter, and the wall of the urinary bladder.
#Specimens were abdominal and thoracic fluids, blood, oral cavity swabs, lymph nodes, vaginal swabs, transtracheal wash fluid, and milk.
**Procedures performed before infection occurred.

12 months (*25*). Future studies with larger datasets might investigate the effect of varying time frames with respect to antimicrobial drug administration.

In dogs, identification of intravenous catheterization as a risk factor for MRSA infection was not unexpected. Intravenous catheterization has been associated with increased rates of MRSA infections in humans (*26*) and has been significantly associated with death of horses with MRSA infections (*27*). In previous studies as well as ours, however, intravenous catheterization might reflect a consequence of MRSA infection rather than a risk factor for development of MRSA infection.

Overall and with respect to outcome (discharged vs. euthanized), no significant differences between MRSA and MSSA infections were found. This finding is relevant for counseling clients, particularly considering the publicity regarding MRSA and the possible perception that MRSA infections are untreatable or carry a poor prognosis. Numerous studies in human medicine have compared mortality rates associated with MRSA and MSSA infections, but the results have been conflicting (*6*,*8*,*10*,*11*). Wang et al. (*6*) were unable to detect an association between higher mortality rates in patients with community-associated MRSA infections than in those with community-associated MSSA infections. Melzer et al. (*10*) were unable to demonstrate that mortality rates for patients with hospital-associated MRSA infections were significantly higher than those for patients with hospital-associated MSSA infections. Conversely, results from a retrospective cohort study conducted by Wang et al. (*8*), indicated that the mortality rate for patients with hospital-associated MRSA bacteremia was 1.78× higher than that for hospital-associated MSSA bacteremia.

In our study, inadequate epidemiologic definitions and veterinary surveillance data prevented us from classifying MRSA and MSSA infections as hospital or community as-

Table 4. Clinical outcome characteristics for dogs with MRSA and MSSA infections, United States and Canada, 2001–2007*

Variable	MRSA, no. (%) dogs, n = 40	MSSA, no. (%) dogs, n = 80†	Odds ratio (95% CI)	p value
Surgery required because of infection				
No	24 (60)	46 (57.5)	Ref	
Yes	16 (40)	34 (42.5)	0.89 (0.37–2.12)	0.84
Outcome				
Discharged	36/39 (92.3)	69/77 (89.6)	Ref	
Euthanized	3/39 (7.7)	8/77 (10.4)	0.63 (0.06–4.10)	0.71

*MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; CI, confidence interval; Ref, referent category. Dogs with MRSA (case-patients) and MSSA (controls) infections were matched for veterinary referral hospital and date of admission.

sociated. Nevertheless, other possible explanations as to why mortality rates between case-patients and controls did not differ significantly might in part be the predominant infection types and the retrospective aspect of the study. Most MRSA and MSSA infections were pyodermas and otitis externa or interna infections, which are superficial, rarely become invasive, and seldom result in death. Consequently, infection types for which death would be a more realistic possible outcome were limited, resulting in a corresponding limitation in statistical power. Comparison of mortality rates between patients with MRSA or MSSA infections would be best performed among only those with invasive infections and should be considered for future studies. Here, mortality rate information was obtained retrospectively and only recorded up to the time of discharge. Thus, whether dogs died from their infections after discharge from the referral hospital, causing an underestimate of deaths, is unknown.

Although our study was larger than previous studies, the power was still limited despite enrollment of 2 MSSA controls per each MRSA case-patient. Additional limitations were enrollment of case-patients and controls from referral hospitals and the use of matching. Because dogs in this study were from referral hospitals, extrapolation of results to the general dog population might be biased. In general veterinary practice, antimicrobial drug use, hospitalization, surgical procedures, and specific medical and surgical cases might differ considerably from those in referral hospitals. The incomplete medical records that accompanied case-patients and controls from referral hospitals might have affected responses to questions regarding previous medical or surgical procedures and antimicrobial drug use, all of which might have affected the results. Other potential risk factors such as underlying illnesses, admitting service, hospitalization locations (i.e., intensive care unit vs. hospitalization ward), and treatment cost were not investigated but could play a role in the development and outcome of MRSA infections in dogs. Finally, by using matching to control for potential confounders, the matched factors—date of admission and referral hospital—precluded the investigation of these variables as potential risk factors for a MRSA infection.

Despite these limitations, however, we found no identifiable differences between MRSA and MSSA infections in dogs with regard to signalment, types of infections, and clinical outcome. The prognosis for a dog with a MRSA infection is reasonably good. However, when determining that prognosis and when counseling owners, veterinarians should focus on the location and severity of infection rather than the bacterium involved. Furthermore, administration of β-lactams and fluoroquinolones were significant risk factors for the development of a MRSA infection. This finding strengthens the need for veterinarians to consider prudent antimicrobial drug–use guidelines and to restrict the use of fluoroquinolones as empirical or first-line therapy. Guidelines should recommend identification and susceptibility of the causal bacterial pathogen by performing a culture and susceptibility test. On the basis of susceptibility results, antimicrobial drugs should be dispensed at the proper dosage and duration for treatment and, in the absence of clinical disease, should not be prescribed.

Although only 4 risk factors were identified as being significantly associated with MRSA infection, results from the univariable analyses isolated several risk factors that have considerably large odds ratios or p values slightly greater than 0.05. With the exception of fluoroquinolones and β-lactams, measure of association for all other antimicrobial drug classes was reasonably higher for those dogs given specific antimicrobial drugs compared with those that were not. Because of the small sample size, however, the power of these associations was limited.

Our study shows that MRSA is an emerging pathogen in dogs, and risk factors for MRSA infection are similar to those identified in humans. Results from larger studies in the future might indicate that other classes of antimicrobial drugs, previous hospitalization and surgery, age, and the presence of a urinary catheter are also significantly associated with MRSA infections. Only larger sample sizes will provide more information on MRSA and MSSA infections and will determine more accurately other risk factors associated with MRSA infections in dogs.

Dr Faires is a veterinarian and a PhD student in the Department of Population Medicine at the Ontario Veterinary College, University of Guelph. Her primary research interests include the epidemiology of MRSA in people and animals.

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Address for correspondence: Meredith C. Faires, Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1, Canada; email: mfaires@uoguelph.ca

Actinobaculum schaalii, a Common Uropathogen in Elderly Patients, Denmark

Steffen Bank, Anders Jensen, Thomas M. Hansen, Karen M. Søby, and Jørgen Prag

Actinobaculum schaalii can cause urinary tract infections and septicemia but is difficult to identify by cultivation. To obtain a fast diagnosis and identify *A. schaalii*, we developed a TaqMan real-time quantitative PCR. Routine urine samples were obtained from 177 hospitalized patients and 75 outpatients in Viborg County, Denmark, in 2008–2009. The PCR detected *A. schaalii* in 22% of samples from patients >60 years of age. This assay showed that *A. schaalii* is more common than implied by routine cultivation. In 90% of PCR-positive urine samples, other common uropathogens were identified. This finding suggests that *A. schaalii* is a common, undetected, bacterial pathogen. Our results suggest that *A. schaalii* may be a more common pathogen than previously thought, especially in patients with unexplained chronic urinary tract infections, who are often treated with trimethoprim or ciprofloxacin, to which *A. schaalii* is resistant.

Actinobaculum schaalii was first described in 1997 and named after Klaus P. Schaalii, a German microbiologist specializing in actinomycete microbiology. The genus *Actinobaculum* includes *A. schaalii*, *A. suis*, *A. massiliae*, and *A. urinale* and is closely related to the genera *Actinomyces* and *Arcanobacterium* (*1*).

These bacteria are small, gram-positive, facultative anaerobic, CO_2-requiring coccoid rods. They grow as dimorphic gray colonies <1 mm in diameter, are nonmotile and non–spore forming, and show weak β-hemolysis on agar plates containing 5% horse or sheep blood after 3–5 days of growth. They are catalase, oxidase, and urease negative and resistant to trimethoprim and ciprofloxacin (*2*). Their habitat is probably the human genital or urinary tract (*1*).

Because of its slow growth and resemblance to the normal bacterial flora on skin and mucosa, *A. schaalii* is often overlooked or considered a contaminant. Furthermore, it is often overgrown by faster-growing commensal and pathogen bacteria. Most laboratories incubate urine samples only overnight in ambient air, which further impedes isolation of *A. schaalii* (*2*).

Difficulties identifying *A. schaalii* by using traditional phenotypic tests have obscured its pathologic role for many years. However, *A. schaalii* can cause urinary tract infections (UTIs), some of which lead to serious illnesses such as urosepsis, osteomyelitis, and septicemia, mainly among the elderly and patients predisposed to UTIs (*1*–*6*). We developed a TaqMan real-time quantitative PCR (qPCR) specific for the gyrase B (*gyrB*) gene for fast and sensitive detection of *A. schaalii* from urine and blood samples.

Materials and Methods

Patient and Control Groups

From October 2008 through January 2009, a total of 252 routine urine samples were randomly selected from patients of all ages from 3 hospitals and 150 medical practitioners in Viborg County, Denmark (population ≈230,000 persons). Seventy percent of patients were from hospitals. Urine collection was midstream, from bedpans, from catheters, or unspecified in 41%, 19%, 18%, and 21% of cases, respectively. A total of 38 control urine samples were obtained from patients before they underwent elective surgery of hips or knees. These patients were 63–81 years of age and had negative results for leukocyte esterase and nitrate by a urine dipstick test (Roche Diagnostics Ltd., Burgess Hill, UK).

Author affiliations: Viborg Hospital, Viborg, Denmark (S. Bank, T.M. Hansen, K.M. Søby, J. Prag); and Aarhus University, Aarhus, Denmark (A. Jensen)

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Cultures and Wet Smear Microscopy of Urine Samples

Samples tested by using PCR were simultaneously analyzed by using standard laboratory tests. These tests were wet smear microscopy and incubation on 5% Columbia sheep blood agar (Becton Dickinson, Heidelberg, Germany) in an atmosphere of 5% CO_2 at 35°C for 1 or 2 days.

Extraction of DNA

Bacteria were incubated anaerobically on 5% Columbia sheep blood agar in an atmosphere of CO_2 at 35°C for 2 days before harvesting. DNA was purified by taking a swab of bacteria from the agar plate and transferring it to 1 mL of saline. The DNA from bacteria was extracted from 800 μL of saline by using the Kingfisher mL magnetic particle processor (Thermo Electron Corporation, Waltham, MA, USA) according to the manufacturer's instructions, eluted in 100 μL elution buffer, and stored at 4°C until use. DNA was also obtained from 800-μL urine samples as described above.

Sequencing

Fourteen *A. schaalii* strains, including reference strain CCUG 27420, were used for sequencing. Universal primer pair UP-1 and UP-2r was used to amplify the *gyrB* gene from *A. schaalii* (Table 1). PCR was performed as described by Yamamoto and Harayama. (*7*). The PCR product was then gel purified by using the QIAquick Gel Extraction Kit (QIAGEN, Hilgen Germany) and sequenced in an ABI 3130 XL genetic analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. Sequencing primers UP-1S and UP-2Sr (Table 1) were used to sequence the purified PCR product in both directions. Primers were synthesized by DNA Technology (Aarhus, Denmark).

Primers and Probe

Sequence alignment editor BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) programs were used to design a primer and probe specific for *A. schaalii* by multiple alignment of *gyrB* sequences from 14 *A. schaalii* strains, including reference strain CCUG 27420. Potential primers and probe were analyzed for the requirements imposed by real-time PCR by using PrimeQuest (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) and mfold (www.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi) programs. Selected primers and probe were analyzed for specificity against GenBank sequences by using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The primer pair A.s-forward 5′-GGCCATGCAG TGGACCTC-3′ and A.s-reverse 5′-GCACATCATCA CCGGAAAGA-3′ amplified a 185-bp fragment. The probe 5′-TCCGAATCGGTCAATACCTTCGC-3′ was labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with Black Hole Quencher 1. Primers and probe were synthesized by Sigma-Aldrich (St. Louis, MO, USA).

TaqMan qPCR

PCR amplification was performed by using a Mx3000P Real Time PCR System (Stratagene, La Jolla, CA, USA) in a 25-μL reaction volume. The PCR mixture contained 12 μL of 2× Brilliant QPCR Master Mixture (Stratagene), 2.5 μL of 100 nmol/L (final concentration) TaqMan probe, 2 μL of 200 nmol/L (final concentration) forward and reverse primers, and 5 μL of template DNA. An internal control containing 1.25 μL of internal PCR control primer/probe mixture and 0.25 μL of internal PCR control DNA (Applied Biosystems) was also used. Samples were incubated for 1 cycle at 95°C for 2 min and 50 cycles at 95°C for 30 s and 60°C for 60 s. All samples were run in duplicate. DNA from *A. schaalii* CCUG 27420 was used as a positive control and was included in each PCR. Sterile water was used as a negative control. Results were analyzed by using the Mx3000P software package (Stratagene).

Detection Limit and Quantification

The detection limit of the *A. schaalii gyrB* assay was determined by using a 10-fold serial dilution of known concentrations (1.5×10^1 to 1.5×10^8 CFU/mL) of *A. schaalii* CCUG 27420. Quantification of *A. schaalii* in urine samples was performed by using the same dilution series.

Analytical Specificity

To determine the analytical specificity of the assay, we tested 36 clinical strains of *A. schaalii* and strain CCUG 27420. Phylogenetically related (*1*) and clinically relevant bacterial strains, including several *Actinomyces* spp., *Ar-*

Table 1. Sequences of primers and probe used for identification of *Actinobaculum schaalii*, Denmark, 2008–2009

Primer or probe	Sequence (5′ → 3′)
UP-1	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA
UP-2r	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT
UP-1S	GAAGTCATCATGACCGTTCTGCA
UP-2Sr	AGCAGGGTACGGATGTGCGAGCC
A.s-forward	GGCCATGCAGTGGACCTC
A.s-reverse	GCACATCATCACCGGAAAGA
A.s-probe	TCCGAATCGGTCAATACCTTCGC

canobacterium spp., and reference strains *A. suis* CCUG 19026, *A. urinale* CCUG 46093, and *A. massiliae* CCUG 47753, were also tested (Table 2).

Verification of TaqMan qPCR Assay Results

To verify results of this assay, 6 PCR products were sequenced. The first 15 PCR-positive urine samples were cultivated, and isolates were identified as described by Reinhard et. al. (*2*). Identity of isolated *A schaalii* strains was confirmed by using a qPCR.

Purification of DNA from Blood Cultures

Ten milliliters of blood and 1 mL of culture containing 2×10^7, 2×10^5, 2×10^3, and 2×10^1 CFU/mL of *A. schaalii* reference strain CCUG 27420 were added to aerobic and anaerobic BACTEC culture vials (Becton Dickinson). DNA from bacteria-positive blood cultures was extracted from 800 μL of aerobic or anaerobic media and purified by using the Kingfisher processor as described above.

Because BACTEC culture vials contain sodium polyanetholesulfonate (SPS), a known PCR inhibitor, either DNA must be purified from BACTEC culture vials by using specific purification methods or purified DNA must be diluted to prevent the SPS from inhibiting the PCR (*8*). Ten-fold serial dilutions of purified DNA from positive BACTEC culture vials were made and tested by using the qPCR as described above. DNA was extracted from an anaerobic BACTEC culture vial from a patient sample from which *A. schaalii* had been isolated by cultivation.

Statistical Analysis

The χ^2 test was used to analyze differences in detection of *A. schaalii*. Statistical analyses were performed by using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Cultivation of PCR-Positive Samples

Isolates were obtained from 7 of the 15 urine samples cultured. The 7 isolates were confirmed positive by our real-time PCR.

Detection Limit and Analytical Specificity

Assay results were linear at bacterial concentrations from 1.5×10^4 to 1.5×10^8 CFU/mL with an R^2 value of 1.000 ($Y = -3.296 \times \log(X) + 25.96$). The detection limit of the assay was between 1.5×10^3 and 1.5×10^4 CFU/mL, which corresponds to 7.5–75 CFU/reaction. The assay amplified DNA from all 37 isolates of *A. schaalii* tested. No PCR amplification signal was detected when other species were tested (Table 2).

DNA Sequencing Analysis

The 6 PCR products amplified from bacteria-positive urine samples had the expected size. Sequence alignment of the 6 PCR products showed homology to the sequenced *gyrB* gene from *A. schaalii* strains.

Identification of *A. schaalii* from Blood Cultures

The 2 anaerobic BACTEC culture vials to which 1 mL of 2×10^7 CFU/mL and 2×10^5 CFU/mL had been added and 1 aerobic BACTEC culture vials to which 1 mL of 2×10^7 CFU/mL had been added showed

Table 2. Species used to test analytical specificity of gyrase B real-time PCR for *Actinobaculum schaalii*, Denmark, 2008–2009

Species	Source
Actinobaculum spp.	
A. schaalii	CCUG 27420*
A. schaalii†	Clinical isolates‡
A. massiliae	CCUG 47753
A. suis	CCUG 19026
A. urinale	CCUG 46093
Actinomyces spp.	
A. gerencseriae	Clinical isolates
A. graevenizii	Clinical isolates
A. israelii	Clinical isolates
A. meyeri	Clinical isolates
A. naeslundii	Clinical isolates
A. neuii	Clinical isolates
A. odontolyticus	Clinical isolates
A. radingae	Clinical isolates
A. turicencis	Clinical isolates
A. urogenitalis	Clinical isolates
A. viscosus	Clinical isolates
Arcanobacterium spp.	
A. bernardiae	Clinical isolates
A. hemolyticum	Clinical isolates
A. pyogenes	Clinical isolates
Other spp.	
Gardnerella vaginalis	Clinical isolates
Rothia dentocariosa	Clinical isolates
Common uropathogens	
Alcaligenes faecalis	Clinical isolates
Candida albicans	Clinical isolates
Citrobacter koseri	Clinical isolates
Escherichia coli	Clinical isolates
Hemolytic streptococcus group A	Clinical isolates
Hemolytic streptococcus group B	Clinical isolates
Klebsiella oxytoca	Clinical isolates
K. pneumoniae	Clinical isolates
Nonhemolytic streptococci	Clinical isolates
Proteus mirabilis	Clinical isolates
Proteus vulgaris	Clinical isolates
Pseudomonas aeruginosa	Clinical isolates
Staphylococcus aureus	Clinical isolates
Staphylococcus epidermidis	Clinical isolates

*GenBank accession no. FJ209064.
†Thirty-six isolates.
‡GenBank accession nos. FJ518817–FJ518825.

positive results in the BACTEC 9240 blood culture system. There was no growth recorded with lower inoculum concentrations.

PCR with undiluted and 10-fold diluted DNA was inhibited, probably by SPS. However, the 100-fold dilution of purified DNA from the 2 anaerobic and 1 aerobic BACTEC culture vials was PCR positive. The 100-fold dilution of purified DNA from a positive anaerobic BACTEC culture vial (patient specimen) was also PCR positive.

Analysis of Urine Samples

Of 252 urine samples, 41 (16%) were PCR positive with bacterial concentrations $>10^4$ CFU/mL. Of 155 urine samples from patients >60 years of age, 34 (22%) were PCR positive (Table 3), of which 31 (91%) harbored other common uropathogenic bacteria in addition to *A. schaalii* (Table 4). Species distribution of these common uropathogenic bacteria was comparable to that found in our microbiology department throughout the year. Treatment with antimicrobial drugs before specimens were obtained was reported by 19% of the patients.

The 41 PCR-positive urine samples were collected midstream from 37% of patients, from bedpans for 27%, from catheters for 12%, and by an unspecified method for 24%. Among 177 hospitalized patients, 18% of samples from 104 patients >60 years of age and 10% of samples from 73 patients ≤60 years of age were PCR positive ($p = 0.133$). Among 75 urine samples obtained by practitioners, 30% of samples from 51 patients >60 years of age and none of the samples from 24 patients ≤60 years of age were PCR positive ($p = 0.002$). There was no significant difference in the presence of *A. schaalii* by sex of the patients ($p = 0.485$). When the control group (patients who had had hip or knee surgery) was compared with patients >60 years of age, no significant difference in the presence of *A. schaalii* was found ($p = 0.227$). In addition, we did not find any detectable differences between PCR-positive and PCR-negative results for hospitalized patients concerning underlying urinary tract pathologic changes and concurrent conditions such as hypertension and diabetes.

Discussion

The real-time PCR assay confirmed that infection with *A. schaalii* increases with age (*2*). More than 1 of 5 urine samples from patients >60 years of age were PCR positive, and *A. schaalii* was most common in patients who visited medical practitioners and who had an infection with ordinary urinary pathogens. In comparison, culture findings in a study in our laboratory showed that 0.4% of cultured urine samples from patients >60 years of age had *A. schaalii* and that these patients had a broad spectrum of UTIs (*2*).

The present study shows that bacteria species, especially anaerobic or slow-growing species, are more common than what culture results indicate. Most likely, other pathogen bacteria exist that are even more difficult to identify by cultivation than is *A. schaalii*. Molecular biologic techniques such as real-time PCR can be valuable tools for identification of these organisms. Pathogenic bacteria that are difficult to cultivate or identify by cultivation should not be underestimated.

Other common uropathogens were identified by cultivation in 9 of 10 PCR-positive urine samples (Table 4). This finding indicates that *A. schaalii* is probably a common, undetected bacterial copathogen in many UTIs. Because most PCR-positive samples were from persons with multiple infections, determining which microorganism caused the UTI is difficult. However, results from our study support findings in case reports (*2*,*3*,*6*) in which *A. schaalii* was often found in monoculture for patients who had UTIs and therefore considered the causative agent. Furthermore, PCR showed that *A. schaalii* is a more common pathogen than previously thought. However, it will be difficult to fulfill the last of Koch's criteria and prove with animal experiments that *A. schaalii* is a uropathogen.

Clinical microbiologists, clinicians, and medical practitioners should be aware of *A. schaalii* in patients predis-

Table 3. Distribution of *Actinobaculum schaalii* in 252 urine samples, Denmark, 2008–2009*

Age of sample donors, y	No. (%) samples	95% CI	CFU/mL of *A. schaalii* in PCR-positive samples			
			10^4–10^5	$>10^5$–10^6	$>10^6$–10^7	$>10^7$
0–10	12 (0)		0	0	0	0
11–20	16 (6)		0	0	0	1
21–30	21 (5)		1	0	0	0
31–40	15 (0)		0	0	0	0
41–50	11 (9)		1	0	0	0
51–60	22 (18)		2	2	0	0
61–70	52 (15)		4	3	0	1
71–80	54 (20)		4	4	1	2
>80	49 (31)		3	4	2	6
≤60	97 (7)	3–14	4	2	0	1
>60	155 (22)	16–29	11	11	3	9
Healthy controls	38 (13)	4–28	2	3	0	0

*CI, confidence interval.

Table 4. Uropathogens identified by cultivation of 155 urine samples from patients >60 y of age, Denmark, 2008–2009

Characteristic	*Actinobaculum schaalii*	
	PCR positive	PCR negative
Total no. samples	34	121
No growth	3	45
Uropathogens* $\geq 10^4$ CFU	31	76
Escherichia coli	13	38
Other *Enterobacteriaceae*	13	14
Other organisms		
Gram-negative aerobic rods	2	6
Enterococcus faecalis	0	6
Coagulase-negative staphylococci	0	4
Aerococci spp.	1	3
Streptococcus spp.	2	3
Yeast	0	2
≥3 species	4	8

*Two species were identified in 4 of 34 PCR-positive samples and in 8 of 121 PCR-negative samples.

posed for UTIs or unexplained chronic UTIs, especially if initial findings of wet smear microscopy for bacterial rods and leukocytes differ from negative growth under commonly used aerobic cultivation methods. For patients with suspected infections, urine should be sent to a department of clinical microbiology and incubated in an atmosphere of 5% CO_2 for 2 to 3 days.

For patients with clinically verified UTIs who do not respond to treatment with ciprofloxacin or trimethoprim, infection with *A. schaalii* should be suspected. If *A. schaalii* is the cause of the infection, treatment with β-lactams, such as ampicillin or cephalosporins, should be given. The optimal duration of antimicrobial drug treatment with β-lactams is not clearly defined but several weeks of treatment may be required in severe cases.

Because *A. schaalii* can be difficult to identify even when cultured in an atmosphere of 5% CO_2, the real-time PCR described in this report can be used for identification in urine and blood cultures. Alternatively, if the bacteria can be isolated by cultivation, the API Coryne and Rapid ID32A test systems (bioMérieux, Marcy l'Etoile, France) can be used for identification, as described by Reinhard et. al. (*2*). In conclusion, *A. schaalii* is an underestimated opportunistic copathogen that probably causes UTIs and urosepsis, particularly in elderly patients or patients predisposed for UTIs.

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Dr Bank is a physician in the Department of Clinical Microbiology at Viborg Hospital, Viborg, Denmark. His primary research interests are real-time PCR and urinary tract infections.

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Address for correspondence: Steffen Bank, Department of Clinical Microbiology, Viborg Hospital, Heibergs Allé 4, DK-8800 Viborg, Denmark; email: stb@mb.au.dk

Norovirus Gastroenteritis Outbreak with a Secretor-independent Susceptibility Pattern, Sweden

Johan Nordgren, Elin Kindberg, Per-Eric Lindgren, Andreas Matussek, and Lennart Svensson

Norovirus (NoV) is recognized as the commonest cause of acute gastroenteritis among adults. Susceptibility to disease has been associated with histo-blood group antigens and secretor status; nonsecretors are almost completely resistant to disease. We report a foodborne outbreak of GI.3 NoV gastroenteritis that affected 33/83 (40%) persons. Symptomatic disease was as likely to develop in nonsecretors as in secretors (odds ratio [OR] 1.41, 95% confidence interval [CI] 0.46–4.36 vs. OR 0.71, 95% CI 0.23–2.18, p = 0.57). Moreover, no statistical difference in susceptibility was found between persons of different Lewis or ABO phenotypes. The capsid gene of the outbreak strain shares high amino acid homology with the Kashiwa645 GI.3 strain, previously shown to recognize nonsecretor saliva, as well as synthetic Lewis a. This norovirus outbreak affected persons regardless of secretor status or Lewis or ABO phenotypes.

Norovirus (NoV) is the leading cause of nonbacterial, acute gastroenteritis among adults and is responsible for numerous outbreaks worldwide (*1–4*). The virus is frequently associated with contaminated food, causing ≥50% of all food-related outbreaks (*5*). Several studies (*6–11*) have associated norovirus susceptibility with the presence of an α1,2-linked fucose on histo-blood group antigens (HBGAs), which is determined by the *FUT2* gene (*12*,*13*). Persons carrying ≥1 functional *FUT2* allele, and thus expressing α1,2 fucosyltransferase 2 (FucT-II), are termed secretor positive (secretors), and can express the A and B blood group antigens as well as H-type 1 and Lewis b (Le^b) antigens on mucosa and in secretions. Persons lacking FucT-II are termed secretor negative (nonsecretors) and have been shown to be highly protected from infections with several NoV genotypes, including the common GII.4, as well as the Norwalk virus prototype strain (GI.1) (*6–11*).

Saliva-binding studies have demonstrated that different NoV strains exhibit different binding patterns (*14–16*), with the Norwalk virus (GI.1) mainly recognizing saliva from secretors with blood groups A and O, while exhibiting low or no binding to saliva to nonsecretors and carriers of blood group B, suggesting protection against infection among the latter 2 groups. Virus-like particles (VLPs) of the common GII.4 strains have been found to mainly bind saliva from secretors irrespective of blood group (*16*), although binding to nonsecretor saliva has been described for VLPs of some GII.4 strains (*17*).

Although NoV infections of secretors are well documented (*18*) and a few cases of infected nonsecretors have been reported (*19*,*20*), no virus has been identified in authentic outbreaks that is completely secretor or Lewis antigen independent, where homozygous carriers of the nonsense G428A mutation in *FUT2* are at similar or higher risk for infection than are secretors. We describe a foodborne NoV outbreak in which persons were infected regardless of secretor status or Le phenotypes; and no difference was observed between nonsecretor (Le^{a+b-}) persons and secretors regarding risk of symptomatic norovirus infection. Our data provide new knowledge about susceptibility factors and NoV genotypes and suggest that additional studies of host genetic receptor factors and NoV are needed.

Author affiliations: University of Linköping, Linköping, Sweden (J. Nordgren, E. Kindberg, P.-E. Lindgren, L. Svensson); National Board of Forensic Medicine, Linköping (E. Kindberg); County Hospital Ryhov, Jönköping, Sweden (P.-E. Lindgren, A. Matussek); and Capio St Görans Hospital, Stockholm, Sweden (A. Matussek)

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Materials and Methods

Outbreak Data and Sample Collection

In October 2007, a NoV gastroenteritis outbreak occurred in Jönköping, Sweden, at a seminar for healthcare improvement (October 25–27), attended by 112 healthcare workers from different parts of Sweden. The healthcare workers were asked to take part in this case–control study, and 83 persons, including 4 employees of the restaurant that provided food service, decided to participate. Thirty-three of these 83 persons acquired acute gastroenteritis during or shortly after the seminar. Saliva samples were collected from all 83 participants in the study and stored at –20°C until further use. Stool samples (n = 4) were obtained from the cook, 2 employees, and 1 participant of the conference with symptoms of NoV gastroenteritis. Epidemiologic investigations indicated that the lunch on the first day was contaminated with NoV and was subsequently the cause of the outbreak. The cook was ill 4 days before the outbreak started, and 3 days later other employees of the restaurant became ill, suggesting the restaurant employees as the probable source of NoV contamination in the food. NoV disease was identified by at least 1 of the following signs or symptoms: vomiting, diarrhea, or nausea combined with stomach ache ≈12–60 hours after ingesting the meal. Description of symptoms was obtained through a questionnaire sent to all participants in the study. The study was approved by the local ethics committee (M205-04 T48-08).

DNA Extraction from Saliva

Genomic DNA from 200 μL saliva was extracted by using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer (Blood and Body Fluid Spin Protocol). Extracted DNA was stored in AE buffer (QIAGEN) at –20°C until PCR amplification.

PCR Amplification of *FUT2* and Determination of *FUT2* 428 Genotype

The *FUT2* gene amplification by PCR was performed as previously described (*6*). Genotyping of the G428A mutation in the *FUT2* gene was performed as previously described (*6*,*7*,*21*). These methods can distinguish between carriers of the homozygous wild-type, heterozygous, and homozygous mutated genotype.

Detection of Histo-Blood Group Antigens in Saliva

The ABO histo-blood group phenotype of secretor-positive persons and the Lewis phenotype of all 83 persons were determined by a saliva-based ELISA, essentially as described by Bucardo et al. (*6*) and Rydell et al. (*22*). Protein concentration was determined in boiled (5 min) and centrifuged (5 min, 10,000 rpm) saliva by means of a Bradford assay. ELISA plates (NUNC 96F Maxisorp; Thermo Fisher Scientific, Roskilde, Denmark) were coated with saliva, diluted to a final protein concentration of 1 μg/mL in coating buffer (0.1 M carbonate–bicarbonate buffer, pH 9.6); plates were incubated for 2 h at 37°C followed by 4°C overnight. The following day, the plates were washed 4 times with washing buffer (0.9% NaCl, 0.05% Tween 20 [Sigma-Aldrich, St. Louis, MO, USA]), and then incubated for 1.5 h at 37°C with antibodies α-A (ABO1 clone 9113D10), α-B (ABO2 clone 9621A8) (Diagast, Loos Cedex, France), α-Le^a (Seraclone, LE1 clone 78FR 2.3), and α-Le^b (Seraclone LE2 clones LM129-181 and 96 FR2.10) (Biotest AG, Dreieich, Germany). Antibodies were diluted 1:5000 in phosphate-buffered saline with 10% fetal bovine serum (Invitrogen AB, Lidingö, Sweden) and 0.05% Tween 20 (Sigma-Aldrich). After 4 washes, horseradish peroxidase–conjugated goat anti-mouse IgG (heavy plus light chain) (Bio-Rad Laboratories, Hercules, CA, USA), diluted 1:7,500, was added, and plates were incubated for another 1.5 h at 37°C and subjected to 4 final washes. The reaction was developed using 3′,3′,5′,5-tetramethylbenzidine (DakoCytomation, Carpinteria, CA, USA), and stopped by addition of 2M H_2SO_4. The plate was read at 450 nm in a spectrophotometer. The cutoff value was twice the mean level of 6 known negative samples. The α-Le^b antibody cross-reacted weakly with Le^a; this signal was subtracted from the Le^b values read in Le^a-positive persons.

Virus RNA Extraction and Reverse Transcription

RNA extraction from the 4 collected stool specimens was performed by using the EZ1 robot (QIAGEN) according to the manufacturer's instructions and stored at –80°C until used for reverse transcription. Reverse transcription was performed as previously described (*6*,*23*), by using random hexamer primers (GE Healthcare, Uppsala, Sweden) and Illustra Ready-To-Go RT-PCR beads (GE Healthcare).

Norovirus Detection with Real-Time PCR

NoV detection and quantification were performed with a real-time PCR specific for the open reading frame (ORF) 1–ORF2 junction, as described by Nordgren et al. (*24*). This real-time PCR assay can semiquantify and distinguish between NoVs GI and GII (*24*). PCR amplification of the N-terminal and shell (N/S) region was performed on a PTC-100TM thermal cycler (MJ Research Inc., South San Francisco, CA, USA) in a 50-μL mixture composed of 1.33 U of Expand High Fidelity polymerase (Boehringer Mannheim GmbH, Mannheim, Germany), 5 μL of the supplied buffer (including 1.5 mmol/L $MgCl_2$; Boehringer Mannheim GmbH), 100 μM GeneAmp dNTP mixture with dTTP (Applied Biosystems, Branchburg, NJ, USA), 200 nM forward primer NVG1f1b (5′-CGY TGG ATG CGN TTC CAT

GA-3′) (*24*), 200 nM reverse primer G1SKR (5′-CCA ACC CAR CCA TTR TAC A-3′) (*25*), and 5 μL template DNA.

Nucleotide Sequencing of the Norovirus N/S Region and Virus Genotyping

Nucleotide sequencing of the N/S region was performed by Macrogen Inc. (Seoul, South Korea). The sequencing reaction was based on BigDye chemistry; NVG1f1b forward primer (*24*) and G1SKR reverse primer (*25*) were used as sequencing primers. The amplicons were sequenced twice in each direction. Sequence alignment of the Jönköping (JKPG) strain and reference NoV genotypes was performed by using the ClustalW algorithm, version 1.8 (www.ebi.ac.uk/clustalw), with default parameters, on the European Bioinformatics Institute server. We performed phylogenetic analysis using the MEGA 4.0 software package (www.megasoftware.net), and the phylogenetic tree was constructed using the neighbor-joining and Kimura 2-parameter methods. Significance of the taxonomic relationships was obtained by bootstrap resampling analysis (1,000 replications). Assignment of genotypes used reference strains described by Zheng et al. (*26*).

PCR Amplification of the Norovirus Capsid Gene

To amplify the gene encoding the NoV capsid, we set up a PCR mixture containing 2.5 μL 10× native *Pyrococcus furiosus* (*pfu*) polymerase buffer (Invitrogen AB, Lidingö, Sweden), 200 μM GeneAmp dNTP mix with dTTP (Applied Biosystems), 200 nM forward primer CapGI3fw (5′-GAT CTC CTG CCC GAT TAT GTA AAT GAT GAT G-3′, this study), targeting the end of ORF1 and beginning of ORF2, 200 nM reverse primer CapGI3rv (5′-CAT TAT GAT CTC CTA ATT CCA AGC CTA CGA GC-3′, this study), specific for the end of ORF2 and beginning of ORF3, 5 μL cDNA, 2.5 U native *pfu* DNA polymerase (Stratagene, La Jolla, CA, USA), and 36 μL RNAse-free water. After initial denaturation at 94°C for 5 min, PCR amplification was performed with 40 cycles of 94ºC for 1 min, 58ºC for 1 min, and 72ºC for 2 min, and thereafter a final elongation at 72ºC for 10 min. The PCR products were visualized by electrophoresis on a 2% agarose gel, using staining with ethidium bromide and UV transillumination.

Cloning of the Norovirus Capsid Gene and Nucleotide Sequencing

The capsid fragment was cloned into a pPCR-Script Amp SK(+) vector and transformed into XL10-Gold Kan ultracompetent cells, using the Stratagene PCR-Script Amp Cloning Kit (Stratagene) according to the manufacturer's instructions. After overnight incubation of 2 separate colonies from each transformation reaction, plasmid DNA was extracted and purified, using the Plasmid Miniprep Kit (QIAGEN) according to the manufacturer's instructions. Nucleotide sequencing was performed on 2 separate plasmid extractions from each sample (n = 2) by Macrogen Inc., by using the BigDye chemistry with M13 forward and reverse primers. The nucleotide sequences for the N/S region or the complete capsid gene of the JKPG isolates are available under GenBank accession nos. FJ711163, FJ711164, and FJ711165.

Statistical Analysis

Categorical data were analyzed using the Fisher exact test with 2-tailed significance. Unadjusted odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using SPSS 14.0 for Mac (SPSS Inc., Chicago, IL, USA).

Results

Outbreak Description

A total of 83 persons responded to the questionnaire and participated in the study. Among them, 33 (40%) were symptomatic, and 50 (60%) reported no symptoms. The latter group may include exposed asymptomatic as well as nonexposed persons. The onset of symptoms varied from 1 through 3 days (mean 36 h) after ingestion of the contaminated meal (Figure 1); mean duration of symptoms was 35 h. The most common symptoms were vomiting (23/32, 72%), diarrhea (20/32, 63%), joint pain (18/32, 56%), and headache (14/32, 44%). Most symptomatic persons (n = 30) had diarrhea, vomiting, or both, whereas the remaining 3 persons had nausea and stomach ache.

Distribution of ABO and Lewis Phenotypes and Secretor Status

To investigate whether persons associated with the outbreak had a skewed HBGA profile, we determined the ABO, Lewis, and secretor status distributions among symptomatic and asymptomatic/nonexposed persons and compared them with results from earlier investigations of the population in Sweden (Table 1). The ABO, Lewis, and secretor status distributions were in the normal ranges compared with those investigations (Table 1), with the exception of the AB and Lewis negative phenotypes. Furthermore, we observed that all HBGAs investigated, except AB (n = 1), were found among asymptomatic/nonexposed and symptomatic persons. Sixty-one persons were secretor and Lewis positive; of these, 52 (85%) were positive for Le^a and Le^b in saliva. The 4 persons from whom NoV was isolated were all secretors, having ALe^{a-b+}, ALe^{a-b+}, OLe^{a-b-}, and OLe^{a-b+} HBGA profiles, respectively.

Difference in Susceptibility to Symptomatic Infection between Secretors and Nonsecretors

Previous studies have shown a strong correlation between symptomatic NoV infections and the secretor-positive

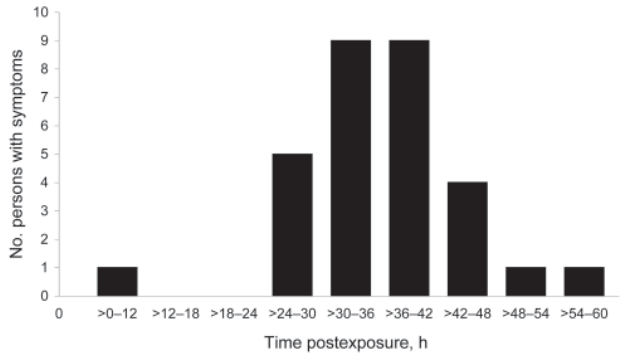

Figure 1. Time to onset of symptoms after patient exposure to norovirus-contaminated food (n = 30), Sweden. Zero indicates the time point for serving and ingesting the contaminated food.

phenotype (*7–10*). To investigate whether secretor and Lewis status were associated with susceptibility in this study, secretor and Lewis status were determined by genotyping and phenotyping of all persons. We observed that 7/15 (47%) nonsecretors were symptomatically infected, compared with 26/68 (38%) secretors (Table 1). Although the calculated OR for nonsecretors was ≈2× that of secretors (OR 1.41 vs. 0.71), the differences were not significant (Table 2). Thus, in the group studied, nonsecretors were as likely as secretors to be symptomatically infected by norovirus. The same pattern was observed for the Lewis phenotypes; no statistical difference was found between persons with Le^{a+b-}, Le^{a-b+}, or Le^{a-b-} regarding risk for symptomatic infection (Table 2). None of the Lewis-negative nonsecretors (n = 3) were symptomatically infected. The *FUT2* G428A genotyping did not show any significant differences between heterozygous secretors and homozygous secretors (OR 1.20, 95% CI 0.49–2.95 vs OR 0.67, 95% CI 0.27–1.65; Table 2).

Association between ABO Blood Types and Symptomatic Infection

Previous studies have shown that ABO blood types are associated with susceptibility to symptomatic NoV infections, with persons having blood type B at lower risk of infection when challenged with Norwalk virus (GI.1) (*8*,*28*). In this outbreak, we found that symptoms developed in 2/12 (17%) of persons with blood group B (Table 1). Although persons with blood group B were infected to a lesser extent than persons with other blood groups, this reduction was not significant (OR 0.27, 95% CI 0.05–1.33; Table 2). Furthermore, no significant differences were found when comparing symptomatic and nonsymptomatic persons with blood types A and O (OR 1.56, 95% CI 0.58–4.16, and OR 1.39, 95% CI 0.50–3.89, respectively) (Table 2). Thus, no blood type provided complete protection or was associated with a higher or lower risk for disease.

Association between Blood Type, Secretor Status, and Clinical Symptoms

A recent study suggested that blood type can have an influence on clinical symptoms after NoV infection (*29*). To investigate whether this would apply in this outbreak, blood types, secretor status, and clinical symptoms were compared. We did not find any correlation between blood type and secretor status with clinical symptoms (Table 3).

Similarity of JKPG and Kashiwa645 Strains in the P2 Domain and Putative Receptor Binding Sites

NoV GI was detected by real-time PCR in all collected stool specimens (n = 4); three of these isolates (881–883) were subsequently genotyped by nucleotide sequencing of the N/S region. The fourth sample could not be genotyped because of low virus concentration in the stool sample. Phylogenetic analysis clustered the 3 isolates with NoV

Table 1. Distribution of histo-blood group antigens phenotypes and secretor status among 83 participants in a case–control study of a norovirus gastroenteritis outbreak in Sweden, 2007*

Antigen and secretor status	No. (%) symptomatic persons, n = 33	No. (%) asymptomatic/ nonexposed persons, n = 50†	Total no. (%) persons, n = 83	Normal distribution, % (95% CI)†‡
Blood type (n = 68)				
A	14 (44)	18 (56)	32 (47)	47 (37–56)
B	2 (17)	10 (83)	12 (18)	15 (8–22)
O	10 (43)	13 (57)	23 (34)	33 (23–40)
AB	0	1 (100)	1 (1)	7 (2–12)
Lewis (n = 83)				
Le^{a+b-}	7 (58)	5 (42)	12 (14)	18 (11–26)
Le^{a-b+}	23 (38)	38 (62)	61 (73)	76 (68–84)
Le^{a-b-}	3 (30)	7 (70)	10 (12)	6 (1–10)
Secretor status (n = 83)				
Secretor§	26 (38)	42 (62)	68 (82)	80 (72–88)
Nonsecretor	7 (47)	8 (53)	15 (18)	20 (12–28)

*ABO blood group could only be determined for the 68 secretor-positive persons (26 symptomatic and 42 asymptomatic). CI, confidence interval.
†$p \geq 0.15$.
‡See Larsson et al. (*27*).
§Homozygous and heterozygous.

Table 2. Influence of secretor status, *FUT2* polymorphism, and histo-blood group antigens on risk for norovirus GI.3 symptomatic infection, Sweden*

Secretor status	OR (95% CI)	p value
Secretor, n = 68	0.71 (0.23–2.18)	0.57
Nonsecretor, n = 15	1.41 (0.46-4.36)	0.57
FUT2 428 polymorphism		
G/G (secretor), n = 35	0.67 (0.27–1.65)	0.50
G/A (secretor), n = 33	1.20 (0.49–2.95)	0.82
A/A (nonsecretor), n = 15	1.41 (0.46–4.36)	0.57
Histo-blood group antigens		
Blood type,† n = 68		
A, n = 32	1.56 (0.58–4.16)	0.46
B, n = 12	0.27 (0.05–1.33)	0.11
O, n = 23	1.39 (0.50–3.89)	0.60
AB, n = 1	Not applicable‡	1.0
Lewis, n = 83		
Le^{a+b-}, n = 12	2.42 (0.70–8.42)	0.21
Le^{a-b+}, n = 61	0.73 (0.27–1.95)	0.61
Le^{a-b-}, n = 10	0.61 (0.15–2.57)	0.73

*FUT, fucosyltransferase; OR, odds ratio; CI, confidence interval.
†Compared between secretors. ABO blood group could only be determined for the 68 secretor-positive persons.
‡No carrier of blood type AB was symptomatically infected with norovirus.

GI.3 strains (data not shown). The entire capsid gene was subsequently sequenced from 2 isolates and compared with reference strains (Figure 2). The closest amino acid similarity (98.0%) of the complete capsid gene was found with strain PD196-DEU (GI.3), isolated in Germany 2000, and with the Kashiwa645 (GI.3) strain (97.8%), used in an earlier VLP binding study (*14*).

We then investigated the amino acid composition of the capsid P2 domain of the outbreak strain and compared it with the Kashiwa645 and Norwalk strains. Although the JKPG strain differed by 4 aa at positions 344, 367, 377, and 397 (97.1% homology) compared with Kashiwa645, it shared only ≈50% aa positions with the GI.1 Norwalk strain.

Discussion

Previous studies have shown a strong (*6*–*11*) but not absolute (*19*,*20*) association between nonsecretors and protection from symptomatic NoV disease. In contrast to these observations, we report a foodborne NoV outbreak affecting persons regardless of secretor, Lewis, or ABO phenotype.

Because the host genetic observation of this outbreak was unexpected, attempts were made to compare the HBGA frequencies of the participating persons with those of the population in Sweden. The ABO, secretor, and Lewis phenotype frequencies in this study agreed with results from earlier investigations from the population in Sweden (*21*,*27*) (Table 1), with the exception of the AB and Lewis negative phenotypes, probably due to their low prevalence in combination with the small sampling set. Seven (8%) secretor and 3 (4%) nonsecretor persons were Lewis negative and hence lacked Lewis antigen in saliva. Genotyping of the *FUT2* G428A nonsense mutation confirmed secretor-negative genotype of all Le^{a+b-} persons and the secretor-positive genotype of Le^{a-b+} persons.

Comparison of secretor and Lewis phenotypes regarding susceptibility to symptomatic NoV infection showed that nonsecretors were as susceptible to symptomatic disease as secretors. Consistent with the lack of secretor association, no significant difference in susceptibility was noted between Le^{a+b-} and Le^{a-b+} persons (OR 2.42, 95% CI 0.70–8.42 vs. OR 0.73, 95% CI 0.27–1.95). None of the nonsecretors who were also Lewis negative (n = 3), hence lacking the Le^a antigen and ABO in saliva, were symptomatically infected. These findings indicate but do not prove that the Le^a antigen is a putative receptor for this norovirus strain. The disease pattern of this outbreak is consistent with the findings by Shirato et al. (*14*), who observed strong binding to synthetic Le^a and saliva from secretors and nonsecretors with VLPs from the GI.3 Kashiwa645 strain, which shares high homology with the JKPG strain in the P2 domain. A mechanistic virus–saliva binding study with the authentic virus would have been desirable, but limited amounts of virus restricted our attempts to investigate if the outbreak virus binds to saliva both from secretors and nonsecretors.

No ABO phenotype provided protection or was associated with a higher risk of disease, although persons with blood type B exhibited a low (17%) frequency of symptomatic infection (Tables 1, 2). Blood type B has previously

Table 3. Relationship between clinical symptoms of norovirus infection and secretor status and blood type distribution among 83 participants in a case–control study of a norovirus gastroenteritis outbreak in Sweden, 2007*

	No. (%) persons reporting symptom			
Data	Diarrhea	Vomiting	Joint pain	Headache
Blood type				
A, n = 14	9 (64)	12 (86)	9 (64)	7 (50)
B, n = 2†	1 (50)	0 (0)	0 (0)	0(0)
O, n = 9‡	6 (67)	6 (67)	4 (44)	5 (56)
Secretor, n = 25‡	16 (64)	18 (72)	13 (52)	12 (48)
Nonsecretor, n = 7	4 (57)	5 (71)	5 (71)	2 (29)
Total	20 (63)	23 (72)	18 (56)	14 (44)

*HBGA, histo-blood group antigen.
†One person with HBGA type B experienced only nausea and stomachache.
‡One secretor-positive person with HBGA type O did not provide a description of symptoms.

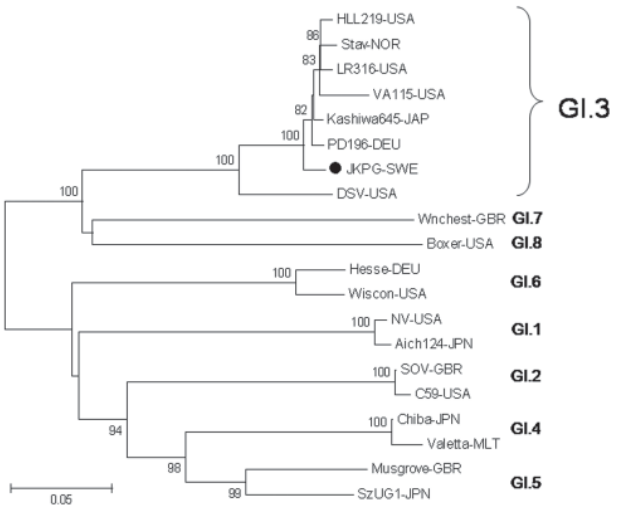

Figure 2. Phylogenetic analysis of amino acids of the norovirus capsid gene from the gastroenteritis outbreak in Jönköping, Sweden (JKPG, •) and reference strains. The tree was constructed using the neighbor-joining and Poisson correction methods, with MEGA 4.0 software (www.megasoftware.net). Bootstrap values are shown at the branch nodes (values <70% are not shown). Reference sequences were collected from Genbank and represent the 8 genotypes of GI as described by Zheng et al. (*26*). Scale bar indicates nucleotide substitutions per site. NV-USA [M87661], Aich124-JPN [AB031013], SOV-GBR [L07418], C59-USA [AF435807], HLL219-USA [AF414403], Stav-Nor [AF145709], LR316-USA [AF414405], VA115-USA [AY038598], Kashiwa645-JAP [BD011871], PD196-DEU [AF439267], JKPG-SWE [FJ711163] DSV-USA [U04469], Chiba-JPN [AB042808], Valetta-MLT [AJ277616], Musgrove-GBR [AJ277614], SzUG1-JPN [AB039774], Hesse-DEU [AF093797], Wiscon-USA [AY502008], Wnchest-GBR [AJ277609], Boxer-USA [AF538679].

been associated with protection from disease when challenged with the Norwalk virus (*28*) and was also supported by in vitro binding studies with VLPs (*16*). Shirato et al. (*14*) found that the Kashiwa645 (GI.3) VLP bound weaker to saliva from blood type B-positive persons compared with types A and O, which agrees with the disease pattern observed in this outbreak. It is possible that the α-gal in the blood type B structure partly covers an epitope needed for binding and hence decreases the ability of the JKPG strain to infect carriers of blood type B.

One limitation of our study is that some of the asymptomatic persons may not have been exposed to the virus. This possibility could result in sampling artifacts because symptoms could have developed in the unexposed (and thus seemingly protected) persons if they had been exposed. However, this possibility is unlikely to influence the main findings of this study, namely the secretor- and HBGA-independent infection pattern, because symptomatic persons were found in all HBGA groups.

An advantage with genotyping compared with phenotyping is that the roles of heterozygosity and homozygosity in disease susceptibility can be investigated. In this study, we did not observe any significant differences between heterozygous and homozygous secretors (OR 1.20, 95% CI 0.49–2.95 vs. OR 0.67, 95% CI 0.27–1.65) (Table 2), which is in agreement with earlier observations (*7*,*9*).

By comparing our strain with a reference strain of the same cluster (Kashiwa645) used in earlier binding studies (*14*), we aimed to elucidate structural similarities or differences that could explain the unique disease profile of the outbreak. The JKPG strain investigated in this outbreak shares high amino acid homology with the GI.3 Kashiwa645 strain (Figure 2). Shirato et al. (*14*) found that the Kashiwa645 strain bound to the same extent to secretor and nonsecretor saliva. However, another consideration is that Asian nonsecretors in the study (*14*) were identified as carriers of a missense mutation at nt 385 (A→T) and thus are incomplete nonsecretors, producing small amounts of ABO and Le^b HBGA in secretions. The similarities between the binding profile of the Kashiwa645 strain and the disease profile of the JKPG strain indicate that saliva binding may be used to assess susceptibility patterns for individual NoV strains.

In conclusion, we report a foodborne NoV outbreak infecting persons irrespective of Lewis and secretor status, with Le^{a+b-} persons homozygous for the *FUT2* G428A nonsense allele being symptomatically infected at similar rates compared with secretors. Our observed disease pattern is in concordance with saliva binding specificities of VLP based on the Kashiwa645 strain, sharing high homology in the P2 domain with the JKPG strain. Increased knowledge of susceptibility factors for norovirus disease will be helpful in the development of preventive or therapeutic measures for infection.

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Dr Nordgren works primarily with enteric viruses at the Division of Molecular Virology, Linköping University. His main research interest is norovirus epidemiology.

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Address for correspondence: Lennart Svensson, Division of Molecular Virology, Department of Clinical and Experimental Medicine, University of Linköping, 581 85 Linköping, Sweden; email: lennart.t.svensson@liu.se

Food Reservoir for *Escherichia coli* Causing Urinary Tract Infections

Caroline Vincent, Patrick Boerlin, Danielle Daignault, Charles M. Dozois, Lucie Dutil, Chrissi Galanakis, Richard J. Reid-Smith, Pierre-Paul Tellier, Patricia A. Tellis, Kim Ziebell, and Amee R. Manges

Closely related strains of *Escherichia coli* have been shown to cause extraintestinal infections in unrelated persons. This study tests whether a food reservoir may exist for these *E. coli*. Isolates from 3 sources over the same time period (2005–2007) and geographic area were compared. The sources comprised prospectively collected *E. coli* isolates from women with urinary tract infection (UTI) (n = 353); retail meat (n = 417); and restaurant/ready-to-eat foods (n = 74). *E. coli* were evaluated for antimicrobial drug susceptibility and O:H serotype and compared by using 4 different genotyping methods. We identified 17 clonal groups that contained *E. coli* isolates (n = 72) from >1 source. *E. coli* from retail chicken (O25:H4-ST131 and O114:H4-ST117) and honeydew melon (O2:H7-ST95) were indistinguishable from or closely related to *E. coli* from human UTIs. This study provides strong support for the role of food reservoirs or foodborne transmission in the dissemination of *E. coli* causing common community-acquired UTIs.

Extraintestinal infections caused by *Escherichia coli* cause serious illness and death. Every year, 6–8 million cases of uncomplicated urinary tract infections (UTI) occur in the United States and 130–175 million cases occur globally; >80% are associated with *E. coli* (*1*,*2*). The urinary tract is the most common source for *E. coli* causing bloodstream infections, which cause 40,000 deaths from sepsis each year in the United States (*1*). Uncomplicated UTIs alone are responsible for an estimated $1–$2 billion of direct healthcare costs in the United States annually (*1*,*2*). Antimicrobial drug resistance among extraintestinal *E. coli* is further adding to the cost of treating these infections (*3*). Drug-resistant infections often require more complicated treatment regimens and result in more treatment failures.

The immediate reservoir of *E. coli* that causes extraintestinal infections is the intestinal tract of the person. Although extraintestinal infections caused by *E. coli* are not usually associated with outbreaks, mounting evidence shows that extraintestinal *E. coli* may be responsible for community-wide epidemics. For instance, in 2001, we reported the discovery of *E. coli* O11/O77/O17/O73:K52:H18-ST69. This clonal group caused 11% of all *E. coli* UTIs and 49% of all trimethoprim/sulfamethoxazole-resistant *E. coli* UTIs in 1 California community over a 4-month period (*4*). It caused antimicrobial drug–resistant UTIs in Michigan, Minnesota, and Colorado (*5*), and pyelonephritis in several states (*6*). Other outbreaks of UTIs caused by *E. coli* have been described, including a large *E. coli* O15:K52:H1 outbreak in South London (*7*), clusters of cases in Copenhagen, Denmark, caused by *E. coli* O78:H10 (*8*), and cases in Calgary, Alberta, Canada, caused by an extended-spectrum β-lactamase-producing *E. coli* (*9*).

Identification of these outbreak strains has suggested that environmental sources, possibly contaminated meat and other foods, may play a role in the local spread of closely related *E. coli* strains. If there is a food animal reservoir for extraintestinal *E. coli*, then the use of antimicrobial agents in food animal production may select for antimicrobial drug–resistant forms of extraintestinal *E. coli* (*10*,*11*). Links between antimicrobial resistance and specific strains of extraintestinal *E. coli* in animal food products, specifically chicken meat, and human infections have been observed (*12*–*16*). In a previous study, we noted an increase

Author affiliations: McGill University, Montréal, Québec, Canada (C. Vincent, C. Galanakis, P.-P. Tellier, P.A. Tellis, A.R. Manges); University of Guelph, Guelph, Ontario, Canada (P. Boerlin, R.J. Reid-Smith); Public Health Agency of Canada, Saint-Hyacinthe, Québec (D. Daignault, L. Dutil); INRS-Institut Armand-Frappier, Laval, Québec (C.M. Dozois); and Public Health Agency of Canada, Guelph (R.J. Reid-Smith, K. Ziebell)

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in antimicrobial drug–resistant UTIs among women who report frequent chicken and pork consumption (*17*).

Evidence showing that food can be a reservoir for extraintestinal *E. coli* includes 1) community-based outbreaks of extraintestinal infections caused by epidemic strains of *E. coli* causing uncomplicated UTIs (*4*,*18*) and other severe infections (*6*,*19*,*20*); 2) the determination that these epidemic strains share antimicrobial drug susceptibility patterns and genotypes with isolates from retail meat (*12*–*15*); and 3) the epidemiologic association between retail meat consumption and intestinal acquisition of antimicrobial drug–resistant *E. coli* causing UTIs (*17*). On the basis of these observations, we hypothesize that retail chicken is the main reservoir for *E. coli* causing human extraintestinal infections.

Methods

Study Design

E. coli isolates from human clinical samples, restaurant/ready-to-eat foods, and retail meat were systematically sampled over the same period. Human clinical isolates and restaurant/ready-to-eat isolates were obtained from Montréal, Québec, Canada. Retail meat isolates from Québec and Ontario were included because women with infections were primarily from these regions. We hoped to maximize the probability that matching genotypes between *E. coli* from these 3 sources could be identified. *E. coli* isolates from each source were cultured and processed separately to prevent cross-contamination. The study protocol was approved by the McGill University Institutional Review Board (A01-M04-05A).

Sampling of *E. coli* Causing Human UTIs

E. coli isolates from women with UTIs in Montréal from June 1, 2005, to May 30, 2007, were included. Women 18–45 years of age with a suspected UTI were enrolled. UTI was defined as the presence ≥2 relevant symptoms including dysuria, increased urinary frequency or urgency, pyuria, and hematuria and $>10^2$ colony-forming units of *E. coli* per milliliter of clean-catch urine (*21*). A total of 1,395 consecutive UTI samples were obtained. Details about specimen culture and bacterial identification of *E. coli* are provided in Manges et al. (*18*). One *E. coli* isolate from each urine culture was arbitrarily selected for further analysis. If a woman had had recurrent UTIs, only the isolate from the first infection was included. The study sample (n = 353) of *E. coli* isolates was assembled in the following manner. All cephalothin-resistant *E. coli* (n = 19) were included. Isolates known to be members of a clonal group (n = 46) found to be closely related to or indistinguishable from other *E. coli* causing UTI in unrelated women were included (*4*,*18*,*22*) because we hypothesized that these *E. coli* would be more likely to be associated with food sources. A random sample of *E. coli* isolates resistant to ≥1 antimicrobial agents was assembled (n = 172). We chose to oversample resistant *E. coli*, as antimicrobial resistance has been associated with possible outbreaks of extraintestinal *E. coli* infections. A random sample of fully susceptible *E. coli* isolates (n = 116) was selected.

Sampling of *E. coli* from Retail Meat

A total of 417 *E. coli* isolates from fresh, raw retail chicken, beef, and pork products were selected from the collection of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), which monitors antimicrobial resistance in bacteria from meat obtained from grocery and other retail stores in several provinces in Canada (*23*). Isolates collected by the CIPARS in Montréal, areas of Québec outside Montréal, and parts of Ontario from January 1, 2005, to July 31, 2007, were included as follows. All CIPARS isolates from Montréal were included because all cases of UTI occurred in Montréal (n = 197). All CIPARS nalidixic acid–resistant *E. coli* from all regions of Canada were included (n = 24); these isolates have been associated with reduced susceptibility to fluoroquinolones. Randomly selected susceptible and resistant isolates from outside Montréal, including other regions of Québec and Ontario, were selected to better represent the possible sources of retail meat exposure for the UTI cases. The overall sampling fraction for retail chicken meat-associated isolates was ≈60%, given that our primary hypothesis focused on retail chicken meat. The sampling fraction for retail beef was 20% and for retail pork 20%. A strong association between extraintestinal *E. coli* clonal groups and antimicrobial resistance has been reported (*4*,*7*,*9*,*18*). Our targeted sampling fraction for antimicrobial resistance was 60% for each retail meat category; however, only 25% of retail beef isolates were resistant.

Sampling of *E. coli* from Restaurant/ Ready-to-Eat Food Sources

We included all 74 *E. coli* isolates from restaurant and ready-to-eat food sources for Montréal collected from January 1, 2005, to December 31, 2007, by the Division de l'Inspection des Aliments (*24*,*25*). These isolates were recovered from a range of prepared and ready-to-eat foods, including meat, fruit, vegetables, and other items. Isolates were collected as part of routine surveillance activities and from complaint-related inspections of restaurants and establishments offering ready-to-eat foods.

Antimicrobial Drug Susceptibility

We determined the minimum inhibitory concentration values for 15 antimicrobial agents for all *E. coli* isolates by the broth microdilution method (*26*), using the Sensititre

Automated Microbiology System (Trek Diagnostic Systems Ltd., Cleveland, OH, USA). National Antimicrobial Resistance Monitoring System (NARMS) susceptibility panel CMV1AGNF was used for *E. coli* testing. Human clinical and restaurant/ready-to-eat isolates were also evaluated for resistance to cephalothin and nitrofurantoin by a standard disk diffusion method (*27*). Isolates were defined as resistant, intermediate, or susceptible according to Clinical and Laboratory Standards Institute and NARMS guidelines (*23*). Isolates exhibiting intermediate resistance were interpreted as susceptible.

Multilocus Variable Number Tandem Repeat Analysis

We performed multilocus variable number tandem repeat analysis (MLVA) on all isolates using capillary electrophoresis methods as described previously in Manges et al. (*28*). Essentially, 8 loci were amplified in separate PCRs by using fluorescent primers. Raw fragment lengths for each locus were binned manually using a minimum threshold of ± 3 bp to distinguish alleles. *E. coli* CFT073, K12, and O157:H7 were used as positive controls. The set of 8 alleles for each isolate was defined as the MLVA profile.

Enterobacterial Repetitive Intergenic Consensus Sequence 2 PCR Fingerprinting

E. coli isolates exhibiting indistinguishable MLVA profiles were compared by enterobacterial repetitive intergenic consensus sequence 2 PCR (ERIC2 PCR) fingerprinting (*29*). Isolates with fingerprints that were indistinguishable on visual inspection were grouped and selected for further typing.

Clonal Group Definition

A clonal group was defined as ≥2 *E. coli* isolates exhibiting indistinguishable MLVA and ERIC2 PCR patterns. We focused only on groups identified by MLVA and ERIC2 PCR that contained members from >1 source. Groups containing isolates from retail meat and restaurant/ready-to-eat food sources were included to determine whether related extraintestinal *E. coli* from retail meat isolates could be identified in prepared food. These groups were given a designation that included the serogroup and multilocus sequence type (MLST), as in serogroup O25:H4 and ST131 (O25:H4-ST131). Selected isolates representing each clonal group were chosen and evaluated by pulsed-field gel electrophoresis (PFGE), serotyping, MLST, and phylogenetic typing to confirm the identities of these clonal groups and to define their within-group variability.

Pulsed-Field Gel Electrophoresis

The standard Centers for Disease Control and Prevention protocol for molecular subtyping of *E. coli* O157:H7 by PFGE was used (*30*). PFGE of *Xba*I- and *Not*I-digested DNA was performed on selected isolates belonging to each clonal group. Isolates exhibiting identical PFGE patterns were considered genetically indistinguishable, those exhibiting 1–3 band differences were considered closely related, and those exhibiting 4–6 band differences were considered possibly related (*31*).

Serotyping

The Public Health Agency of Canada Laboratory for Foodborne Zoonoses performed O- and H-serotyping using established protocols. Isolates that did not react with O antiserum were classified as nontypeable (ONT), and those that were nonmotile were denoted NM.

MLST and Phylotyping

MLST on selected *E. coli* isolates was performed as previously described (*32*). Gene amplification and sequencing were performed by using the primers specified at the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli). Allelic profile and sequence type determinations were assigned according to this website's scheme. Determination of the major *E. coli* phylogenetic groups (A, B1, B2, and D) was performed by multiplex PCR (*33*).

Statistical Analyses

Proportions and 95% confidence intervals for proportions were estimated. Differences in proportions were assessed by χ^2 tests; statistical significance was defined as a p value <0.05. All analyses were conducted using Stata version 9.0 (StataCorp LP, College Station, TX, USA).

Results

Final Sample Assembly

We analyzed 844 *E. coli* isolates obtained from human UTIs (n = 353), retail meat (n = 417), and restaurant/ready-to-eat foods (n = 74). Table 1 contains details regarding the year of isolation, geographic location, and specific meat or food source.

Clonal Group Identification and Characterization

Seventeen clonal groups were identified (containing a total of 72 isolates). Eleven groups contained isolates from human infections and retail meat sources; 5 groups contained isolates from retail meat and restaurant/ready-to-eat food sources; and 1 group contained isolates from restaurant/ready-to-eat food and human infections. Fifty-seven representative isolates were selected for evaluation by PFGE, MLST, serotyping, and phylotyping (Table 2).

On the basis of PFGE patterns, we identified 2 clonal groups (group 1 and group 2) that contained genetically indistinguishable isolates and 1 clonal group (group 3)

Table 1. Sources of 844 *Escherichia coli* isolates collected and analyzed in Canada, by year and location, 2005–2007*

Source	Total no. (%) isolates	Year, no. (%) isolates			Location, no. (%) isolates		
		2005	2006	2007	Quebec	Ontario	Other†
Clinical							
UTI	353 (42)	103 (29)	175 (50)	75 (21)	353 (100)	0	0
Retail meat							
All	417 (49)	178 (43)	158 (38)	81(19)	264 (63)	139 (33)	14 (3)
Chicken	253 (61)	107 (42)	101 (40)	45 (18)	141 (56)	99 (39)	13 (5)
Beef	82 (20)	37 (45)	26 (32)	19 (23)	81 (99)	1 (1)	0
Pork	82 (20)	34 (41)	31 (38)	17 (21)	42 (51)	39 (48)	1 (1)
Restaurant/ready-to-eat foods							
All	74 (9)	19 (26)	33 (45)	22 (30)	74 (100)	0	0
Chicken	21 (28)	7 (33)	6 (29)	8 (38)	21 (100)	0	0
Beef	13 (18)	3 (23)	6 (46)	4 (31)	13 (100)	0	0
Pork	5 (7)	0	4 (80)	1 (20)	5 (100)	0	0
Fish/seafood	6 (8)	2 (33)	2 (33)	2 (33)	6 (100)	0	0
Other meat‡	9 (12)	1 (11)	7 (78)	1 (11)	9 (100)	0	0
Other food§	20 (27)	6 (30)	8 (40)	6 (30)	20 (100)	0	0
Total	844 (100)	300 (36)	366 (43)	178 (21)	691 (82)	139 (16)	14 (2)

*UTI, urinary tract infection.
†British Columbia (n = 4) and Saskatchewan (n = 10).
‡Bison, lamb, duck, and snail.
§Fruits (honeydew melon), vegetables, cheese, rice, couscous, and pasta.

that contained closely related isolates from food sources and human UTIs. Group 1 contained *E. coli* characterized as O25:H4-ST131, which was identified in 1 sample of retail chicken meat and in 2 cases of human infection. The *Xba*I PFGE patterns of the human isolate (MSHS 161) and the retail chicken isolate (EC01DT06-1737-01) were indistinguishable, and the second human isolate (MSHS 1134A) differed by 1 band from the other 2 patterns (Figure 1, panel A). The *Not*I PFGE patterns of the 2 human isolates, which were indistinguishable, differed from the retail chicken isolate by a single band (Figure 1, panel B). The retail meat isolate from this group was susceptible to all antimicrobial agents tested, while 1 of the 2 isolates from human infections was resistant to cephalothin and the second was resistant to ampicillin, streptomycin, sulfisoxazole, and tetracycline.

Group 2 contained *E. coli* characterized as O2:H7-ST95; one isolate was from a restaurant/ready-to-eat food source (a honeydew melon) and 8 isolates were from cases of human infection. The *Xba*I PFGE patterns were indistinguishable for 3 of the human infection isolates (MSHS 100, 186, and 811) and the restaurant/ready-to-eat food isolate (68616.01); the other 5 O2:H7-ST95 isolates differed by 1 band (MSHS 1229), two bands (MSHS 95 and MSHS 1062), and 4 bands (MSHS 782 and MSHS 819) from the food source isolate, respectively (Figure 1, panel A). The *Not*I PFGE patterns for MSHS 100 and MSHS 186 were indistinguishable from the restaurant/ready-to-eat isolate, and the other human infection isolates differed by 1 to 7 bands (Figure 1, panel B). The *E. coli* isolate from the food source was fully susceptible, as were most isolates from the human infections, except for 2 (one was resistant to ampicillin, and the second to ampicillin, sulfisoxazole, and trimethoprim/sulfamethoxazole).

Group 3 contained *E. coli* characterized as O114:H4-ST117; one isolate was from retail chicken meat and the second was from a human UTI. The *Xba*I PFGE patterns of the human infection isolate (MSHS 1014A) and retail meat isolate (EC01DT05-0789-01) differed by 5 bands (Figure 2). The *Not*I PFGE patterns differed by >6 bands (Figure 2). Both isolates were fully susceptible. In addition to shared PFGE patterns, these 3 groups of *E. coli* shared the same MLSTs, serotypes, and phylotypes.

The clonal group characterized as *E. coli* O17/O73/O77:H18-ST69, also known as clonal group A (*4*), was identified in human and retail meat samples, although closely related PFGE patterns were not observed (group 4, Table 2). Three other groups (groups 5–7, Table 2), characterized as *E. coli* O4:H5-ST493, O36:NM-ST401, and O172:H16-ST295, exhibited shared MLSTs, serotypes, and phylotypes, but the PFGE patterns were not related.

Discussion

We report the identification of *E. coli* isolates from retail chicken and other food sources that are indistinguishable from or closely related to isolates from human UTIs. Our a priori hypothesis, based on results from previous studies, suggests that retail meat, specifically retail chicken meat, could be a reservoir for *E. coli* causing human extraintestinal infections. This study provides strong support for this hypothesis on the basis of genetic similarities between food and human clinical isolates.

Johnson et al. have proposed that antimicrobial drug–resistant *E. coli* from human feces (and human bloodstream

infections) tend to be more similar to antimicrobial-resistant and -susceptible *E. coli* from retail poultry meat sources (*14*,*15*). These observations indicate that the selection of resistant *E. coli* is more likely to occur in the animal food reservoir than in humans. In this study, we observed that genetically related *E. coli* from food sources and human infections tended to be susceptible, suggesting that both resistant and susceptible isolates causing UTIs in women may be transmitted through the food supply. Our study also identified members of the O2:H7-ST95 group, previously associated with extraintestinal disease in both humans and avian hosts (*34*). The O2:H7-ST95 food source isolate from this study was from a honeydew melon. Potential origins of this *E. coli* contamination could include human or food animal sources.

The *E. coli* O25:H4-ST131 clonal group, also identified in this study, has been associated with extended spectrum β-lactamase production and fluoroquinolone resistance and has been found across Europe and in Canada (*18*,*35*–*37*). The *E. coli* O25:H4-ST131 isolates identified in this study are susceptible; however, because this clonal group may be found in a food animal reservoir and transmitted by

Table 2. Characteristics of *Escherichia coli* clonal groups identified within isolates from 3 types of samples, Canada, 2005–2007*†

Group and strain	Type of sample	Isolate source	Location‡	Year	Genotype			MLST	Serotype
					MLVA	ERIC2	*Xba*I PFGE	ST	
1									
EC01DT06-1737-01	Retail meat	Chicken	Montreal	2006	1.033	33.01	33A.0	131	O25:H4
MSHS 161	Clinical	Human	Montreal	2005	1.033	33.01	33A.0	131	O25:H4
MSHS 1134A	Clinical	Human	Montreal	2007	1.033	33.01	33A.1	131	O25:H4
2									
68616.01	RTE	Honeydew	Montreal	2005	1.018	18.01	18A.0	95	O2:H7
MSHS 100	Clinical	Human	Montreal	2005	1.018	18.01	18A.0	95	O2:H7
MSHS 186	Clinical	Human	Montreal	2005	1.018	18.01	18A.0	95	O2:H7
MSHS 811	Clinical	Human	Montreal	2006	1.018	18.01	18A.0	95	O2:H7
MSHS 1229	Clinical	Human	Montreal	2007	1.018	18.01	18A.1	95	O2:H7
MSHS 95	Clinical	Human	Montreal	2005	1.018	18.01	18A.2	95	O2:H7
MSHS 1062	Clinical	Human	Montreal	2007	1.018	18.01	18A.2	95	O2:NM
MSHS 782	Clinical	Human	Montreal	2006	1.018	18.01	18A.4	95	O2:H7
MSHS 819	Clinical	Human	Montreal	2006	1.018	18.01	18A.4	95	O2:H7
3									
EC01DT05-0789-01	Retail meat	Chicken	Ontario	2005	1.023	23.01	23A.0	117	O114:H4
MSHS 1014A	Clinical	Human	Montreal	2007	1.023	23.01	23A.5	117	O114:H4
EC01DT05-0224-01	Retail meat	Chicken	Ontario	2005	1.023	23.01	23B	117	ONT:NM
EC01DT06-1887-01	Retail meat	Chicken	Montreal	2006	1.023	23.01	23C	117	O143:H4
EC01DT07-0956-01	Retail meat	Chicken	Other	2007	1.023	23.01	23D	117	O53:H4
EC01DT05-1700-01	Retail meat	Chicken	Quebec	2005	1.023	23.01	NT	117	O160:H4
EC01DT07-1050-01	Retail meat	Chicken	Ontario	2007	1.023	23.01	NT	117	O45:H4
EC01DT07-1090-01	Retail meat	Chicken	Montreal	2007	1.023	23.01	NT	117	O24:H4
MSHS 133	Clinical	Human	Montreal	2005	1.023	23.01	NT	117	O24:NM
4									
EC01DT06-0649-01	Retail meat	Pork	Montreal	2006	1.116	116.01	116A	69	O17/73/106:H18
MSHS 719	Clinical	Human	Montreal	2006	1.116	116.01	116C	69	O44:H18
MSHS 956	Clinical	Human	Montreal	2007	1.116	116.01	116D	69	ONT:H18
5									
EC01DT05-1012-01	Retail meat	Pork	Ontario	2005	1.102	102.01	102A	493	O4:H5
MSHS 769	Clinical	Human	Montreal	2006	1.102	102.01	102B	493	O4:H5
6									
EC01DT06-1265-01	Retail meat	Beef	Montreal	2006	2.107	107.01	107A	401	O36:NM
76083.08	RTE	Chicken	Montreal	2007	2.107	107.01	107B	401	O36:NM
7									
EC01DT06-0274-01	Retail meat	Chicken	Quebec	2006	2.097	97.01	97A	295	O172:H16
79287	RTE	Chicken	Montreal	2007	2.097	97.01	97B	295	O172:H16

*MLST, multilocus sequence typing; MLVA, multilocus variable number tandem repeat analysis; ERIC2, enterobacterial repetitive intergenic consensus sequence 2; PFGE, pulsed-field gel electrophoresis; ST, sequence type; RTE, restaurant/ready-to-eat foods; NT, nontypeable; ONT, serogroup nontypeable; NM, non-motile; UNK, unknown. An expanded version of Table 2 containing all isolates is available online at www.cdc.gov/EID/content/16/1/88-T2.htm.

†All isolates in groups 1, 2, and 5 were phylotype B2; all isolates in groups 3, 4, and 8 were phylotype D; all isolates in groups 6, 9, 10, and 11, as well as isolate MSHS 689 in group 17, were phylotype A; all isolates in groups 7, 12, 13, 14, 15, and 16, as well as isolate EC01DT05-0469-01 from group 17, were phylotype B1.

‡Other locations were Saskatchewan or British Columbia.

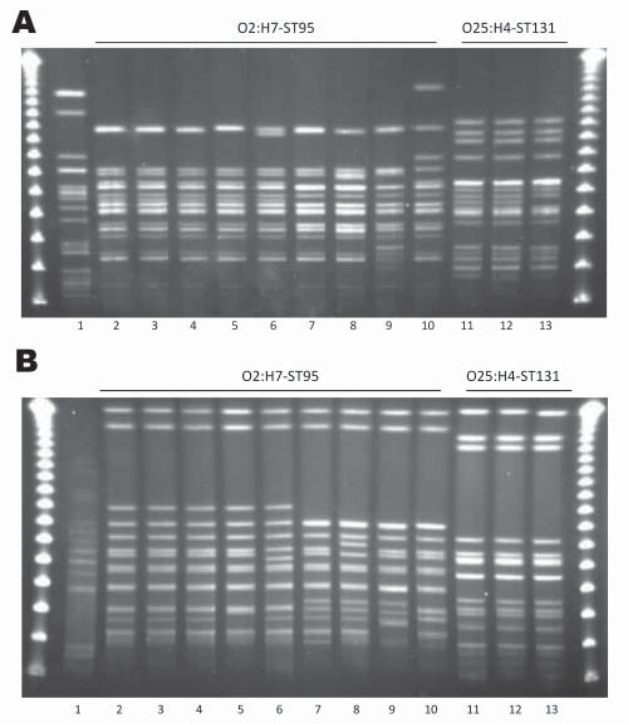

Figure 1. Pulsed-field gel electrophoresis patterns for *Escherichia coli* O2:H7-ST95 and *E. coli* O25:H4-ST131. A) *Xba*I; B) *Not*I. Lane 1 is the positive control *E. coli* O11:H18-ST69 (SEQ102); lane 2 is an *E. coli* O2:H7-ST95 isolate from a restaurant sample of honeydew melon (68616.01); lanes 3–10 are isolates from human urinary tract infection cases (UTIs; lane 3, MSHS 100; lane 4, MSHS 186; lane 5, MSHS 811; lane 6, MSHS 1229; lane 7, MSHS 95; lane 8, MSHS 1062; lane 9, MSHS 782; lane 10, MSHS 819); lane 11 is an *E. coli* O25:H4-ST131 isolate from a retail chicken sample (EC01DT06-1737-01); and lanes 12 and 13 are *E. coli* isolates from human UTIs (lane 12, MSHS 161; lane 13, MSHS 1134A). Outer lanes are pulsed-field molecular weight markers.

food, amplification and transmission of these highly resistant organisms could be possible. Extended spectrum β-lactamase-producing *E. coli* have not yet been identified by CIPARS (*23*,*38*,*39*).

This study was ecologic in design and presents several limitations. Epidemiologic information on the UTI cases was not available. Information on travel, history of antimicrobial drug use, dietary information, and other factors would have been useful to describe the study population and to assess the significance of other possible transmission routes that might explain our results. The study also oversampled retail chicken meat and consequently undersampled isolates from retail pork and beef. It is possible that closely related clonal groups could be identified that contain isolates from both human infections and pork or beef samples. Because of insufficient power in our sampling strategy we could not exclude the existence of these groups; additional sampling of isolates from retail pork and beef are underway to address this question. Despite oversampling isolates from retail chicken meat, we observed that 82% (a greater fraction than the 60% sampling fraction) of *E. coli* belonging to the 17 clonal groups were associated with retail chicken meat. We also oversampled antimicrobial drug-resistant isolates; however, most (53%) isolates that belonged to a clonal group were fully susceptible. Even though the size and scope of this study was limited, we were able to detect several instances of groups containing closely related isolates from human and food sources. It is therefore probable that a food reservoir exists and that foodborne transmission of extraintestinal *E. coli* is common.

The identification of 2 clonal groups containing isolates from retail chicken meat and human infections supports our a priori hypothesis. We cannot exclude the possibility that food source isolates were present because of human contamination during food production, processing or handling, even though it is very unlikely. Subsequent research will help determine whether these *E. coli* occur in a food animal reservoir or whether transfer of these *E. coli* results from contamination during food processing or preparation and reflects human-to-human transmission by food.

This study demonstrates that some *E. coli* from retail chicken meat and other food sources are closely related to *E. coli* causing human UTIs. Since a food animal reservoir apparently exists for *E. coli* that cause urinary tract and other extraintestinal infections, this further reinforces the need for responsible antimicrobial drug stewardship in

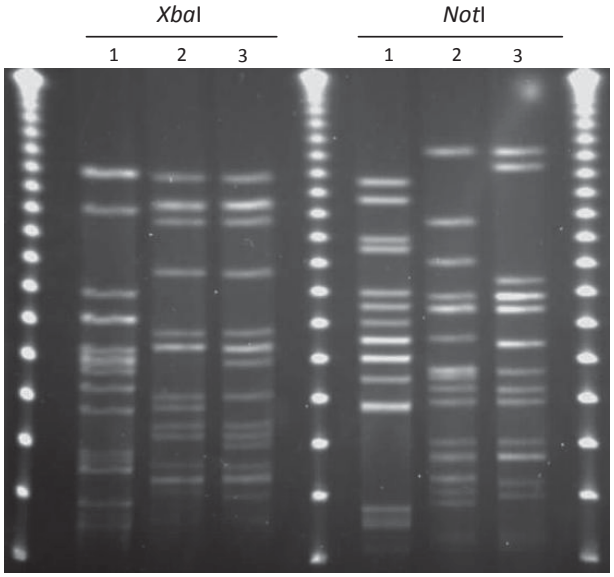

Figure 2. *Xba*I and *Not*I pulsed-field gel electrophoresis patterns for *Escherichia coli* O114:H4-ST117 (lanes 2 and 3). Lane 1 is the positive control *E. coli* O11:H18-ST69 (SEQ102), lane 2 is an *E. coli* O25:H4-ST131 isolate from a retail chicken sample (EC01DT06-1737-01), and lane 3 is an *E. coli* isolate from a human urinary tract infection case (MSHS 1014A). Outer and center lanes are pulsed-field molecular weight markers.

veterinary medicine and food animal production as well as in human medicine.

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Ms Vincent is a graduate student in the Department of Microbiology and Immunology at McGill University in Montréal, Québec. Her research interests include bacteriology and molecular epidemiology and she is currently investigating the possible foodborne transmission of *E. coli* causing urinary tract infections.

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Address for correspondence: Amee R. Manges, Department of Epidemiology, Biostatistics and Occupational Health, McGill University, 1020 Pine Ave W 36B, Montréal, Québec H3A 1A2, Canada; email: amee.manges@mcgill.ca

Serologic Cross-Reactivity with Pandemic (H1N1) 2009 Virus in Pigs, Europe

Constantinos S. Kyriakis, Christopher W. Olsen, Susy Carman, Ian H. Brown, Sharon M. Brookes, Jan Van Doorsselaere, and Kristien Van Reeth

We tested serum samples from pigs infected or vaccinated with European swine influenza viruses (SIVs) in hemagglutination-inhibition assays against pandemic (H1N1) 2009 virus and related North American SIVs. We found more serologic cross-reaction than expected. Data suggest pigs in Europe may have partial immunity to pandemic (H1N1) 2009 virus.

Pandemic (H1N1) 2009 virus is a reassortant of ≥2 circulating swine influenza viruses (SIVs) (*1*). Six gene segments, including the gene encoding a classical H1 hemagglutinin (HA), are similar to ones found in triple reassortant SIVs circulating in pigs in North America. The neuraminidase (NA) and matrix genes originate from Eurasian avian-like H1N1 SIV. This specific reassortant had never been found in swine when it was first detected in humans. However, during May–October 2009, pandemic (H1N1) 2009 virus was isolated from swine in Canada, Argentina, Australia, Singapore, (Northern) Ireland, Norway, the United States, Japan, and Iceland (*2*). Humans were suspected to be the source of infection; pigs did not contribute to spread of the virus in humans.

Recent experimental studies confirmed pandemic (H1N1) 2009 virus can become established in swine populations (*3*,*4*). A key question is whether immunity to circulating SIVs, which differ antigenically and genetically in different parts of the world, protects pigs against pandemic (H1N1) 2009 virus. Three SIV subtypes are cocirculating in European swine populations: an avian-like subtype H1N1 virus introduced from wild birds in 1979, a human-like subtype H3N2 reassortant with HA and NA genes acquired from descendants of Hong Kong/68 pandemic virus, and a subtype H1N2 reassortant virus, which acquired H1 from human influenza (H1N1) viruses in the 1980s (*5*). We aimed to clarify whether pigs infected or vaccinated with these viruses have cross-reactive antibodies against pandemic (H1N1) 2009 virus or related North American SIVs, both of which have an antigenically distinct, classical H1 HA.

Author affiliations: Ghent University, Ghent, Belgium (C.S. Kyriakis, K. Van Reeth); University of Wisconsin, Madison, Wisconsin, USA (C.W. Olsen); University of Guelph, Guelph, Ontario, Canada (S. Carman); Veterinary Laboratories Agency-Weybridge, Addlestone, UK (I.H. Brown, S.M. Brookes); and KATHO Catholic University of South-West Flanders, Roeselare, Belgium (J. Van Doorsselaere)

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The Study

We tested 66 swine sera obtained from previous experimental studies (*6*). All pigs were negative for SIV antibodies at the start of the experiments. Serum samples were collected 3–4 weeks after the last infection or immunization. Immunization categories, experimental groups, and influenza virus strains used are shown in the Table.

Twenty-seven pigs were intranasally (IN) inoculated with 7.0 $\log_{10}$ 50% egg infectious doses of swine/Belgium/1/98 (avian-like swine influenza [H1N1]), swine/Gent/7625/99 (reassortant influenza [H1N2] with human-lineage HA), swine/Flanders/1/98 (reassortant influenza [H3N2] with human-lineage HA), or some combination of the three. These viruses are representative of prevailing SIVs in western Europe. Two groups received a single inoculation with either SIV (H1N1) or SIV (H1N2). Three groups received dual, consecutive inoculations with 2 SIV subtypes at a 4-week interval.

Twenty-four pigs received 2 intramuscular (IM) doses of commercial, inactivated SIV vaccine, at a 4-week interval. One vaccine contained a classical swine-lineage subtype H1N1 virus, A/New Jersey/8/76; the remaining 3 contained various avian-like subtype H1N1 SIVs (Table). The vaccines contained various types of adjuvants.

Six pigs were first inoculated IN with swine/Belgium/1/98 (H1N1) followed by a single IM administration of New Jersey/8/76-based SIV vaccine 5 weeks later. Nine pigs were hyperimmunized against various European SIVs (Table) by IN inoculation, followed by an IM innoculation with the same virus in combination with Freund's complete adjuvant 4 weeks later.

All serum samples were examined in hemagglutination-inhibition (HI) assays against the European subtype H1N1, H1N2, and H3N2 viruses listed above; 3 North American SIVs with a classical H1; and A/California/04/2009, a prototype pandemic (H1N1) 2009 virus (*7*). The North American SIVs included swine/Iowa/H04YS2/2004 (triple reassortant influenza [H1N1]), swine/Ontario/11112/04 (reassortant influenza [H1N1]), and swine/Indiana/9K035/99 (triple reassortant influenza [H1N2]). Low amino acid homology was present in the HA1 region of the HA gene between the European H1 SIVs used for infection of pigs and the North American H1 SIVs (range 70%–75%) or the pandemic (H1N1) 2009 virus (69%–72%). When compared with avian-like in-

fluenza (H1N1) strains in European vaccines, the New Jersey/8/76 vaccine strain was more closely related to North American SIVs (77%–81% vs. 92%–94%) and pandemic (H1N1) 2009 virus (72%–75% vs. 90%).

HI antibodies induced by a single infection with European subtype H1N1 or H1N2 SIVs did not cross-react with North American H1 SIVs or with pandemic (H1N1) 2009 virus. In contrast, consecutive infections with 2 European subtypes frequently induced cross-reactive antibodies, even though European viruses do not contain a classical swine H1 HA.

Three European vaccines also induced cross-reactive antibodies to North American H1 SIVs, and 2 induced antibody titers ≥20 to pandemic (H1N1) 2009 virus in most pigs. HI titers against pandemic virus were lower with A/New Jersey/8/76-based vaccine than with vaccine containing an avian-like SIV (H1N1).

A single vaccination with A/New Jersey/8/76-based vaccine induced only minimal HI antibody titers in influenza-naive pigs, but existing antibody titers (data not shown) to European avian-like subtype H1N1 SIV in pigs previously infected with this virus dramatically increased, even though these viruses contain HA proteins of the avian and classical swine lineages respectively. All pigs had antibodies to the viruses with a classical swine H1 HA, including pandemic (H1N1) 2009 virus.

Finally, hyperimmunization with European SIVs of subtype H1N1, H3N2, or H1N2 resulted in high titers to the homologous viruses. Cross-reactions with viruses with a classical H1 were consistently observed with serum samples from pigs hyperimmune to avian-like European influenza (H1N1) viruses, but they were rare or absent after

Table. Serologic cross-reactivity between North American H1 SIVs and pandemic (H1N1) 2009 virus after experimental infection and/or immunization of pigs by various methods with European SIVs*

Group	No. pigs	Range of antibody titers of positive animals (no. pigs with HI antibody titers ≥10)						
		European SIVs			North American H1 SIVs			
		Swine/ Belgium/ 98 (H1N1) avH1†	Swine/ Gent/99 (H1N2) huH1†	Swine/ Flanders/98 (H3N2) huH3†	Swine/ Iowa/2004 (H1N1) cH1†	Swine/ Ontario/ 2004 (H1N1) cH1†	Swine/ Indiana/99 (H1N2) cH1†	Pandemic (H1N1) 2009 cH1†
Infection‡								
H1N1 (avH1†)	6	40–160 (6)	–§	–	–	–	–	10 (1)
H1N2 (huH1†)	6	–	40–160 (6)	–	–	–	–	–
H1N1 (avH1†)- 4w - H1N2 (huH1†)	5	80–160 (5)	20–40 (5)	–	20 (4)	10–20 (5)	10–20 (5)	20–40 (5)
H1N2 (huH1†)- 4w - H1N1 (avH1†)	4	80–160 (4)	160–320 (4)	10 (3)	10–40 (4)	20–80 (4)	20–40 (4)	20–160 (4)
H1N1 (avH1†)- 4w - H3N2 (huH3†)	6	40–160 (6)	–	20–80 (6)	–	10–40 (3)	10–20 (4)	10–40 (6)
Double vaccination¶								
New Jersey/8/76 (H1N1, cH1†)	6	10–40 (6)	10 (1)	10–80 (5)	80–160 (6)	20–80 (6)	80–320 (6)	10–20 (5)
Swine/Netherlands/25/ 80 (H1N1, avH1†)	6	10–40 (6)	10 (1)	10–160 (6)	20–40 (5)	10–80 (6)	10–40 (6)	10 (3)
Swine/Belgium/230/92 (H1N1, avH1†)	6	20–640 (6)	10–20 (2)	20–320 (6)	10–320 (5)	10–320 (6)	10–160 (5)	10–640 (5)
Swine/Haselunne/2617/ 2003 (H1N1, avH1†)	6	10–40 (6)	40–160 (6)	20 (6)	–	20 (1)	10–20 (5)	10 (1)
Infection# followed by single vaccination								
H1N1 (avH1†)- 4w - New Jersey/8/76	6	640–2,560 (6)	10 (3)	10 (1)	40–80 (6)	40–80 (6)	80–320 (6)	40–80 (6)
Hyperimmunization**								
H1N1 (avH1†)	3	320–1,280 (3)	10–20 (2)	–	40–160 (3)	40–160 (3)	40–80 (3)	40–160 (3)
H1N2 (huH1†)	3	40 (1)	320–1,280 (3)	–	20 (1)	10–80 (2)	40 (1)	80 (1)
H3N2 (huH3†)	3	–	–	160–640 (3)	–	–	–	–

*SIV, swine influenza virus.
†Phylogenetic lineage of the hemagglutinin gene: c, classical swine; hu, human; av, avian.
‡Infection with swine/Belgium/1/98 (H1N1), swine/Gent/7625/99 (H1N2), or swine/Flanders/1/98 (H3N2).
§Antibody titers <10 in all pigs.
¶Only the influenza (H1N1) vaccine component is shown. The first 3 vaccines are bivalent (subtypes H1N1, H3N2); the fourth is trivalent (subtypes H1N1, H3N2, H1N2).
#Infection with swine/Belgium/1/98 (H1N1).
**Each individual pig was hyperimmunized with a different European SIV isolate: subtype H1N1 viruses were swine/Finistère/2899/82, swine/Belgium/1/98, and swine/Gent/132/2005; subtype H1N2 viruses were swine/Scotland/410440/94, swine/Gent/7625/99, and swine/Gent/177/2002;

hyperimmunization with subtype H1N2 or H3N2 strains.

Conclusions

A large antigenic and genetic distance exists between European H1 SIVs and viruses with a classical H1 HA (*8*,*9*). Nevertheless, pigs infected or vaccinated with European SIVs frequently have cross-reactive HI antibodies to pandemic (H1N1) 2009 virus and related North American SIVs. Two factors predispose for serologic cross-reactivity: 1) elevated antibody titers to European avian-like subtype H1N1 SIVs, as in hyperimmune swine serum and some postvaccination serum; and 2) infection with 2 European SIV subtypes.

Consecutive experimental infection of pigs with the antigenically distinct SIVs of subtypes H1N1 and H1N2 causes a strong boost of already existing HI antibody titers to the first infecting virus, as shown by longitudinal investigations (*6*). These pigs may even develop low levels of cross-subtype-reactive HI antibodies to SIV (H3N2). In humans and in mice, sequential infections with influenza virus variants seemingly lead to a predominant antibody response against cross-reactive epitopes on the HA, which are shared with the first infecting virus, whereas the response to strain-specific epitopes may be lower (*10*). Such cross-reactive antibodies may cause cross-recognition of viruses with a classical H1 HA in our dually infection–immune pigs, but further studies with monoclonal antibody escape mutants are needed to understand cross-reactivity at the molecular level. Multiple SIV infections have become common in swine-dense regions of Europe since the introduction of the H1N2 subtype in the late 1990s (*11*).

The HI test will clearly fail to differentiate here between established SIVs and pandemic (H1N1) 2009 virus. This conclusion is further supported by preliminary HI tests on sera collected from finishing pigs in Belgium during 2007. Of 172 serum samples, 35% and 36%, respectively, were positive against swine/Iowa/2004 (H1N1) and swine/Indiana/99 (H1N2); 95% of these positives had antibodies to >1 European SIV subtype.

Besides serum antibody to the HA, an infection with live influenza virus also stimulates mucosal immunity and cellular immune responses to highly conserved viral epitopes (*12*). These responses mean partial protection against a subsequent infection with an antigenically unrelated strain may occur in the absence of cross-reactive antibodies (*13*). In contrast, protection offered by inactivated influenza vaccines is almost entirely dependent on serum HI antibody titers. Two of the 4 vaccines used induced HI antibody titers against pandemic (H1N1) 2009 virus that are considered protective. Unexpectedly, avian influenza (H1N1) SIV-based vaccine gave higher antibody titers than the A/New Jersey/76-based vaccine. The amino acid homology between vaccine and test strains is thus a less reliable predictor of serologic cross-reactivity than one would assume. Our data suggest that preexisting immunity to established SIV strains may partially protect pigs in Europe against pandemic (H1N1) 2009 virus, but the extent of such protection needs to be assessed in well-controlled challenge experiments. Pandemic (H1N1) 2009 infection in pigs in Europe has so far been limited to countries where SIVs are absent or circulating at low levels. Whether the virus will become established in countries where SIVs are widespread remains to be seen.

The divergence between H1 of contemporary seasonal influenza (H1N1) viruses of humans and pandemic (H1N1) 2009 virus is approximately the same as that between SIVs in Europe and North America. However, cross-reactive HI antibody responses to pandemic (H1N1) 2009 virus have been almost exclusively detected in humans born before 1950 (*14*). These persons have been exposed to older variants of seasonal influenza (H1N1) viruses that are more closely related to classic swine influenza (H1N1) viruses and pandemic (H1N1) 2009 virus. The present epidemiologic situation in humans thus differs from that in pigs. Two antigenically distinct influenza (H1N1) viruses—seasonal and the 2009 virus—could cocirculate in humans in the future. This cocirculation will likely broaden serologic responses and protection against influenza but may also complicate interpretation of HI test results.

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Dr Kyriakis was a PhD student at the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, from 2004 to 2009. He has studied the efficacy of influenza vaccines for pigs and was involved in the European Surveillance Network for Influenza in Pigs 2.

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Address for correspondence: Kristien Van Reeth, Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium; email: kristien.vanreeth@ugent.be

Hospitalizations for Pandemic (H1N1) 2009 among Maori and Pacific Islanders, New Zealand

Ayesha Verrall, Katherine Norton, Serena Rooker, Stephen Dee, Leeanne Olsen, Chor Ee Tan, Sharon Paull, Richard Allen, and Timothy K. Blackmore

Community transmission of influenza A pandemic (H1N1) 2009 was followed by high rates of hospital admissions in the Wellington region of New Zealand, particularly among Maori and Pacific Islanders. These findings may help health authorities anticipate the effects of pandemic (H1N1) 2009 in other communities.

Spread of influenza A pandemic (H1N1) 2009 virus coincided with the Southern Hemisphere winter, leading to outbreaks in Australia and New Zealand (NZ) (*1*). Quantifying the number of admissions and the age, ethnicity, and medical conditions of patients admitted to hospitals in the Wellington region because of acute illness during the first wave of pandemic (H1N1) 2009 provides valuable information to health authorities anticipating the impact of the pandemic in other regions.

The Study

Patients suspected of having influenza and requiring hospital assessment were tested with real-time reverse transcription–PCR by using protocols from the US Centers for Disease Control and Prevention (*2*). This analysis included only persons (case-patients) with confirmed pandemic (H1N1) 2009 who were admitted to public hospital beds in the NZ Wellington and Hutt Valley regions. The results of viral testing were not available at the time of admission and therefore did not affect decisions about admission.

We identified case-patients by matching laboratory and hospital patient information systems. Sex, age, clinical service, inpatient mortality, and self-reported ethnicity were available, as was limited information about medical conditions. Ethnicity data were aggregated as NZ European, Maori, Pacific Islanders, or other (i.e., all other and unspecified ethnicities combined). Admission rates per 100,000 persons were calculated using regional denominator data from the 2006 NZ Census (*3*) and age standardized by a direct method using 10-year age groups.

Testing for pandemic (H1N1) 2009 began on April 24, 2009, and local community transmission was detected in the Wellington region early in June. During June 8–August 31, pandemic (H1N1) 2009 was identified in 229 hospitalized case-patients. Hospitalizations began in June, peaked in July, and then declined rapidly (Figure 1). The mean age of admitted persons was 26 years (range 0–82 years); 62% were <30 years of age. A total of 117 (51%) admissions were under adult medical services, 79 (34%), under pediatric medical services, and 15 (7%), under obstetric services. Mean duration of admission was 6.1 days (range 0–24 days) and 111 (48%) patients stayed in hospital for <72 hours (Figure 2). Nineteen (8%) case-patients were admitted to intensive care or high dependency units for at least 1 night. Five (2%) case-patients died during their hospital stay; all of these deaths were attributed to pandemic (H1N1) 2009.

Seventy-four (32%) case-patients were NZ European, 70 (31%) were Maori, 64 (28%) were Pacific Islanders, 17 (7%) were other, and 4 (2%) were unspecified. The rate of admission for NZ Europeans in the Wellington region was 25.6 per 100,000. Age-adjusted rates for Pacific Islanders and Maori were 180 and 128 per 100,000, respectively.

Ninety-one (40%) of the total group had a chronic lung condition; of these, 58 (64%) had asthma. For 116 (51%), a preexisting medical condition was described in the discharge summary. Eighteen women were either pregnant or immediately postpartum during their hospital stays. Rates of chronic respiratory conditions were lower for Pacific Islanders than for Maori or all others (28%, 41%, and 48%, respectively) (Table). Ethnicity and presence of a chronic

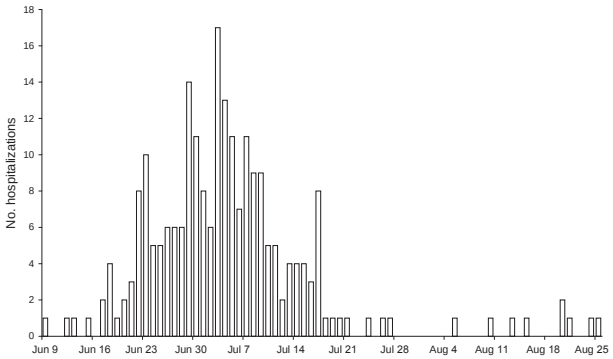

Figure 1. Number of hospitalizations for pandemic (H1N1) 2009, by date of admission, Wellington region, New Zealand, June–August 2009.

Author affiliations: Capital and Coast District Health Board, Wellington, New Zealand (A. Verrall, K. Norton, S. Rooker, L. Olsen, C.E. Tan, S. Paull, R. Allen, T.K. Blackmore); Hutt Valley District Health Board, Lower Hutt, New Zealand (S. Dee); and University of Otago, Wellington (A. Verrall)

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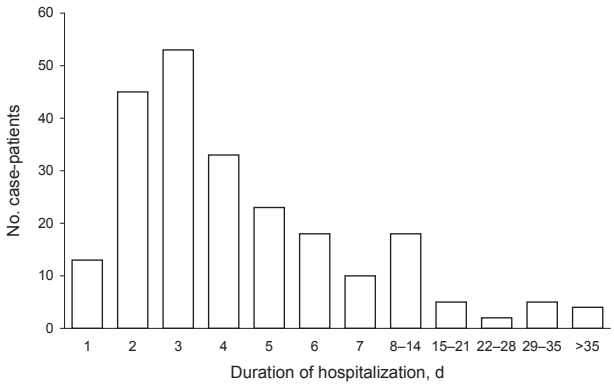

Figure 2. Duration of hospitalization for case-patients with pandemic (H1N1) 2009, Wellington region, New Zealand, June–August 2009.

respiratory condition were dependent ($\chi^2 = 6.22$, 2 df, p = 0.044). Rates of other medical conditions for Pacific Islanders, Maori, and all others were 56%, 41%, and 54% (independent $\chi^2 = 3.67$, 2 df, p = 0.16).

Conclusions

The Wellington region had higher population-adjusted hospitalization rates for pandemic (H1N1) 2009 than did New Zealand and countries overseas (*4*). In Australia, the reported rate of hospitalizations was one third that in our region (*5*). Local testing or referral patterns may have contributed to more admissions in a variety of ways. First, our rates of laboratory confirmation may be higher because of ready access to timely PCR results (*6*), facilitating investigation and case detection in persons admitted to a hospital. NZ hospitals are unlikely to have a lower threshold for admission than Australian hospitals. Although both countries have free access to hospital care for pandemic (H1N1) 2009, NZ hospitals are always busy in the winter, so clinical services attempt to manage patients in the community wherever possible.

Our study shows that Pacific Islanders and Maori were 7 and 5 times more likely, respectively, than NZ Europeans to require hospital admission. These findings are consistent with observations from previous influenza epidemics. During the 1918 pandemic, the death rate was 7-fold higher in Maori than in the NZ Europeans (*7*). In Samoa in 1918, 80% of the population was considered infected, and 7,264 died from a total population of 36,405 (*8*). Other indigenous communities, such as Aboriginals in Australia and Inuit in Canada, also appear to have higher rates of severe influenza-related illness (*9*).

Factors other than disease severity may contribute to admissions. A higher incidence of pandemic (H1N1) 2009 in Pacific Islanders or Maori households is likely to have led to greater representation across the spectrum of clinical manifestations, including severe disease (Environmental Science and Research, unpub. data). This explanation is supported by national surveillance, which demonstrated higher rates of confirmed pandemic (H1N1) 2009 in Maori and Pacific Islanders. However, this finding depends heavily on community testing, which was never uniformly available across NZ and ceased to be offered routinely once NZ switched to a manage-it phase (*10*). An alternative cause of higher rates in Pacific Islanders or Maori may be a greater readiness to self-refer to an emergency department for assessment when unwell.

This study does not address why severe influenza is more frequently observed in Pacific Islanders and Maori. We did not observe higher rates of preexisting medical conditions in the hospitalized Maori and Pacific Islanders and noted lower rates of chronic respiratory conditions in Pacific Islanders. However, other conditions were detected only on discharge summary review and not by screening or systematic questioning; thus other undocumented conditions may contribute to ethnic disparity in hospitalizations. A comparison of seropositivity rates in representative sections of high- and low-risk communities is needed to ascertain whether high hospitalization rates resulted from high rates of community transmission, preexisting conditions, or higher rates of complications of influenza. Future control strategies, including primary prevention or improved access to timely treatment, could then be refined to the needs of Maori and Pacific Islanders. In any case, targeting vaccination to the elderly and persons with known comorbid conditions will fail to protect a substantial minority of persons who need hospital care and will particularly disadvantage Pacific Islanders.

The observed age distribution is consistent with other reports describing acute pandemic (H1N1) 2009 (*11*). Most admissions were for only a few days, but a substantial minority of hospitalized case-patients required care for sev-

Table. Characteristics of 229 persons hospitalized with influenza A pandemic (H1N1) 2009 (case-patients), Wellington region, New Zealand, June–August 2009

Ethnic group	No. case-patients	Age-adjusted hospitalization rate per 100,000	Median age, y	No. (%) case-patients with chronic lung condition	No. (%)case-patients with other medical condition
Pacific Islanders	64	180	15.5	18 (28)	36 (56)
Maori	70	128	23.5	29 (41)	29 (41)
Other*	95	25.6	29	45 (47)	44 (46)

*All other and unspecified ethnicities combined.

eral weeks. Most new admissions were confined to 1 month at the start of winter.

Pandemic (H1N1) 2009 has resulted in hospital admissions and severe illness in our study population more frequently than reported elsewhere. What proportion is due to good access to nucleic acid-based testing, local disease transmission dynamics, or intrinsic susceptibility of our population to the virus is not clear. Nonetheless, our findings will interest those planning health services internationally, including other areas serving Pacific Islanders and indigenous communities.

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Dr Verrall is an infectious diseases registrar at Wellington Hospital. Her research interests include infectious diseases epidemiology and ethics.

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Address for correspondence: Timothy K. Blackmore, Infectious Diseases Physician and Microbiologist, Wellington Hospital, Private Bag 7902, Wellington, New Zealand; email: timothy.blackmore@ccdhb.org.nz

Pandemic (H1N1) 2009 Surveillance and Prevalence of Seasonal Influenza, Singapore

Yee-Sin Leo, David C Lye, Timothy Barkham, Prabha Krishnan, Eillyne Seow, and Angela Chow

On April 25, 2009, Singapore implemented strict containment measures for pandemic (H1N1) 2009 with enhanced surveillance and hospital isolation. In the first month, seasonal influenza, predominantly virus subtype H3N2, was diagnosed for 32% of patients with acute febrile respiratory illness. Our findings underscore the high prevalence of seasonal influenza in Singapore.

Tropical countries experience influenza year round, with 2 peaks corresponding to the rainy seasons (*1*). Despite this year-round activity, seasonal influenza is often neglected in tropical countries in terms of clinical diagnosis, treatment, and vaccination (*2*). In Singapore, influenza activity usually peaks in June and December (*3*). The annual all-cause death rate from seasonal influenza in Singapore has been estimated at 14.8/100,000 person-years; the proportion of deaths among persons ≥65 years of age is 11.3× higher than that among the general population (*4*). In addition, previous pandemic influenza–related excess deaths in Singapore are comparable to those in temperate countries (*5*).

In April 2009, a novel influenza A virus (H1N1) of swine origin emerged in the United States (*6*) and triggered alarm about its pandemic potential (*7*). On June 11, 2009, the World Health Organization announced that the virus had become pandemic; it is now referred to as pandemic (H1N1) 2009 virus (*8*). We report on enhanced influenza surveillance in Singapore that was implemented before the first case of pandemic (H1N1) 2009 was detected on May 26, 2009, and we describe the transition in Singapore from influenza cases caused predominantly by seasonal influenza to cases caused exclusively by pandemic (H1N1) 2009 virus.

The Study

This surveillance study was approved by the Chairman of Medical Board, Tan Tock Seng Hospital (TTSH), Singapore. The clinical study was approved by the Domain Specific Review Board, National Healthcare Group, Singapore.

On April 25, 2009, Singapore's Ministry of Health initiated containment measures in response to pandemic (H1N1) 2009. Travelers returning from affected countries to Singapore with acute febrile respiratory illness were screened at the TTSH emergency department, the designated screening center for pandemic (H1N1) 2009. Thermal screening was conducted at all border entry points, and the mass media publicized nationwide that persons with a risk for pandemic (H1N1) 2009 virus infection based on travel history, fever, and respiratory symptoms should go to the TTSH emergency department for screening. Combined nasal and throat swab specimens were tested with an in-house, gel-based PCR for influenza A, H1, H3, H5, N1, and N2. All specimens with subtype H1N1–positive test results underwent in-house, probe-based influenza (H1N1) 2009 PCR, and partial sequencing of the matrix gene was conducted to confirm positive PCR results.

From April 27 through May 24, 2009, a total of 300 persons (244 travelers returning from affected countries with a respiratory illness and 56 symptomatic contacts) were screened for influenza infection: 24.0% had subtype H3N2, 1.6% had seasonal subtype H1N1, and 2.7% had influenza B. The median age of patients was 36 years (range 10–70 years), and 14.7% had other illnesses (asthma [52.3% of those with other illnesses], diabetes mellitus [29.5%], heart disease [11.4%], cancer [4.5%], and HIV [4.5%]). Fever was reported for 92.9% of all screened persons, cough for 82.4%, sore throat for 57.6%, rhinorrhea for 62.4%, myalgia for 25.9%, headache for 16.5%, and gastrointestinal symptoms for 9.4%.

Two thirds of the patients with influenza-positive PCR results met the criteria for influenza-like illness (ILI) as defined by the US Centers for Disease Control and Prevention (CDC) (*9*). Patients who met these criteria were more likely than those who did not to have influenza-positive PCR results (odds ratio [OR] 8.1, 95% confidence interval [CI] 4.6–14.3, p = 0.0001). Compared with contacts who had no recent travel, travelers returning from North America were less likely to have influenza-positive PCR test results (OR 0.13, 95% CI 0.06–0.27, p = 0.0001).

To enhance detection of pandemic (H1N1) 2009, we obtained nasal and throat swab samples on May 2 and 3, 2009, from all hospitalized patients with clinically suspected pneumonia, regardless of their travel history. The samples were tested by PCR for influenza. A total of 146 patients were screened, of whom 21 (14.4%) were positive for influenza; 10.3% of the 146 patients had H3N2, 1.4% had seasonal H1N1, and 2.7% had influenza B. The median age of these patients was 67 years (range 20–95 years), and their median hospital stay at screening was 1 day (range 0–17 days). Of the patients, 52% were male, and 86% had

Author affiliation: Tan Tock Seng Hospital, Singapore

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other illnesses. At the time of admission, 90% of patients had a fever, 76% had a cough, 24% had a sore throat, 52% had rhinorrhea, 24% had myalgia, and 19% had headache. Findings on chest radiographs were abnormal for 10 patients (48%), of whom 5 had findings consistent with pneumonia. All but 1 patient were treated with antimicrobial drugs; none was given antiviral drug therapy. The median hospital stay was 5 days (range 1–38 days). Two patients died; both had multiple illnesses.

In addition, during May 2–8, 2009, as part of nationwide enhanced influenza surveillance, nasal and throat swab specimens from patients screened at the TTSH emergency department were tested by PCR for influenza virus. The patients had fever or respiratory symptoms but no history of travel to an affected country. Overall, 95 patients were screened, of whom 30 (31.6%) had positive results for influenza; 24.2% of the 95 patients had H3N2 and 7.4% had influenza B. Fever was reported for 69.5% of the 95 patients, cough for 75.8%, sore throat for 56.8%, rhinorrhea for 58.9%, myalgia for 16.8%, and headache for 44.2%. Of the 30 patients with PCR results positive for influenza, 22 (73%) met the criteria for ILI. Compared with patients who did not meet these criteria, patients who did meet them were 3× more likely to have influenza-positive PCR results (OR 3.02, 95% CI 1.17–7.75, p = 0.019). Only 6 (6.3%) of the 95 patients had self-reported influenza vaccination in the preceding 6 months.

Screening of patients with ILI symptoms continued at the TTSH emergency department, and from May 3 through June 13, 2009, only seasonal influenza (predominantly virus subtype H3N2) was detected. Pandemic (H1N1) 2009 was first detected during the week beginning June 14, and the weekly incidence rapidly increased until the week ending July 25, when all influenza cases were caused by pandemic (H1N1) 2009 virus (Figure).

By July 25, 2009, a total of 838 patients with pandemic (H1N1) 2009 virus infection had been seen at the TTSH emergency department. The median age of patients was 22 years (range 10–90 years). Fever was reported for 85.3%, cough for 87.2%, sore throat for 55.4%, rhinorrhea for 41.6%, myalgia for 11.1%, and headache for 11.0%; 57% of patients met the CDC criteria for ILI. Patients with pandemic (H1N1) 2009 were significantly younger (p = 0.0001) than patients with seasonal influenza, but the proportion with ILI in each group was similar (OR 0.65, 95% CI 0.41–1.04, p = 0.071).

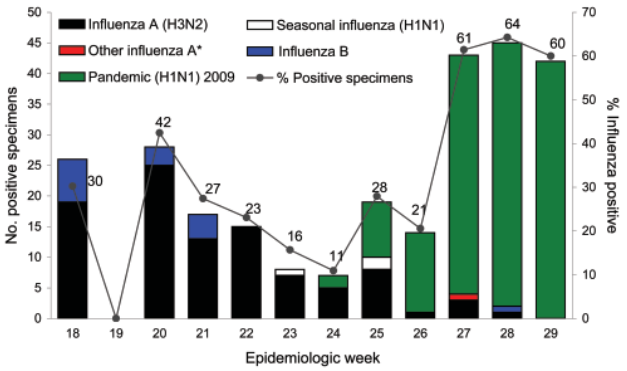

Figure. Influenza-positive test results for surveillance samples obtained at the emergency department, Tan Tock Seng Hospital, Singapore, May 3–July 25, 2009. An epidemiologic week starts on a Sunday and ends on a Saturday (e.g., week 18 started on May 3 and week 29 on July 19). *Undetermined influenza A subtypes.

Conclusions

Each year in the United States, seasonal influenza accounts for >200,000 hospitalizations and 41,000 deaths, and it is the seventh leading cause of death (*10*). The effect of seasonal influenza in the tropics is less well studied. In Thailand, influenza was detected in 11%–12.5% of patients with community-acquired pneumonia and in 23% of outpatients with ILI (*11*,*12*).

Enhanced influenza surveillance in Singapore in response to pandemic (H1N1) 2009 yielded a high prevalence of seasonal influenza: 28.3% (85 of 300) among returning travelers and their contacts with respiratory symptoms, 14.4% (21 of 146) among hospitalized patients with suspected pneumonia, and 31.6% (30 of 95) among patients self-reporting to the TTSH emergency department with fever and respiratory symptoms but without a history of travel to affected countries. These findings reflect the peak local influenza season (*2*,*4*–*5*). The risk of having seasonal influenza was lower among travelers returning from North America, a finding that was consistent with the timing of the peak local influenza season and the noninfluenza season in the Northern Hemisphere.

Of 21 patients with influenza-positive PCR results, only 5 had radiographic evidence of pneumonia despite having a pneumonia diagnosis based on an acute history of fever and cough. No patients, including those with positive test results, were treated with antiviral drugs. This finding, in addition to low influenza vaccine coverage in Singapore, reflects the underappreciation of influenza by doctors in this country.

Of note, 67%–73% of the patients with influenza-positive PCR results met the CDC ILI criteria during the study period; this finding may guide testing for seasonal influenza during peak influenza seasons in May–June and December. Its value in nonpeak seasons, however, requires further evaluation because influenza occurs year-round in the tropics (*1*). Temperature >38°C and either cough or sore throat (*13*) were the most specific screening criteria and had the best positive predictive value; temperature of 37.5°C and either cough, sore throat, or rhinorrhea were the

Table. Performance of influenza screening criteria for travelers returning to Singapore and their contacts, April 27–May 24, 2009

Screening criteria	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %
Temperature ≥37.5°C and cough or sore throat	74.1	71.6	50.8	87.5
Temperature ≥37.5°C and cough, sore throat, or rhinorrhea	81.2	71.2	52.7	90.5
Temperature ≥37.8°C and cough or sore throat (*9*)	67.1	80.0	57.0	86.0
Temperature >38°C and cough or sore throat (*13*)	54.1	87.0	62.2	82.7

most sensitive screening criteria and had the best negative predictive value for influenza (Table).

Our findings highlight a high prevalence of seasonal influenza during peak times in Singapore; the prevalence is comparable to that during typical influenza seasons in temperate countries in the Southern Hemisphere. This prevalence underscores the need for not neglecting seasonal influenza and for a more robust surveillance system to be in place for community and hospital monitoring in Singapore.

Dr Leo is clinical director of the Communicable Disease Centre, Tan Tock Seng Hospital, Singapore. Her research interests include HIV, dengue, influenza, and other emerging infectious diseases.

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Address for correspondence: Yee-Sin Leo, Tan Tock Seng Hospital, 11 Jalan Tan Tock Seng, Singapore 308433; email: yee_sin_leo@ttsh.com.sg

Reemergence of Syphilis in Martinique, 2001–2008

André Cabié, Bruno Rollin, Sandrine Pierre-François, Sylvie Abel, Nicole Desbois, Pascale Richard, Patrick Hochedez, Raphaëlle Théodose, Danielle Quist, Raymond Hélénon, Christian Derancourt, Annick Cavelier, and Bernard Liautaud

Syphilis reemerged in Martinique in 2004 and initially affected 3 HIV-infected patients. By March 2008, syphilis was diagnosed for 37 men and 18 women. As of October 31, 2009, this outbreak had not yet been brought under control. It initially affected mainly men who had sex with men before it spread to heterosexual persons, minority group members, and crack cocaine users.

Syphilis was expected to reemerge in Martinique after outbreaks occurred in large western urban centers in 1998 (*1*,*2*), and cases were reported in Guadeloupe in 2001 (*3*). Soon after the first cases were diagnosed in Martinique, we conducted a study to determine whether these cases represented an outbreak and to identify demographic and social determinants (*4*) of this outbreak.

The Study

In 2001, we increased syphilis screening at University Hospital in Fort-de-France, Martinique. Screening included use of the rapid plasma reagin (RPR) test and the *Treponema pallidum* hemaglutination assay (TPHA). All positive and discordant results were verified by using fluorescent treponemal antibody absorption, which detects *T. pallidum*–specific immunoglobulin (Ig) G and IgM. Darkfield microscopy was used whenever possible. We reviewed medical files of all patients who had received a diagnosis of syphilis during January 1, 2001–March, 31, 2008. Patients were included in the study if they had recent syphilis (primary, secondary, or early latent stage) as defined by the US Centers for Disease Control and Prevention (Atlanta, GA, USA) (*5*).

We investigated the yearly incidence of recent syphilis among HIV-infected patients treated at the infectious diseases unit of the hospital, at the Vernes Sexually Transmitted Disease (STD) Clinic (Fort-de-France, Martinique), and at anonymous voluntary counseling and testing clinics. We also obtained syphilis test results of all persons who were tested at the central laboratory of the hospital. Laboratory definition of active syphilis was an RPR titer >4 and a TPHA titer >80 for an initial screening test, or a 4-fold increase in RPR titer in samples after previously positive results. TPHA screening results for voluntary blood donors were collected at a blood bank.

Recent syphilis was diagnosed for 55 patients at University Hospital during 2001–2008 (Table 1). Patients (37 men and 18 women) had a median age of 41 years (interquartile range [IQR] 36–44 years). Twenty-one (57%) of 37 men were men who have sex with men (MSM), and 9 (43%) of 21 were bisexual. One fourth of the patients never used condoms. Of 36 patients questioned about oral sex, 30 admitted practicing oral sex, of whom only 2 (6.6%) always used condoms. Each patient's median number of sexual partners during the previous 12 months was 2.5 (IQR 1.5–3.5).

One of the first patients to receive a diagnosis in 2004 reported >100 sexual partners, most during a recent stay in Paris. Primary, secondary, and early latent syphilis was diagnosed in 12, 33, and 10 patients, respectively, and 21 patients with secondary syphilis had genital lesions. Cholestatic hepatitis developed in 7 of 29 HIV-positive patients. Six patients had neurosyphilis or ophthalmic syphilis, all of whom also had secondary rashes.

Median RPR titer for the 55 patients was 32. Results of darkfield microscopy were positive for 17 (74%) of 23 patient specimens, 5 from genital mucosa and 12 from skin lesions. All patients had prevention counseling and were successfully treated with penicillin (except for 1 patient who was successfully treated with azithromycin). Seven relapses occurred. More than half of the patients were HIV positive (53%): 22 with a previous diagnosis of HIV infection and 7 with a new diagnosis of HIV infection at the time of syphilis diagnosis. Median duration of HIV infection was 48 months (IQR 21–91 months), and median CD4 lymphocyte count was 516 cells/μL (IQR 340–639 cells/μL).

At the time of syphilis diagnosis, 8 patients were receiving highly active antiretroviral therapy, and 4 had an HIV viral load <50 copies/mL. Twenty-three patients (42%) were crack cocaine users, and 17 patients (7 heterosexual

Author affiliations: Centre Hospitalier Universitaire de Fort-de-France, Fort-de-France, Martinique (A. Cabié, B. Rollin, S. Pierre-François, S. Abel, N. Desbois, P. Hochedez, R. Théodose, D. Quist, R. Hélénon, C. Derancourt, B. Liautaud); Clinical Research Center of French West Indies and French Guiana, Inserm CIE 802 (A. Cabié); Unité de Consultation et de Soins Ambulatoires Centre Médical Pénitentiaire de Fort-de-France (S. Abel, D. Quist); Etablissement Français du Sang, Fort-de-France (P. Richard); and Vernes STD Clinic, Fort-de-France (R. Hélénon, A. Cavelier)

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Table 1. Characteristics of 55 patients with syphilis, University Hospital, Fort-de-France, Martinique, January 1, 2001– March 31, 2008*

Characteristic	Syphilis stage						Total
	Primary		Secondary		Early latent		
	HIV-positive	HIV-negative	HIV-positive	HIV-negative	HIV-positive	HIV-negative	
Total no. patients	1	11	24	9	4	6	55
Sex							
M	1	9	17	6	4	0	37
F	0	2	7	3	0	6	18
MSM	1	1	13	3	3	0	21
Crack cocaine use	0	6	8	6	0	3	23
Precarious conditions	0	5	6	5	0	1	17
HIV diagnosis							
Before syphilis	1	ND	18	ND	3	ND	22
Concomitant with syphilis	0	ND	6	ND	1	ND	7
Darkfield microscopy, no. positive/total	0/1	2/5	11/13	4/4	ND	ND	17/23
Modal RPR titer	16	8	128	16	32	32	32

*MSM, men who have sex with men; precarious conditions, ≥1 of the following: homelessness, lack of welfare, being followed-up in a psychiatry unit, mental deficiency, having paid sex, incarceration in correctional facility >2× in the past 5 years; ND, not determined; RPR, rapid plasma reagin.

men, 2 MSM, and 8 women) (31%) lived in precarious conditions (defined as ≥1 of the following: homelessness, lack of welfare, being followed-up in a psychiatry unit, mental deficiency, having paid sex, incarceration in a correctional facility >2× in the past 5 years).

The first cases of syphilis were diagnosed in 2004, with peaks in number of cases in 2005 and 2007–2008 (Figure 1). Demographic and social characteristics of patients changed rapidly. The first peak in 2005 included mostly HIV-infected MSM, and the second peak in 2007–2008 included almost as many women as men and a larger proportion of persons living in precarious conditions; crack-cocaine use and paid sex were more frequent, but HIV infection was lower (Figure 2).

During 2000–2007, the incidence of syphilis among HIV-infected patients increased from 0/1,000 to 12.1/1,000 patient-years (Table 2). A marked decrease in cases of recent syphilis diagnosed at the STD clinic occurred during 1987–1992, and <5 cases were recorded yearly during 2001–2007. Among persons tested for syphilis in anonymous voluntary counseling and testing clinics, 12 were diagnosed with recent syphilis during 2002–2007 (incidence <5 cases/year).

Among blood donors, incidence of positive TPHA results was 0.3%–0.6% during 2000–2007, and an incidence of 1.04% was observed in 1999. However, the outbreak was too limited to have affected this group. The incidence of active syphilis as a proportion of all cases reported to the central laboratory database increased from 1.6% to 11.6% during 2000–2007 (Table 2).

Conclusions

Infectious syphilis had become so rare in France and in the French Caribbean islands in the 1990s that mandatory notification of this disease was canceled in 2000. That same year, outbreaks were reported by several STD clinics in France (*6*). These outbreaks followed reports of outbreaks among MSM in major urban centers in the United States (*2*,*7*), Canada, Europe, Australia, and New Zealand (*4*). Three epidemiologic profiles of syphilis patients were recently defined on the basis of social determinants (*4*): general populations in developing countries, minority populations with a low socioeconomic status in industrialized countries, and MSM.

In 2001, an outbreak of 58 cases of recent syphilis diagnosed during 1993–2001 (38 cases in 2001) was reported in Guadeloupe (*3*). This outbreak occurred mainly (89%) in the population living in precarious social and economic conditions; 21% had a history of recent imprisonment, and 50% used crack cocaine and had paid sex. The M:F ratio increased from 0.81:1 to 1.37:1. Twenty-six percent of the patients were HIV positive.

A limited but uncontrolled syphilis outbreak is ongoing in Martinique. It started with an MSM epidemiologic profile, then shifted to persons within a specific heterosexual group that included crack cocaine users. This shift is similar to that reported for HIV infection in the Caribbean in the 1980s (*8*–*11*). Bisexuality may play a role by linking different populations during HIV and syphilis epidemics (*8*,*11*,*12*).

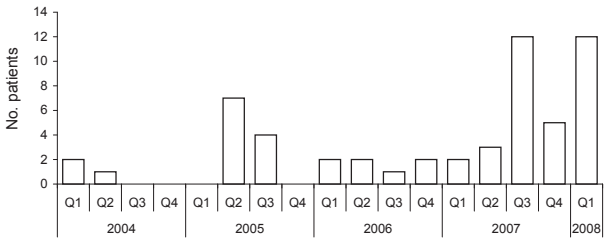

Figure 1. Patients with syphilis at University Hospital, Fort-de-France, Martinique, first quarter of 2004 through first quarter of 2008.

Table 2. Incidence of syphilis, Fort-de-France, Martinique, 2000–2007*

Characteristic	2000	2001	2002	2003	2004	2005	2006	2007
HIV-infected patients at University Hospital								
Total no. patients	479	492	508	544	564	659	724	746
MSM, no. (%)	107 (22)	108 (22)	110 (22)	126 (23)	124 (22)	160 (24)	184 (25)	185 (25)
Incidence of recent syphilis/ 1,000 patient-years, %	0	0	0	0	5.3	15.2	9.6	12.1
Incidence of recent syphilis/ 1,000 patient-years in MSM, %	0	0	0	0	24.2	31.2	21.7	27.1
Vernes STD Clinic								
Syphilis tests, no.	306	559	1,368	1,478	1,266	1,081	976	1,592
Recent syphilis, no.	0	1	2	3	0	0	3	4
A-VCT clinics								
Syphilis tests, no.	ND	ND	93	821	651	1,186	709	1,159
Recent syphilis, no.	ND	ND	0	1	2	4	2	3
University Hospital central laboratory								
Syphilis tests, no.	2,053	1,755	1,876	2,575	2,387	3,187	2,050	3,225
TPHA titer >80, no. (%)	189 (9.2)	171 (9.7)	182 (9.7)	235 (9.1)	200 (8.3)	258 (8.1)	114 (5.6)	217 (6.7)
RPR test titer >4/TPHA titer >80,† %	1.6	1.8	2.2	2.1	5.0	7.0	11.4	11.6
RPR test titer ≥8/TPHA titer >80, %	1.1	0.6	1.1	0.9	3.5	6.6	8.8	9.7
Blood donors								
Syphilis tests, no.	8,161	8,315	8,638	8,914	8,993	8,057	9,199	8,495
TPHA titer >80, no. (%)	45 (0.6)	49 (0.6)	39 (0.5)	42 (0.5)	30 (0.3)	31 (0.4)	33 (0.4)	27 (0.3)
RPR test titer ≥1, no. (%)	10 (0.1)	26 (0.3)	11 (0.1)	12 (0.1)	5 (0.06)	5 (0.06)	4 (0.04)	4 (0.05)
RPR test titer >4, no. (%)	1 (0.01)	1 (0.01)	1 (0.01)	1 (0.01)	0	0	1 (0.01)	0
RPR test titer >4/TPHA titer >80,† %	2.2	2.0	2.6	2.4	0	0	3.0	0

*MSM, men who have sex with men; STD, sexually transmitted disease; A-VCT, anonymous voluntary counseling and testing; TPHA, *Treponema pallidum* hemaglutination assay; RPR, rapid plasma reagin;
†Defines active syphilis.

Although our study was relatively small and retrospective, it offered a unique opportunity to observe an emerging outbreak in a relatively isolated population of 400,000 in a small geographic area. Early detection also provides a unique opportunity for therapeutic and preventive intervention.

Interpretation of syphilis test results is often difficult in asymptomatic patients, especially when new-generation tests are used in low-prevalence countries (*13*). In the Caribbean, although yaws was largely eradicated in the 1960s, small outbreaks continue to be reported, as in Martinique from 1974 through 1985. This finding further complicates interpretation of screening results, particularly for older patients.

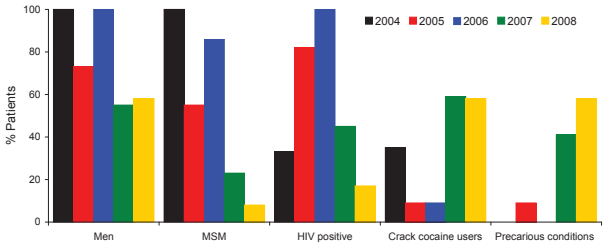

Figure 2. Frequency of syphilis in 55 patients, by group, Fort-de-France, Martinique, 2004–2008. MSM, men who have sex with men; precarious conditions, ≥1 of the following: homelessness, lack of welfare, being followed-up in a psychiatry unit, mental deficiency, having paid sex, incarceration in correctional facility >2× in the past 5 years.

Syphilis outbreaks will be difficult to detect in regions of the Caribbean where the disease is highly endemic and surveillance is poor. Outbreaks of syphilis and congenital syphilis were reported in Trinidad and Tobago and Jamaica in the 1990s (*14*,*15*). Control of outbreaks requires coordinated public health interventions, including new preventive strategies specific for high-risk groups. Preventive messages must be culturally appropriate and must underline the risk for STD transmission by oral sex.

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Dr Cabié is a dermatologist and infectious diseases specialist at the Centre Hospitalier Universitaire de Fort-de-France in Martinique. His research interests include the epidemiology of AIDS and sexually transmitted infections.

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Address for correspondence: André Cabié, Service des Maladies Infectieuses et Tropicales, Centre Hospitalier Universitaire de Fort-de-France, 97200 Fort-de-France, Martinique, French West Indies; email: andre.cabie@chu-fortdefrance.fr

Seagulls and Beaches as Reservoirs for Multidrug-Resistant *Escherichia coli*

Roméo Rocha Simões, Laurent Poirel, Paulo Martins Da Costa, and Patrice Nordmann

A variety of extended-spectrum β-lactamase–producing *Escherichia coli* isolates, with a high rate of cefotaximase-15 resistance, were identified in seagull feces from Porto, Portugal, beaches. Beaches may therefore present a risk to public health because of the potential pathogen-spreading capacity of migratory birds.

The Porto coastline in Portugal, including downtown Porto, has a large seagull population (*Larus fuscus,* the lesser black-backed gull, and *L. cachinnans*, the yellow-legged gull). Seagulls have been reported as a possible reservoir for multidrug-resistant bacteria (*1*). During the past decade, extended-spectrum β-lactamases (ESBL) with growing epidemiologic importance, the cefotaximase (CTX-M) enzymes, have been reported worldwide in *Enterobacteriaceae* from humans (*2*); they are found mostly in *Escherichia coli*. Reasons for the emergence of CTX-M enzymes in human isolates remain unknown. In addition, CTX-M–positive *E. coli* have been identified in poultry (*3*), other farm animals (*4*), and wild animals (birds of prey, foxes [*5*]). *E. coli* strains can be classified into 4 phylogenetic groups (A, B1, B2, and D). The virulent extraintestinal isolates belong mostly to group B2 and, to a lesser extent, group D, whereas most commensal strains belong to groups A and B1. The objective of the study was to evaluate the spread and types of ESBL-positive *E. coli* in feces recovered from wild birds on the beaches of Porto.

The Study

During December 2007 through April 2008, wild seagull (*L. fuscus*, *L. cachinnans*) feces were collected from the Matosinhos and Leça da Palmeira beaches (Porto, Portugal) (20 samples every 2 weeks) using a sterile spatula. Care was taken during sampling to avoid collection of beach sediment. Samples were placed in sterile tubes and processed. Samples were precultured in buffered peptone water (Oxoid, Basingstoke, UK) at a dilution of 1/10 wt/vol and incubated at 37°C. Cultures were injected by streaking 10 μL of the suspensions onto Tergitol BCIG agar (Biokar Diagnostics, Beauvais, France). Another suspension was made in buffered peptone water supplemented with cefotaxime (CTX) at 2 μg/mL and then streaked in a Tergitol BCIG plate supplemented with CTX at 2 μg/mL. The plates were incubated at 37°C overnight. We identified *E. coli* isolates by using the API20E system (bioMérieux, Balmes-les-Grottes, France).

Susceptibility testing was performed by disk diffusion assay (Sanofi-Diagnostic Pasteur, Marnes-la-Coquette, France), as previously described (*3*). MICs were determined by Etest (AB BIODISK; Solna, Sweden) on Mueller-Hinton agar plates at 37°C (*8*). ESBL was detected with a synergy test using disks containing CTX, ceftazidime, and ticarcillin-clavulanic acid (*6*).

Clonal diversity was assessed by pulsed-field gel electrophoresis (PFGE) as described (*3*). Genomic DNA was extracted in situ by treatment with lysozyme (1 mg/mL; Sigma, Saint-Quentin Fallavier, France) and proteinase K (0.5 mg/mL; Sigma) and then restricted with endonuclease *Xba*I (GE Healthcare, Aulnay-sous-Bois, France). We separated resulting fragments by using 1% PFGE-grade agarose gel (Bio-Rad, Hercules, CA, USA) in a CHEF-DR II System (Bio-Rad), with the following protocol: 6 volts/cm, 4–12 s pulse time for 12 h, followed by 15–36 s pulse time for 12 h in 0.5% tris-borate-EDTA buffer at 14°C.

Detection of $bla_{\text{CTX-M}}$ genes was carried out by PCR (*3*). We sequenced the purified PCR products on both strands by using an Applied Biosystems sequencer (ABI 377; Foster City, CA, USA) and analyzed these sequences in the BLAST database (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic grouping of *E. coli* isolates was determined by PCR (*7*) for assignation of phylogenetic groups: group B2, $chuA^+$, $yjaA^+$; group D, $chuA^+$, $yjaA^-$; group B1, $chuA^-$, TspE4C2$^+$; and group A, $chuA^-$, TspE4C2$^-$. Analysis of plasmid content was performed for the $bla_{\text{CTX-M}}$-like positive isolates by using the Kieser technique (*3*). The multilocus sequence typing (MLST) of *E. coli* isolates was determined by sequencing 7 essential genes (*adk, fumC, icd, purA, gyrB, recA,* and *mdh*) as described (*8*), followed by an analysis on the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli) except for *mdh*, *icd*, and *recA* as performed in another study (*9*).

We obtained 139 *E. coli* isolates, of which 45 (32%) displayed an ESBL phenotype. Forty-four (98%) of the 45 ESBL producers carried a $bla_{\text{CTX-M}}$ gene; 1 isolate possessed a $bla_{\text{TEM-52}}$ gene. PCR and sequencing identified the CTX-M ESBL determinants as follows: 8 (18%) were CTX-M-1; 4 (9%), CTX-M-9; 17 (39%), CTX-M-15; and 15 (34%), CTX-M-32.

Author affiliations: Hôpital de Bicêtre, Bicêtre, France (R.R. Simões, L. Poirel, P. Nordmann); and Universidade do Porto, Porto, Portugal (R.R. Simões, P.M. Da Costa)

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PGFE analysis showed a high diversity of genotypes: 8 clones for 8 CTX-M-1–positive isolates, 4 clones for 4 CTX-M-9–positive isolates, 13 clones for 17 CTX-M-15–positive isolates, and 14 clones for 15 CTX-M-32–positive isolates (data not shown). A total of 37% of ESBL producers belonged to virulent extraintestinal groups B2 and D. Of these isolates, 41% expressed CTX-M-15 and 47% expressed CTX-M-32.

MLST identified 25 different types among the 45 *E. coli* isolates. The most commonly identified genotypes were ST1284 (4 isolates), ST131 (4), and ST224 (3). Isolates belonging to ST453, ST86, ST205, ST359, ST165, ST69, ST1152, ST405, ST559, ST1163, ST10, ST58, ST156, ST155, ST10, ST297, ST43, ST58, and ST156 were also identified. Different genotypes carrying the same ESBL determinant were identified; conversely, different ESBL determinants were found among the same genotype. In particular, isolates belonging to the ST131 genotype widely reported among human CTX-M-15–positive *E. coli* producers (*9,10*) harbored either $bla_{\text{CTX-M-32}}$ (2 isolates), $bla_{\text{CTX-M-1}}$ (1), or $bla_{\text{CTX-M-15}}$ (1). Coresistances of the 45 ESBL-positive isolates were as follows: 90% were resistant to tetracycline; 60%, to trimethoprim/sulfamethoxazole; 55%, to nalidixic acid; 50%, to ciprofloxacin; 23%, to gentamicin; and 4%, to chloramphenicol.

Identification of the phylogroups of the different CTX-M producers showed that most belonged to phylogroup B1, and a notable number of isolates belonged to phylogroup D (Figure). In contrast, few isolates belonged to the highly virulent phylogroup B2. Whereas CTX-M-1 producers mostly belonged to phylogroup B1, the CTX-M-15 producers were well distributed among the 4 phylogroups (Figure). Notably, plasmid analysis identified a sizable diversity of plasmid sizes, including *E. coli* isolates producing the same CTX-M type (data not shown).

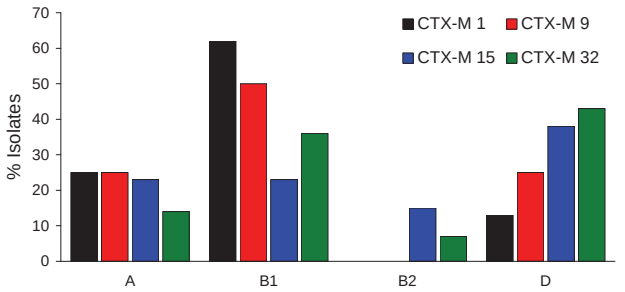

Figure. Distribution of isolates positive for cefotaximase (CTX-M) by phylogenetics group. Vertical axis indicates the percentage for each CTX-M determinant.

Conclusions

CTX-M type β-lactamases are ESBLs of increasing prevalence (*2*). Infections with multidrug-resistant bacilli occur not only in hospitals but also in the community (*11*). Previous studies have reported multidrug-resistance in wild birds (*12*,*13*). Our study provides additional clues that wild seagulls are carriers of ESBL-producing *E. coli,* although at a lower rate than previously reported (*1*). In that study, ESBL determinants were TEM-52, CTX-M-1, CTX-M-14a, and CTX-M-32 (*1*). Another study (focused on poultry) (*3*) reported that the main identified CTX-M determinants were of the CTX-M-1 group (CTX-M-1, CTX-M-15, CTX-M-32), as we also found.

We report that CTX-M-15 was the main CTX-M type identified among birds residing mostly on beaches of downtown Porto, which agrees with CTX-M-15 being the most prevalent ESBL in *E. coli* in Porto-hospitalized patients (*14*). This observation differs from those of a study reporting mostly TEM-52 producers in wild animals in a reserve near Porto, where indirect contacts with humans are less likely (*5*).

Previous studies have reported the association of *E. coli* isolates of groups B2 and D with extraintestinal infections (*15*). Our report shows that 37% of all ESBL isolates belong to B2 or D phylogroup, a higher rate than previously reported (27% of all ESBL) (*1*). This finding could be a matter of concern for human health. However, we showed that the ST131 type known to be frequently isolated in humans and frequently associated with CTX-M-15 production was quite rare (9%). In addition, those ST131 strains were found to harbor diverse CTX-M determinants. In fact, the frequent identification of CTX-M producers here was related neither to the dissemination of a single clone nor to that of a single plasmid.

Our report suggests that beaches may play a major role in dissemination of resistance determinants and may be a source of the CTX-M-15–related community-acquired infections. Migratory birds, such as seagulls crossing an extensive portion of the European coastline between Portugal and Scandinavia, may be reservoirs for these emerging resistance determinants.

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Dr Simões is a veterinary student at Instituto de Ciências Biomédicas Abel Salazar, Porto, and completed this study as the final professional training period of his studies curriculum. His research interests include zoonotic diseases.

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Address for correspondence: Patrice Nordmann, Hopital de Bicetre, Service de Bacteriologie-Virologie, 78, rue du General-Leclerc Le Kremlin, Bicetre 94275, France; email: nordmann.patrice@bct.ap-hop-paris.fr

Cluster of Serogroup W135 Meningococci, Southeastern Florida, 2008–2009

Timothy J. Doyle, Alvaro Mejia-Echeverry, Paul Fiorella, Fermin Leguen, John Livengood, Robyn Kay, and Richard Hopkins

Recently, 14 persons in southeastern Florida were identified with *Neisseria meningitidis* serogroup W135 invasive infections. All isolates tested had matching or near-matching pulsed-field gel electrophoresis patterns and belonged to the multilocus sequence type 11 clonal complex. The epidemiologic investigation suggested recent endemic transmission of this clonal complex in southeastern Florida.

Neisseria meningitidis serogroup W135, which generally accounts for <5% of invasive meningococcal disease in the United States, has frequently been associated with foreign travel and is less often associated with outbreaks than other serogroups (*1*). In Florida during 2004–2007, a total of 337 patients statewide were reported to have meningococcal disease; 6 (1.8%) of these were caused by serogroup W135.

The Study

Meningococcal disease is a reportable condition in Florida. Isolates of *N. meningitidis* from persons with invasive disease are forwarded to the state health department Bureau of Laboratories for serogrouping by slide agglutination. Isolates from outbreaks or clusters are further characterized by pulsed-field gel electrophoresis (PFGE) by using *Nhe*I and *Spe*I restriction enzymes and methods consistent with standard protocols (*2*).

From December 2008 through April 2009, we observed an increase of invasive meningococcal disease caused by serogroup W135 in southeastern Florida totaling 13 patients, of whom 9 had indistinguishable PFGE patterns (Figure 1). Isolates from the 4 other case-patients had PFGE patterns differing by 1 (pattern II), 2 (pattern III), and 5 bands (pattern IV), making them ≥94% related to the dominant pattern. A retrospective review of all 5 W135 isolates in Florida from January 2007 through November 2008 identified 1 additional isolate matching the dominant pattern from a resident of southeastern Florida with illness onset in May 2008. The 4 other isolates from 2007–2008 had PFGE patterns <80% related (Figure 1) and occurred before May 2008 in residents of central and northern Florida.

Of the 14 case-patients with matching or near-matching PFGE patterns (Table 1), 13 denied recent foreign travel. One patient with disease onset in April 2009 was a tourist visiting Miami from the United Kingdom. Eleven (79%) case-patients resided in or were visiting Miami-Dade County, 2 (14%) resided in Broward County, and 1 (7%) was a resident of Palm Beach County. Of the 14 case-patients, the median age was 45 years (range 1–84). Eight (57%) case-patients were female, 7 (50%) were Hispanic; 8 were white, and 6 were black. Twelve patients had a bacteremia syndrome, 2 had meningitis, and 1 had pneumonia in addition to bacteremia. Four of 14 patients died, all with bacteremia. The epidemiologic investigation has not identified any common exposures, social settings, or other connections among patients in this series. Six (43%) case-patients had onset of illness within 10 days of their birthdays (binomial probability, p<0.0001), but no obvious detailed exposures were identified related to this observation.

Illness onset for the 14 W135 patients in this cluster is shown in relation to onset for all 38 meningococcal disease patients with other serogroups identified in Florida during the same 12-month period (Figure 2). Demographic and clinical factors were also compared between these serogroup W135 and non-W135 patients (Table 2). As a group, serogroup W135 patients were older, were more likely to be Hispanic and to reside in or be visiting the southeastern 3-county region, were more likely to have bacteremia, and had higher mortality rates than non-W135 serogroup patients. However, other than Hispanic

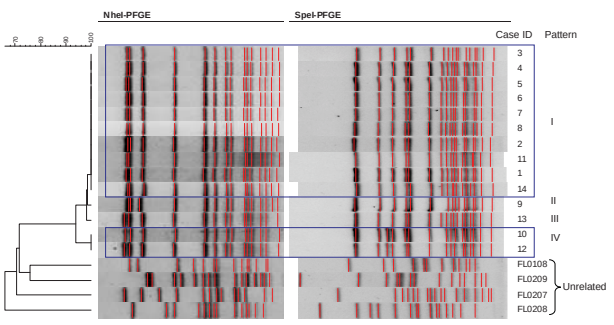

Figure 1. Pulsed-field gel electrophoresis (PFGE) patterns for 14 related and 4 unrelated isolates of *Neisseria meningitidis* serogroup W135, Florida, USA.

Author affiliations: Department of Health, Miami, Florida, USA (T.J. Doyle); Centers for Disease Control and Prevention, Miami (T.J. Doyle); Miami-Dade County Health Department, Miami (A. Mejia-Echeverry, F. Leguen); Department of Health, Jacksonville, Florida, USA (P. Fiorella, R. Kay); Broward County Health Department, Ft. Lauderdale, Florida, USA (J. Livengood); and Department of Health, Tallahassee, Florida, USA (R. Hopkins)

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Table 1. Demographic and clinical characteristics of patients with serogroup W135 meningococcal disease, southeastern Florida, USA, 2008–2009*

Case ID	Age, y/ sex	Race	Hispanic ethnicity	County	Month of onset	Onset near birthday†	Syndrome	Specimen source	Outcome	PFGE group
1	39/M	White	Y	Broward	2008 May	Y	B	Blood	Survived	1
2	1/M	White	Y	Miami-Dade	2008 Dec	Y	M	CSF	Survived	1
3	29/M	White	Y	Palm Beach	2009 Jan	N	M	CSF	Survived	1
4	83/M	White	Y	Miami-Dade	2009 Jan	Y	B, P	Blood	Died	1
5	84/F	White	Y	Miami-Dade	2009 Jan	N	B	Blood	Survived	1
6	26/F	Black	N	Miami-Dade	2009 Jan	Y	B	Blood	Survived	1
7	70/M	White	Y	Miami-Dade	2009 Feb	N	B	Blood	Survived	1
8	50/F	Black	N	Miami-Dade	2009 Feb	N	B	Blood	Survived	1
9	77/F	Black	N	Miami-Dade	2009 Feb	N	B	Blood	Survived	2
10	58/F	Black	N	Broward	2009 Mar	Y	B	Blood	Died	4
11	20/F	White	N	Miami-Dade	2009 Mar	N	B	Blood	Died	1
12	69/F	White	Y	Miami-Dade	2009 Apr	N	B	Blood	Survived	4
13	26/F	Black	N	Miami-Dade‡	2009 Apr	Y	B	Blood	Died	3
14	19/M	Black	N	Miami-Dade	2009 Apr	N	B	Blood	Survived	1

*ID, identification; PFGE, pulsed-field gel electrophoresis; B, bacteremia; M, meningitis; CSF, cerebrospinal fluid; P, pneumonia.
†Within 10 days, before or after.
‡Foreign tourist visitor to Miami-Dade County.

ethnicity and residence in the southeastern region, none of these differences were significant.

Twelve isolates from the Florida cluster were forwarded to Centers for Disease Control and Prevention laboratories for antimicrobial susceptibility testing. All 12 isolates were sensitive to all antimicrobial agents tested (penicillin G, ceftriaxone, ciprofloxacin, rifampin, azithromycin). Seven of these isolates underwent additional molecular characterization with PCR and multilocus sequence typing by using methods previously described (*1,3*). The isolates were confirmed as serogroup W135 by real-time PCR with primers specific for serogroups A, B, C, W135, X, and Y. These isolates were also found to belong to the sequence type (ST)-11/electrophoretic type 37 clonal complex.

The dominant PFGE pattern seen in the Florida cluster is designated H46N06.0068, and is closely related to the pattern observed in the large multicountry outbreak associated with Hajj pilgrims occurring in 2000 (*1*). The 2000 outbreak involved >400 cases and was caused by a single clone of the ST-11 clonal complex. In recent years, increases in serogroup W135 have been noted in northern Argentina (*4*) and southern Brazil (*5*). Among serogroup W135 isolates in these countries, the most common clone has been the hypervirulent ST-11 complex (*4,5*).

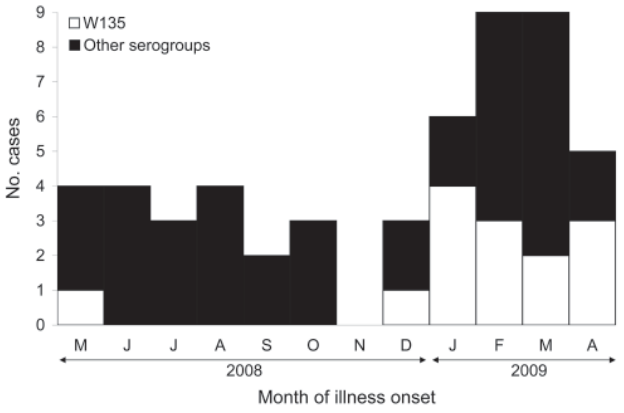

Figure 2. Confirmed meningococcal disease cases, by month of onset, Florida, USA, May 2008–April 2009.

From 1997 through 2006, the annual incidence of meningococcal disease in Florida declined gradually from ≈1.2 to 0.4 cases per 100,000 population (*6*). Historical trends of surveillance data in Florida suggest seasonal peaks of meningococcal disease during November–May, corresponding to the winter dry season, when tourist visitors and part-time residents are most likely to visit. The 3-county region of southeastern Florida in which this W135 cluster occurred had an estimated population in 2008 of >5.5 million inhabitants; 2.4 million of these reside in Miami-Dade County, where >60% of residents are Hispanic. During December 2008 through April 2009, the 32 total patients with meningococcal disease reported in the state represent an annual incidence of 0.41 cases/100,000 Florida residents. The 19 total cases (13 serogroup W135) in the 3-county region during this period represent an annual population incidence of 0.82 cases/100,000 residents in these counties. For the 13 total cases occurring in Miami-Dade County during this period (11 serogroup W135), the annual incidence would be 1.26 cases/100,000 residents in the county. During May 2009, no cases of serogroup W135 meningococcal disease were reported anywhere in the state. In the absence of a more narrowly defined risk group, the cluster of serogroup W135 case-patients described in this report is still far below the threshold for recommending vaccination control efforts (10 cases/100,000 over 3 months) (*7*).

Table 2. Characteristics of patients with serogroup W135 and non-W135 meningococcal disease, Florida, USA, May 2008–April 2009

Variable	No. positive/no. tested (%) W135, n = 14	Non-W135, n = 38	p value*
Female sex	8/14 (57)	22/38 (58)	1.00
White race	8/14 (57)	30/38 (79)	0.16
Hispanic ethnicity	7/14 (50)	7/38 (18)	0.04
Residence in southeast region	14/14 (100)	12/38 (32)	<0.0001
Bacteremia	12/14 (86)	24/38 (63)	0.18
Meningitis	2/14 (14)	13/38 (34)	0.30
Pneumonia	1/14 (7)	5/38 (13)	1.00
Death	4/14 (29)	4/38 (11)	0.19

*By 2-sided Fisher exact test used for categorical variables; Wilcoxon rank-sum test used for age. Median age for W135 patients, 45 years; median age for non-W135 patients, 27 years; p value = 0.07.

Conclusions

Southeastern Florida is considered the gateway to the Americas, with extensive social, cultural, and commercial ties to Central and South America and the Caribbean. The dominant clonal complex observed in the Florida cluster matches the dominant type recently observed in Argentina and Brazil, raising the possibility of introduction to southeastern Florida from South America. Taken together, these observations suggest the possible establishment of a clonal complex of serogroup W135 meningococci in southeastern Florida with subsequent endemic transmission.

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Mr Doyle is a career epidemiology field officer with the Centers for Disease Control and Prevention, assigned to the Florida Department of Health. His interests include applied infectious disease epidemiology and surveillance.

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Address for correspondence: Timothy J. Doyle, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop K72, Atlanta, GA 30333, USA; email: tdoyle@cdc.gov

Human Group A Streptococci Virulence Genes in Bovine Group C Streptococci

Márcia G. Rato, Ricardo Bexiga, Sandro F. Nunes, Cristina L. Vilela, and Ilda Santos-Sanches

Phage-encoded virulence genes of group A streptococci were detected in 10 (55.6%) of 18 isolates of group C streptococci that had caused bovine mastitis. Bovine isolates carried other genetic determinants, such as composite transposon Tn*1207.3*/Φ10394.4 (100%) and antimicrobial drug resistance genes *erm*(B)/*erm*(A) (22.2%), *lin*B (16.6%), and *tet*(M)/*tet*(O) (66.7%), located on mobile elements.

Strains of *Streptococcus dysgalactiae* subsp. *dysgalactiae* are described as α-hemolytic or nonhemolytic (Lancefield group C) and associated only with animal infections (bovine mastitis), a disease with major economic consequences for the dairy industry (*1*). Group A streptococci (GAS)–specific phage-associated virulence determinants encoding pyrogenic exotoxins or superantigens (*speM*, *ssa*), which are strongly associated with severe diseases such as scarlet fever, streptococcal toxic shock syndrome, and rheumatic fever, have been described among human group C streptococci (GCS) or group G streptococci (GGS) (*S. dysgalactiae* subsp. *equisimilis*) (*2*) but not among α-hemolytic GCS (*S. dysgalactiae* subsp. *dysgalactiae*) of bovine origin. In contrast, M protein or M-like proteins were found in human GGS/GCS (*S. dysgalactiae* subsp. *equisimilis*) and in animal GCS (*S. dysgalactiae* subsp. *dysgalactiae*) but only in β-hemolytic strains (*3*).

Composite transposons and other genetic determinants also considered to be located in specific mobile elements such as macrolide (either encoding methylases [*erm* genes] or efflux pumps [*mef* genes]) and tetracycline resistance determinants (*tet* genes) have been found among streptococcal species of human origin. We studied a collection of field isolates of bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* to search for genetic determinants, particularly those carried by mobile elements known to be transferred among human GAS and GGS/GCS.

Author affiliations: Universidade Nova de Lisboa, Caparica, Portugal (M.G. Rato, I. Santos-Sanches); Universidade Técnica de Lisboa, Lisbon, Portugal (R. Bexiga, S.F. Nunes, C.L. Vilela); and Cambridge University, Cambridge, UK (S.F. Nunes)

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The Study

We studied 18 α-hemolytic *S. dysgalactiae* subsp. *dysgalactiae* field isolates of Lancefield group C that had caused bovine subclinical mastitis. Isolates were obtained from 304 milk samples of 248 cows from 8 farms in Portugal that were included in the study. Detailed information regarding isolation methods and identification of field isolates by biochemical methods was described in a study of the subclinical mastitis–associated pathogen *S. uberis* (*4*). To confirm identification of *S. dysgalactiae* subsp. *dysgalactiae*, the 16S rRNA gene was amplified by PCR and sequenced (*5*). *Sma*I/*cfr*9I-digested DNA banding patterns were obtained by pulsed-field gel electrophoresis for clone identification as described (*4*).

All genes analyzed by PCR are shown in the online Appendix Table (available from www.cdc.gov/EID/content/16/1/116-appT.htm). The *emm* gene subtyping was performed as described (www.cdc.gov/ncidod/biotech/strep/M-ProteinGene_typing.htm). Primers used and conditions for PCR were essentially as described elsewhere (online Appendix Table).

Samples without DNA and strains lacking (negative) or carrying (positive) specific genes were used as controls in the PCR. Results were consistent in 2 or 3 PCRs that included these controls. Sequencing of all virulence gene amplicons was performed with the same primers used for amplification (STAB-Vida, Lisbon, Portugal). All sequences were compared with sequences in GenBank by using the BLAST alignment tool (www.ncbi.nlm.nih.gov/BLAST).

Antimicrobial drug resistance against macrolides (erythromycin), lincosamides (pirlimycin), and tetracycline was determined as described (*10*). Macrolide resistance phenotypes identified were M (resistance to macrolides) and MLS_B (resistance to macrolides, lincosamides and streptogramins B).

We detected bacteriophage-associated virulence genes *spe*M, *spe*K, *spe*C, *spd*1, and *spe*L. Overall, *spe*M was found in 10 (55.6%) of 18 bovine GCS isolates, *spe*K in 9 (50%), *spe*C and *spd*1 in 6 (33%), and *spe*L in 4 (22.2%). All but 1 of the PCR products showed expected sizes (online Appendix Table). Tn*1207.3*/Φ10394.4 composite transposon left junction amplicon showed a size of 380 bp instead of 453–6,807 bp as described for GAS (*9*). No amplification was observed for the right junction of this genetic element.

The *emm* gene encoding the antiphagocytic M surface protein was not amplified in any of the 18 bovine GCS isolates; therefore, no *emm* types were obtained. Subsets of isolates were erythromycin and pirlimycin resistant (MLS_B

phenotype) and contained *erm*(B) or *erm*(A) genes (22.2%) or erythromycin susceptible and pirlimycin resistant and contained the *linB* gene (16.6%). All isolates were tetracycline resistant with a subset (66.7%) carrying *tet*(M) or *tet*(O) tetracycline resistance determinants. Distribution of bacteriophage-associated virulence genes and other characteristics of strains are shown in Figure 1.

Sequences of all virulence genes were compared by using the BioEdit sequence alignment editor (www.mbio.ncsu.edu/BioEdit/bioedit.html). One different allele was found for each of the following gene sequences: *spd*1 (among 6 strains), *spe*C (among 6 strains), and *spe*L (among 4 strains). Two alleles were found for *spe*K (among 9 strains) (*spe*K-1 and *spe*K-2), and 4 alleles were found for *spe*M gene sequences (among 10 strains) (*spe*M-1, *spe*M-2, *spe*M-3, and *spe*M-4). Bovine alleles had sizes of 386 bp (*spd*1), 222 bp (*spe*C), 444 bp (*spe*L), 232 bp (*spe*K), and 357 bp (*spe*M). Examples of alignments between bovine virulence gene alleles with sequences from GenBank (only most similar ones) are shown in Figure 2.

Conclusions

Using PCR, we determined that bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* strains (55.6%) carried ≥1 GAS-specific bacteriophage virulence-associated genes (*spd*1, *spe*C, *spe*K, *spe*L, and *spe*M). This finding suggested that bacteriophages may also play a role in the genetic plasticity and virulence of animal GCS.

The *spe*L allele from bovine strains showed higher similarity with the *sze*M allele (99% maximum identity) from *S. equi* subsp. *zooepidemicus* than with the *spe*L allele (97% maximum identity) from *S. pyogenes*. The *sze*M gene encodes a superantigen in *S. equi* subsp. *zooepidemicus*, which is primarily a pathogen of nonhuman animal species. This organism causes mastitis in cows and mares and is most frequently found in horses (*14*). We also observed that 3 of the *spe*M alleles found among bovine strains (*spe*M-1, *spe*M-2, and *spe*M-3) also showed higher similarity with superantigen-encoding gene *sze*L from *S. equi* subsp. *zooepidemicus* than with *spe*M gene sequence from *S. pyogenes*. Another allele (*spe*M-4) showed higher similarity with the *sdm* gene from *S. dysgalactiae* subsp. *dysgalactiae* than with the speM gene from *S. pyogenes*.

The remaining alleles (*spd*1, *spe*C, *spe*K-1, and *spe*K-2) from the GCS *S. dysgalactiae* subsp. *dysgalactiae* bovine strains showed high similarity with *S. pyogenes* superantigen genes (98%–99% maximum identity). This finding supports our hypothesis that GAS prophages may play a role in the genetic plasticity of this pathogen. The *spe*C and *spd*1 genes are known to be localized on the same GAS prophage (*15*), and both genes were detected in 6 bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates in our study.

None of 18 α-hemolytic group C *S. dysglacatiae* subsp. *dysgalactiae* bovine isolates in this study were typed by *emm*-typing because amplification products in the PCR

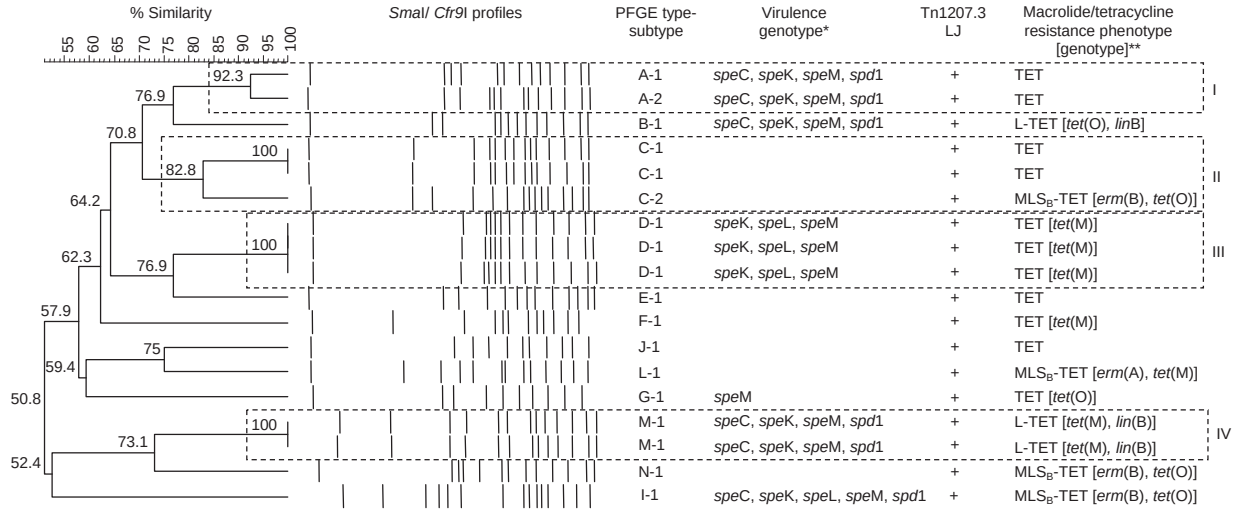

Figure 1. Dendrogram and pulsed-field gel electrophoresis (PFGE) profiles of group C streptococci (*Streptococcus dysgalactiae* subsp. *dysgalactiae*) subclinical mastitis isolates from 8 dairy herds, Portugal. PFGE type-subtype, virulence genotype, antimicrobial drug resistance phenotypes, and genotypes of each isolate are indicated. The dendrogram was produced by using Dice coefficients and unweighted pair group method using arithmetic averages. Default clustering settings of 0.00% optimization (i.e., the relative distance an entire lane is allowed to shift in matching attempts) and 1.5% band position tolerance were used. *All isolates were negative for *spe*A, *ssa*, *spe*H, *speJ*, *spe*I, and *sla*A genes and for Tn*1207.3*/Φ10394.4 element right junction tested by PCR; **All isolates were negative for *mef*A, *tet*(T), *tet*(W), *tet*(L), *tet*(Q), *tet*(S) and *tet*(K) genes tested by PCR; TET, resistance only to tetracycline; MLS_B-TET, resistance to macrolides, lincosamides, streptogramin B, and TET; L-TET, susceptibility to macrolides and resistance to lincosamides (L phenotype) and TET; Tn1207.3 LJ, Tn*1207.3*/Φ10394.4 element left junction. Clusters are shown in roman numerals on the right.

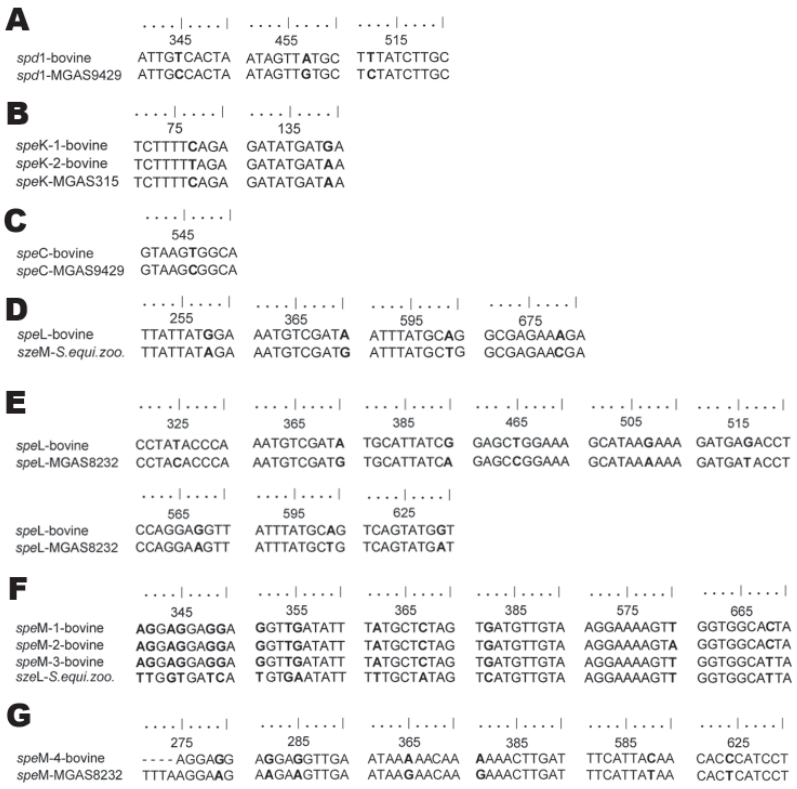

Figure 2. Alignments of bovine group C streptococci (*Streptococcus dysgalactiae* subsp. *dysgalactiae*) alleles of virulence genes from 8 dairy herds, Portugal, with sequences from the National Center for Biotechnology (Bethesda, MD, USA) database showing base differences between sequences. The alignments were created by using BioEdit sequence alignment editor (www.mbio.ncsu.edu/BioEdit/bioedit.html). Nucleotide differences are shown in **boldface**. A) *spd*1 (99% maximum identity); B) *spe*K (99% maximum identity); C) *spe*C (99% maximum identity); D) *spe*L–*sze*M (99% maximum identity); E) *spe*L (97% maximum identity; F) *spe*M alleles 1, 2, and 3–*sze*L (98%–99% maximum identity); G) *spe*M allele 4 (98% maximum identity). *S. equi*. *zoo*., *S. equi* subsp. *zooepidemicus*.

specific for the M surface protein gene *emm* were not obtained. This result is consistent with those of a report that β-hemolytic, but not α-hemolytic, group C *S. dysglacatiae* subsp. *dysgalactiae* isolates of animal origin contained the *emm* gene (*3*).

Amplification (380-bp product) of the left junction of the composite transposon in bovine isolates suggests that this mobile element may be inserted in a similar location, the *comEC* locus, as mapped in *S. pyogenes* and *S. dysglactiae* subsp. *equisimilis*. Absence or unexpected PCR products specific for any of the junctions of this element have been reported in other studies and attributed to possible lack of homology between the target and primers used (*9*). Detection of the *linB* gene carried by a large conjugative plasmid (*13*) in 3 of 18 bovine GCS *S. dysgalactiae* subsp. *dysgalactiae* isolates is indicative of horizontal gene transfer.

Our findings indicate that α-hemolytic bovine GCS isolates, which are known to be environmental or contagious pathogens and a cause of bovine mastitis, may be reservoirs of virulence genes encoded by prophages of human-specific GAS. These genes encode exotoxins, superantigens, and streptodornases, which are responsible for GAS virulence and pathogenesis, and may be transferred to other streptococci of human origin by horizontal genetic transfer. Therefore, α-hemolytic isolates should not be disregarded as putative infectious disease agents in humans.

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Ms Rato is a doctoral candidate in the Department of Life Sciences of the Faculty of Science and Technology, New University of Lisbon, Caparica, Portugal. Her research interests include epidemiology, antimicrobial drug resistance, and virulence mechanisms of streptococci from animal and human origin.

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Address for correspondence: Ilda Santos-Sanches, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus da Caparica, 2829-516 Caparica, Portugal; email: isanches@fct.unl.pt

Perceptions and Reactions with Regard to Pneumonic Plague

G. James Rubin, Richard Amlôt, M. Brooke Rogers, Ian Hall, Steve Leach, John Simpson, and Simon Wessely

We assessed perceptions and likely reactions of 1,005 UK adults to a hypothetical terrorist attack involving pneumonic plague. Likely compliance with official recommendations ranged from good (98% would take antimicrobial drugs) to poor (76% would visit a treatment center). Perceptions about plague were associated with these intentions.

Yersinia pestis, the bacterium that causes plague, is a high-priority bioterrorism agent (*1*). The pneumonic form of plague is of particular concern because it can be transmitted from person to person and is fatal if untreated (*2*). However, interventions such as isolating case-patients, identifying contacts, and providing prophylactic antimicrobial drugs may halt the spread of an outbreak (*3*,*4*). The success of such interventions relies on public cooperation, which should not be taken for granted (*5*). Indeed, various commentators have suggested that future plague outbreaks could result in widespread panic (*2*), mass public fear and civil disruption (*1*), and rioting (*6*).

We used a telephone survey of a sample of the adult population of Great Britain to assess their intended behavioral responses in the event of an outbreak of pneumonic plague. We also assessed their perceptions of pneumonic plague and tested whether perceptions were associated with intentions.

The Study

During September 14–24, 2007, a UK market research company, Ipsos MORI, conducted a random-digit–dial telephone survey. Members of the British population ≥16 years of age were selected by using proportional quota sampling to ensure that the eventual sample of 1,005 participants was representative of the British public (*7*). King's College London's Research Ethics Committee approved the study.

Author affiliations: King's College London, London, UK (G.J. Rubin, M.B. Rogers, S. Wessely); Health Protection Agency, Salisbury, UK (R. Amlôt, S. Leach, J. Simpson); and Health Protection Agency, Wilts, UK (I. Hall)

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The full interview (including several questions not analyzed for this article) and results are in online Technical Appendix 1 (available from www.cdc.gov/EID/content/16/1/120-Techapp1.pdf). The survey was conducted in 4 stages. In stage 1, we asked 7 questions concerning perceptions about pneumonic plague. In stage 2, we asked participants to imagine that 3 persons from their area had received a diagnosis of pneumonic plague. To test whether the origin of an outbreak affects responses, 502 participants were also told that police suspected bioterrorism. This manipulation had no effect on most responses. In stage 3, we informed participants that it was now several days later, that the source of the outbreak had been discovered to be a container deliberately hidden at a train station, and that >100 persons had received a diagnosis of plague. In stage 4, we told participants about a specific public health strategy that was being introduced. We informed 502 randomly selected participants about the setting up of mass treatment centers for persons who had been at the train station and told the other 503 that persons who had been at the train station were being asked to stay home for 7 days and to phone a help line if symptoms developed.

In stages 2 and 3, we asked participants whether they intended to undertake specific spontaneous precautionary behavior (questions 12–19 in online Technical Appendix 1). An extra item in stage 2 asked whether participants would be willing to take prophylactic antimicrobial drugs if asked to (question 25 in online Technical Appendix 1). In stage 4, we asked participants how likely they would be to comply with advice relating to the public health interventions (questions 41–46 in online Technical Appendix 1). Before analysis, all responses were weighted according to participant age, sex, work status, region, and social grade.

As expected, precautionary behavior was more likely to be taken in the stage 3 scenario (Tables 1, 2). In terms of likely compliance with official recommendations, 983 (97.8%) participants reported being very or fairly likely to take antimicrobial drugs if asked to. When asked to imagine that they had been to the affected train station, 379 (75.5%) participants reported that they would visit the treatment center immediately if asymptomatic; slightly fewer (331, 65.9%) reported that they would go immediately if they also had influenza-like symptoms. This decrease appeared to be because participants reported that they would likely first consult a primary care physician, hospital, or medical helpline if they had symptoms. In addition, 88 (9.2%) reported being likely to visit the center even if they had not been at the train station, and 141 (28.1%) said that they were likely to visit if they had not been at the train station but had developed influenza-like symptoms. For participants who had been advised to stay home, 459 (91.3%) reported that they would be likely to comply.

Table 1. Perceptions of and precautionary behavioral responses to a hypothetical pneumonic plague outbreak affecting 3 persons, United Kingdom, September 2007*

Predictor	Variable level, no. responses: Very or fairly likely	Variable level, no. responses: Not very or not at all likely (reference)	Association, adjusted odds ratio (95% confidence interval): Stock up on food, n = 673 (67.2%)†	Leave the area, n = 132 (13.3%)†	Avoid others, n = 746 (74.2%)†	Seek medical advice, n = 667 (66.4%)†	Try to obtain antimicrobial drugs, n = 591 (59.4%)†
If someone catches pneumonic plague, they would feel unwell within 24 h							
	690	149	**1.7 (1.2–2.5)**	1.3 (0.7–2.3)	1.4 (0.9–2.1)	**1.6 (1.2–2.4)**	**1.7 (1.2–2.5)**
There have been cases of pneumonic plague in Britain in the past 10 y							
	228	687	0.9 (0.6–1.2)	0.8 (0.5–1.3)	0.8 (0.6–1.2)	1.1 (0.8–1.6)	0.8 (0.6–1.1)
If you come within 6 feet of someone who had pneumonic plague and was clearly ill, you would probably catch the disease							
	735	237	**2.8 (2.0–3.8)**	1.4 (0.8–2.3)	**2.1 (1.5–2.9)**	**2.1 (1.5–2.9)**	**1.9 (1.4–2.6)**
If you come within 6 feet of someone who had pneumonic plague but who had not yet developed any signs of illness, you would probably catch the disease							
	623	333	**2.0 (1.5–2.7)**	**2.0 (1.3–3.2)**	**2.2 (1.6–3.0)**	**2.1 (1.5–2.8)**	**1.9 (1.4–2.5)**
Unless they receive immediate treatment, then most people who catch pneumonic plague will die from it							
	767	169	**1.9 (1.3–2.7)**	**2.7 (1.4–5.4)**	**1.9 (1.3–2.7)**	**2.1 (1.4–3.0)**	**1.6 (1.1–2.3)**
If antibiotics are administered immediately after a person has been infected, they would probably survive							
	880	69	**0.5 (0.3–1.0)**	0.5 (0.3–1.0)	0.7 (0.4–1.4)	0.7 (0.4–1.3)	0.7 (0.4–1.3)
If someone with plague has been in a room, how long would it take after they leave before it is safe to enter the room?							
<1 d	372		**0.6 (0.4–0.8)**	**0.4 (0.2–0.7)**	**0.4 (0.2–0.6)**	**0.6 (0.4–0.8)**	**0.6 (0.4–0.9)**
1–2 d	226		1.0 (0.6–1.5)	**0.6 (0.4–1.0)**	**0.5 (0.3–0.8)**	0.8 (0.5–1.2)	1.0 (0.7–1.5)
>3 d	237		Reference	Reference	Reference	Reference	Reference

*All odds ratios adjusted for home ownership, ethnicity, sex, age, working status, number of years of education, and social grade. Survey stage 2. **Boldface** indicates significance (p<0.05).
†Very or fairly likely to perform that behavior.

The associations between demographic variables and precautionary behavior are shown in Tables 1 and 2 of online Technical Appendix 2 (available from www.cdc.gov/EID/content/16/1/120-Techapp2.pdf). Associations between perceptions and precautionary behavior were adjusted for relevant demographic variables (Tables 1, 2). In general, participants who perceived pneumonic plague to be more severe, easier to catch, or more persistent in the environment were more likely to engage in precautionary behavior (Tables 1, 2). Table 3 in online Technical Appendix

Table 2. Perceptions of and precautionary behavioral responses to a hypothetical pneumonic plague outbreak affecting >100 persons, United Kingdom, September 2007*

Predictor	Variable level, no. responses: Very or fairly likely	Variable level, no. responses: Not very or not at all likely (reference)	Association, adjusted odds ratio (95% confidence interval): Stock up on food, n = 798 (79.8%)†	Leave the area, n = 223 (22.4%)†	Avoid others, n = 850 (84.6%)†	Seek medical advice, n = 792 (79.4%)†	Try to obtain antimicrobial drugs, n = 724 (72.5%)†
If someone catches pneumonic plague, they would feel unwell within 24 h							
	690	149	**1.8 (1.1–2.7)**	1.6 (1.0–2.5)	1.3 (0.8–2.1)	1.5 (1.0–2.4)	**1.9 (1.2–2.8)**
There have been cases of pneumonic plague in Britain in the past 10 y							
	228	687	1.1 (0.8–1.7)	0.9 (0.6–1.4)	1.0 (0.6–1.5)	1.4 (0.9–2.1)	0.8 (0.5–1.1)
If you come within 6 feet of someone who had pneumonic plague and was clearly ill, you would probably catch the disease							
	735	237	**2.5 (1.8–3.6)**	**1.8 (1.2–2.7)**	**1.8 (1.2–2.6)**	**2.2 (1.5–3.1)**	**2.0 (1.4–2.8)**
If you come within 6 feet of someone who had pneumonic plague but who had not yet developed any signs of illness, you would probably catch the disease							
	623	333	**2.2 (1.6–3.2)**	**1.4 (1.0–2.0)**	**1.5 (1.0–2.1)**	**2.0 (1.5–2.9)**	**1.5 (1.1–2.1)**
Unless they receive immediate treatment, then most people who catch pneumonic plague will die from it							
	767	169	**2.1 (1.4–3.1)**	**2.8 (1.7–4.7)**	**1.8 (1.1–2.8)**	**2.3 (1.5–3.4)**	**2.2 (1.5–3.2)**
If antibiotics are administered immediately after a person has been infected, they would probably survive							
	880	69	0.6 (0.3–1.2)	0.7 (0.4–1.3)	1.1 (0.6–2.2)	0.4 (0.2–1.0)	1.0 (0.6–1.8)
If someone with plague has been in a room, how long would it take after they leave before it is safe to enter the room?							
<1 d	372		**0.6 (0.4–0.9)**	**0.5 (0.4–0.8)**	**0.4 (0.2–0.7)**	**0.6 (0.4–0.9)**	**0.6 (0.4–0.9)**
1–2 d	226		1.3 (0.7–2.1)	**0.6 (0.4–0.9)**	0.6 (0.3–1.0)	0.7 (0.4–1.2)	1.0 (0.6–1.6)
>3 d	237		Reference	Reference	Reference	Reference	Reference

*All odds ratios adjusted for home ownership, ethnicity, sex, age, working status, number of years in education, social grade, number of people at home and parental status. Survey stage 3. **Boldface** indicates significance (p<0.05).
†Very or fairly likely to perform that behavior.

2 shows the associations between demographic characteristics and the likelihood of not complying with public health recommendations. Table 4 in online Technical Appendix 2 shows the equivalent associations for perceptions about plague, after adjustment for relevant demographic variables. Only unnecessary visits to a treatment center were associated with perceptions; participants who felt that there had been cases of plague in the United Kingdom in the past 10 years (odds ratio [OR] 2.3, 95% confidence interval [CI] 1.3–4.0) or who felt that asymptomatic persons might be contagious (OR 2.8, 95% CI 1.5–5.3) were more likely to report that they would visit the treatment center if they had not been to the affected train station, and participants who believed that antimicrobial drugs are an effective treatment for plague were less likely to report that they would visit (OR 0.3, 95% CI 0.2–0.6).

Conclusions

Our survey indicates that should an outbreak of pneumonic plague occur, the inclination of the British public would be to adopt a range of spontaneous precautionary behaviors. Intended compliance with possible public health recommendations ranged from excellent (taking prophylactic antimicrobial drugs) to poor (visiting treatment centers). Some intended behavior we identified might complicate management of an outbreak. In particular, ≈25% of potentially exposed persons would not visit a treatment center, yet ≈10% of unexposed persons would. Given that specific perceptions about pneumonic plague were associated with being likely to engage in precautionary behavior, explicitly, clearly, and repeatedly addressing misperceptions during the early stages of an outbreak might help reduce public anxiety and help with decision making (*8*). However, perceptions showed few associations with willingness to comply with explicit public health advice.

Several caveats should be considered with regard to our methods. First, the large number of statistical tests that we conducted and the wide confidence intervals for some of our results make type 1 and type 2 errors likely. Second, our sample probably underrepresented groups who might be more vulnerable in the context of an outbreak, e.g., those who do not have access to a telephone or do not speak English. Our sample also consisted solely of persons who complied with a request to participate in a survey and who might therefore be more likely to comply with official advice during an outbreak. Our results may therefore overestimate likely compliance during an outbreak. Finally, respondents' difficulty in predicting how they would react to this hypothetical scenario also creates difficulty in assessing validity of results. We therefore caution readers to treat our results as suggestive of the broad level of compliance and precautionary behavior that might occur during an outbreak of pneumonic plague, not as precise predictions.

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Dr Rubin is a lecturer at the Department of Psychological Medicine, King's College London, and an honorary research fellow at the Centre for Emergency Preparedness and Response in the UK Health Protection Agency. His primary research interests lie in understanding the causes and consequences of persons' concerns about the health effects of modern life, including public reactions to chemical, biological, or radiologic incidents.

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Address for correspondence: G. James Rubin, King's College London, Institute of Psychiatry, Department of Psychological Medicine, James Black Centre, 125 Coldharbour Lane, Camberwell, London SE5 9NU, UK; email: g.rubin@iop.kcl.ac.uk

Rapid Displacement of Dengue Virus Type 1 by Type 4, Pacific Region, 2007–2009

Dong-sheng Li, Wen Liu, Aurélie Guigon, Emerald Stewart, Candice Mostyn, Richard Grant, and John Aaskov

Since 2000–2001, dengue virus type 1 has circulated in the Pacific region. However, in 2007, type 4 reemerged and has almost completely displaced the strains of type 1. If only 1 serotype circulates at any time and is replaced approximately every 5 years, DENV-3 may reappear in 2012.

During the past 10–15 years in the Pacific island nation states, sustained transmission of only 1 serotype of dengue virus (DENV) has occurred at any given time (*1*). This single serotype is in marked contrast to all 4 serotypes that cocirculate in many countries in Southeast Asia where dengue is endemic. During 1997–2000 in the Pacific region, the serotype recovered from patients was almost exclusively DENV-2, but during 2000–2001, <1 year, DENV-2 was displaced by multiple genotypes of DENV-1 (*2*). We describe rapid replacement of DENV-1 by DENV-4 during 2008.

The Study

In May 2008, an outbreak of a dengue-like illness began on the island of Tarawa in Kiribati (Figure 1). Immunochromatographic and ELISA assays (PanBio, Brisbane, Queensland, Australia) detected anti–dengue virus immunoglobulin (Ig) M or high titers (≥1,280) of anti–dengue virus IgG in serum from 5 of 18 patients. DENV-4 transmission had not been reported in the Pacific region for >1 decade; however, after serum was cultured with *Aedes albopictus* C6/36 cells, DENV-4 was recovered from 5 of the 13 serum samples from patients who had no detectable anti-DENV IgG or IgM (*2*).

In July 2008, a similar outbreak began in Samoa. Serum from 87 of 469 patients with suspected dengue contained anti-DENV IgM or high titers of anti-DENV IgG (ELISA; PanBio). Serum from 7 of the 87 patients with anti-DENV IgM contained no detectable anti-DENV IgG, suggesting a primary infection. DENV-4 was recovered from 42 of the 382 seronegative patients when serum was cultured with C6/36 cells.

From June 2007 through February 2008 in Tonga, small numbers of dengue cases, confirmed by ELISA, had been reported; the only DENV isolates recovered from these patients were 4 isolates of DENV-1 (J. Aaskov, unpub. data). However, in December 2008 and January 2009, only 4 DENV-4 isolates were recovered from 55 serum samples collected from patients with suspected dengue in Tonga.

In November 2008 in New Caledonia, DENV-4 transmission was detected after the virus had been introduced by residents returning from Vanuatu; and in February 2009, DENV-4 transmission was detected in French Polynesia after it had been introduced there by travelers from New Caledonia. In June 2009, DENV-1 and DENV-4 were cocirculating in New Caledonia and French Polynesia.

DENV-4 was reportedly recovered from a traveler returning to Taiwan from the Solomon Islands in 2007 (*3*) and from a resident of the Solomon Islands in April 2008 (Alyssa Pyke, pers. comm.). DENV-1 had been circulating in the Solomon Islands until at least 2002, preceding the 2003 arrival of a multinational peacekeeping force composed of persons from Australia, Papua New Guinea, New Zealand, Fiji, and Tonga. The chronology of these and other reports of dengue outbreaks involving DENV-4 are shown in the Table.

During some of these outbreaks, the envelope (E) protein genes of DENV-4 recovered from patients were amplified by reverse transcription–PCR (*2*) by using forward primer 5′-GGATTCGCTCTCTTGGCAGGATTTATG-3′ and reverse primer 5′- GCTTCCACACTTCAATTCTTTCCCACTCCA-3′, corresponding to regions in the premembrane and nonstructural protein gene 1, respectively, and the consensus nucleotide sequences of the resultant cDNA determined by Dye Terminator Cycle Sequencing on an automated sequencer (ABI Prism, Australian Genome

Figure 1. Island nation states of the Pacific region.

Author affiliations: Queensland University of Technology, Brisbane, Queensland, Australia (D. Li, Stewart E., J. Aaskov); Australian Army Malaria Institute, Brisbane (W. Liu, C. Mostyn, R. Grant, J. Aaskov); and Institut Pasteur, Noumea, New Caledonia (A. Guigon)

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Table. Chronology of appearance of dengue virus type 4, Pacific region

Date of appearance	Country	Source/reference
2007	Solomon Islands	(*3*)
2008 Apr	Nauru	T. Bryar, pers. comm.*
2008 May	Kiribati	This study
2008 Jul	Samoa	This study
2008 Jul	American Samoa	T. Bryar, pers. comm.*
2008 Jul	Palau	T. Bryar, pers. comm.*
2008 Aug	Cook Islands	T. Bryar, pers. comm.*
2008 Aug	Fiji	T. Bryar, pers. comm.*
2008 Aug	Niue	T. Bryar, pers. comm.*
2008 Oct	Vanuatu	T. Bryar, pers. comm.*
2008 Dec	Tonga	This study
2008 Nov	New Caledonia	This study
2009 Feb	French Polynesia	T. Bryar, pers. comm.*
2009 Jun	Chile (Easter Island)	ProMED-mail†

*Western Pacific Regional Office, World Health Organization.
†www.promedmail.org/pls/otn/f?p=2400:1202:1356950393966944::NO::F2400_P1202_CHECK_DISPLAY,F2400_P1202_PUB_MAIL_ID:X,78506

Research Facility, Brisbane, Queensland, Australia). Sequencing was performed by using the primers above as well as 5′-AACACAGCATGGGACAACAGT-3′ and 5′-GACTCAAACATCTTACCAATGGAG-3′. Sequences were analyzed by using ClustalW, Seqboot, DNADist, Kitsch, and Consense software (www.angis.org.au) from the Australian National Genome Information Service of the University of Sydney. Phylogenetic analyses (Human Research Ethics Approval QUT-0700000910) of the nucleotide sequences of the E genes of DENV-4 that we recovered from patients in Kiribati, Samoa, and Tonga and those of strains of DENV-4 recovered by others showed that all isolates from this recent outbreak in the Pacific were closely related but distinct from other DENV-4 isolates for which sequences were available (Figure 2), including isolates recovered in the Pacific region during the 1970s and 1980s.

The chronology of the recovery of DENV-4 from patients in the region and the phylogenetic analyses suggest that DENV-4 was introduced from Indonesia/Malaysia into the Pacific region, possibly into the Solomon Islands, sometime before 2007. The 3 genotypes of DENV-1 responsible for the earlier outbreak also originated in Southeast Asia (Philippines, Malaysia, Myanmar/Thailand) (*2*). The relative genetic homogeneity of the DENV-4 recovered during this most recent outbreak in the Pacific region (24 variable nucleotide sites in the E genes of 20 isolates resulting in 9 variable amino acid sites) suggests introduction of a single genotype rather than introduction of multiple genotypes and to different locations, as was the case with DENV-1 (*2*). The E proteins of all recent DENV-4 isolates from the Pacific region had isoleucine at position E365 rather than the threonine that was found at this position in earlier DENV-4 isolates. All Pacific region isolates except DENV4 Kiribati08.278 also had isoleucine at E335 rather than valine, which was found at this position in most earlier isolates. These 2 aa changes occurred in a region of domain III of the E protein of flaviviruses rich in epitopes recognized by serum from dengue patients and by neutralizing monoclonal antibodies (*4–6*); they occurred adjacent to the change at E390 in DENV-2, which was associated with the appearance of dengue hemorrhagic fever in South America (*7*).

Conclusions

Outbreaks of dengue in the Pacific region are initiated by the introduction of DENV, usually from Southeast Asia, but the populations of most Pacific island nation states are too small to sustain transmission of a single DENV serotype for >4–5 years. The interisland mobility of the human population in this region ensures rapid spread of any newly introduced viruses.

That the spread of dengue virus serotypes through the Pacific should be so synchronized is remarkable. This synchronization may reflect the relatively small populations of most island states (≈250,000 residents), high attack rates, and a high birth rate (≈30% of the population is <14 years of age). If only 1 DENV serotype circulates at any time,

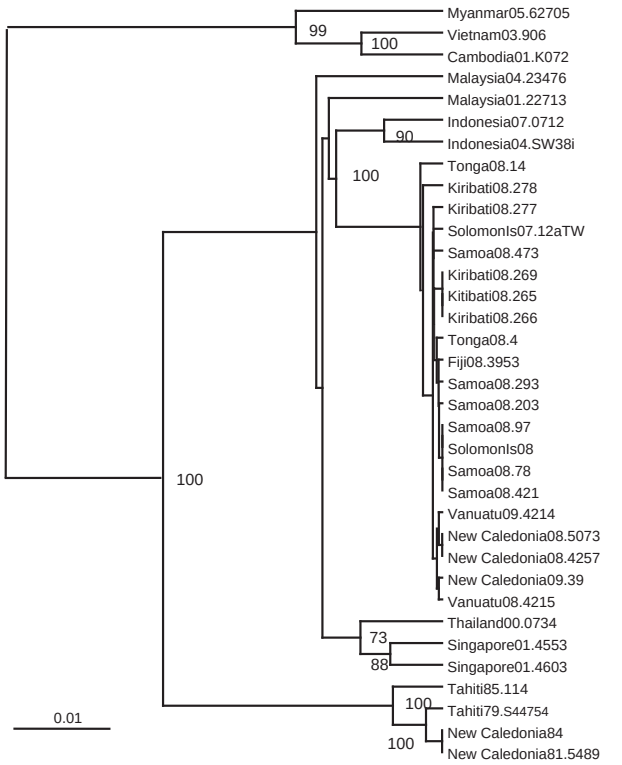

Figure 2. Phylogenetic relationships between the envelope (E) protein genes of dengue type 4 viruses recovered from patients from Pacific island nation states during 2007–2009 outbreaks and from dengue type 4 viruses from Southeast Asia and earlier outbreaks in the Pacific region. Bootstrap values are shown for key nodes. Scale bar represents 0.01 nt changes per site.

and serotype replacement occurs approximately every 5 years, these data suggest that ≈30% (75,000) of 250,000 susceptible hosts are sufficient in these settings to support a serotype replacement and that DENV-3 may reappear in the Pacific island states in ≈2012.

At this stage of study, data are insufficient for drawing conclusions about a role for the amino acid changes at E335 and E365 in the reemergence of DENV-4 in the Pacific region. There may be value in delineating the factors that appear to enable multiple DENV serotypes to circulate in urban areas of more developed Pacific nations (e.g., French Polynesia, New Caledonia, Australia) but that appear to prevent cocirculation of DENV serotypes in nations that are less developed but rapidly becoming urbanized. Such a study, however, would require more robust and comprehensive dengue surveillance programs than exist in many of these nations.

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Dr Li has worked in several virology research programs, most recently at the Institute of Health and Biomedical Innovation at the Queensland University of Technology. His research focuses on molecular epidemiology of flaviviruses and how the evolution of theses viruses affects their structure and function.

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Address for correspondence: John Aaskov, Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Brisbane, Queensland 4059, Australia; email: j.aaskov@qut.edu.au

Hepatitis E Epidemic, Uganda

Eyasu H. Teshale, Christopher M. Howard, Scott P. Grytdal, Thomas R. Handzel, Vaughn Barry, Saleem Kamili, Jan Drobeniuc, Samuel Okware, Robert Downing, Jordan W. Tappero, Barnabas Bakamutumaho, Chong-Gee Teo, John W. Ward, Scott D. Holmberg, and Dale J. Hu

In October 2007, an epidemic of hepatitis E was suspected in Kitgum District of northern Uganda where no previous epidemics had been documented. This outbreak has progressed to become one of the largest hepatitis E outbreaks in the world. By June 2009, the epidemic had caused illness in >10,196 persons and 160 deaths.

Hepatitis E virus (HEV) infection causes large epidemics of liver disease in developing countries (*1*–*3*). In epidemic settings, HEV is transmitted by the fecal–oral route, and the most commonly attributed source of infection is feces-contaminated drinking water (*4*). The incubation period after exposure ranges from 3 to 8 weeks (mean 40 days) and is dose dependent (*5*,*6*). Illness is generally self-limited, with death rates ≤4% in the general population (*7*), but a strikingly high death rate (10%–25%) has been reported among pregnant women (*8*).

In October 2007, an epidemic of hepatitis E was suspected in northern Uganda, where no previous epidemics had been documented. However, outbreaks of hepatitis E had occurred in neighboring Sudan and Chad in 2004 (*9*,*10*). Beginning in the Madi Opei subcounty of Kitgum District, this outbreak has progressed to become one of the largest hepatitis E outbreaks in Africa and globally. By June 2009, a year after the study we report here, the epidemic involved all 19 subcounties of Kitgum and had caused illness in >10,196 persons and 160 deaths (local surveillance, unpub. data). This report describes the results of a case finding and seroprevalence survey in 2 subcounties of Kitgum District, Madi Opei and Paloga.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (E.H. Teshale, C.M. Howard, S.P. Grytdal, T.R. Handzel, V. Barry, S. Kamili, J. Drobeniuc, C.-G. Teo, J.W. Ward, S.D. Holmberg, D.J. Hu); Ministry of Health, Republic of Uganda, Kampala, Uganda (S. Okware); Global AIDS Program, Kampala (R. Downing, J.W. Tappero); and Ugandan Virus Research Institute, Entebbe, Uganda (B. Bakamutumaho)

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The Study

Two subcounties, Madi Opei and Paloga, were selected for a census and seroprevalence survey. In June 2008 (at the time of this investigation), the 2 subcounties represented different stages of the epidemic: Madi Opei was the first subcounty to experience the epidemic; the epidemic appeared to affect Paloga relatively later. No differences in demographic and socioeconomic characteristics were evident between the residents of the 2 subcounties. Trained village health team members conducted a hut-to-hut census. The census was completed using a standardized data collection instrument. Histories of jaundice and jaundice-related death were also obtained.

For the seroprevalence survey, a random sample of residents was identified from the list created during the census. Persons who consented to participate had blood collected by venipuncture. Blood specimens were tested for immunoglobulin (Ig) M and IgG against HEV (MP Biomedicals Asia Pacific Pte Ltd, Singapore), HEV RNA (by an in-house reverse transcription–PCR assay), and serologic markers of infection by hepatitis A virus, hepatitis B virus, and hepatitis C virus. A subset was analyzed to determine the HEV genotype. The HEV sequence from this outbreak strain was compared with other HEV genotype 1 strains isolated from past epidemics. Because the 2 sites were in different stages of the outbreak as determined by the different durations and peaks of the epidemic at the time of the investigation (June 2008), HEV attack and death rates were calculated separately by site and in aggregate. All statistical analyses were performed with SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA).

A total of 19,098 persons were counted in Madi Opei (10,535) and Paloga (8,563) during the census. In Madi Opei, there were 2,137 families and an average of 4.9 persons per household. In Paloga, there were 1,884 families and an average of 4.5 persons per household. Figure 1 shows the distribution of cases of jaundice in Kitgum District, by week of report, October 2007 through January 2009 (data from facility-based passive surveillance). The overall symptomatic hepatitis E attack rate, based on hut-to-hut case finding, in the 2 subcounties was 25.1%. However, at the time of the investigation, the epidemic had peaked (Figure 2, panel A) in Madi Opei and was still increasing in Paloga: 30.1% of Madi Opei residents reported jaundice, but only 18.9% of Paloga residents reported jaundice by the time of the investigation. Symptomatic cases reached their height in April 2008 in Madi Opei but did not peak in Paloga until June 2008 (Figure 2, panel B).

Of the 10,535 Madi Opei residents, jaundice was reported by 3,170 (30.1%). In Paloga, jaundice was reported by 1,619 (18.9%) of 8,563 residents. The number of symptomatic cases was higher for women (28%) than

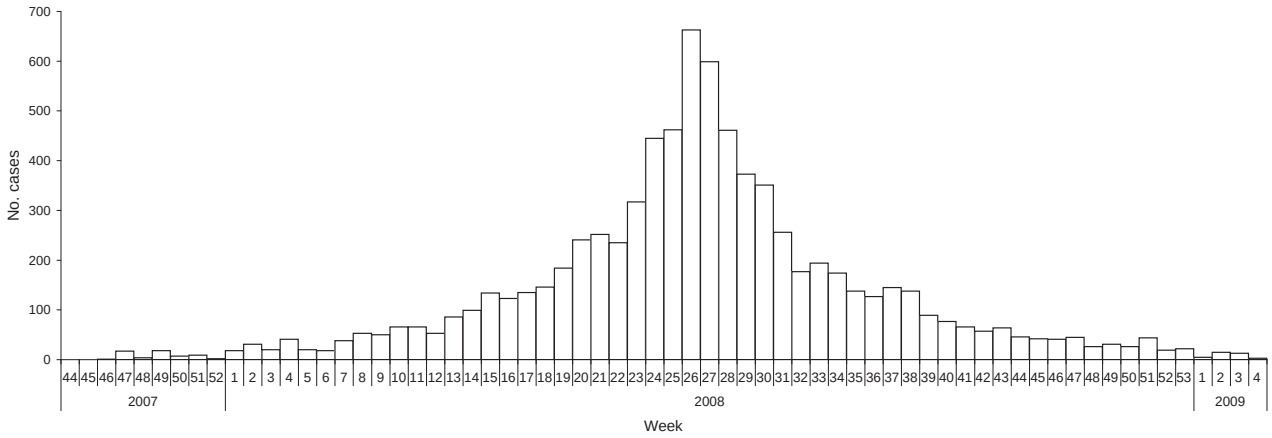

Figure 1. Distribution of cases of jaundice during an epidemic of hepatitis E in Kitgum District, Uganda (N = 7,919), by week of report, October 2007 through January 2009.

for men (22%; p<0.001) (Table). The symptomatic attack rate was lowest for children <2 years of age (6.9%) and highest for pregnant women (80.7%). In the 2 subcounties, 72 deaths were reported among 4,789 persons with jaundice, yielding an estimated case-fatality rate among jaundice cases of 1.5%. Among the 72 jaundice-related deaths, a disproportionate number occurred in children <2 years of age (12/92, 13%) and in pregnant women (13/189, 6.9%).

Sixty-six percent of 720 randomly selected residents agreed to participate in the survey and blood draw. Of the total tested, 305 (64.4%) were positive for IgM or IgG against HEV or both. In a subset of 142 specimens selected at random from among the participants of the survey

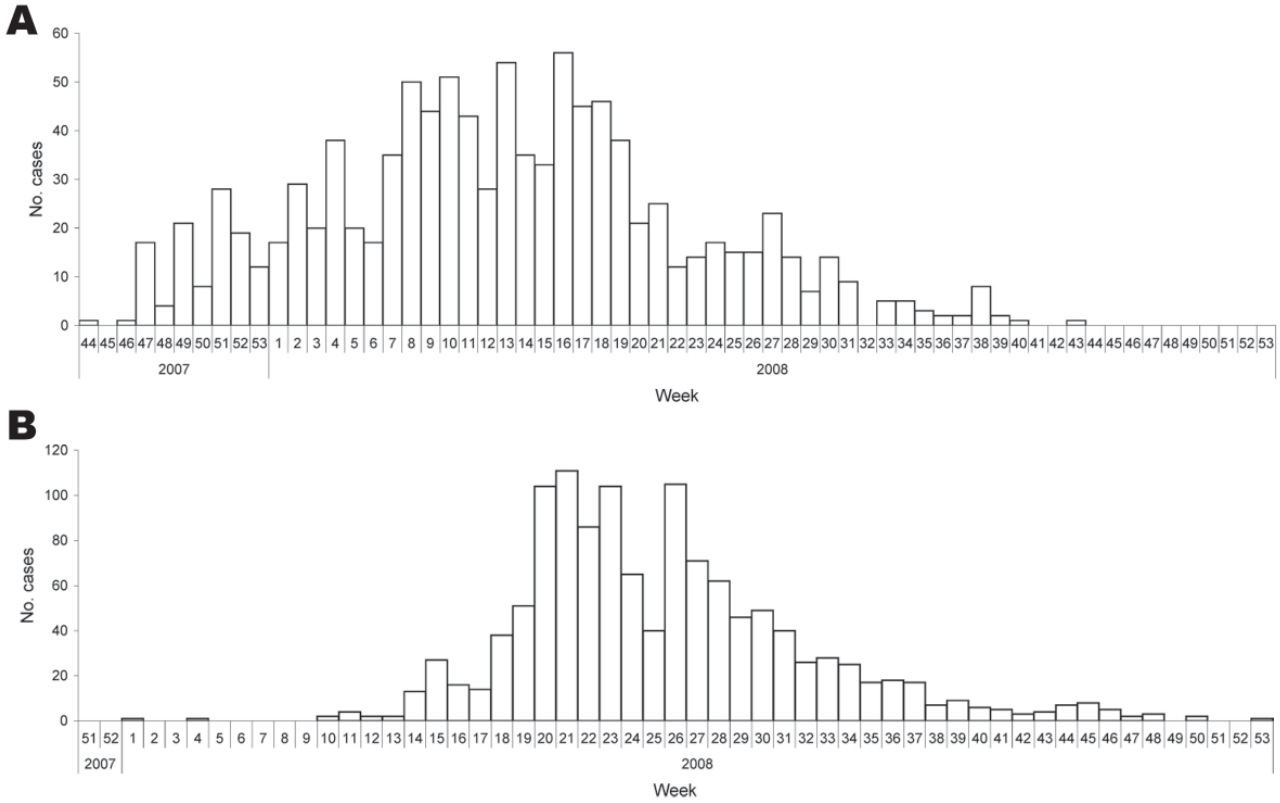

Figure 2. Distribution of cases of jaundice during an epidemic of hepatitis E in A) Madi Opei subcounty (n = 1,026) and B) Paloga subcounty (n = 1,248), by week of report, Kitgum District, Uganda, October 2007 through January 2009. Data are from facility-based passive surveillance.

Table. Symptomatic and serologic evidence of HEV infection, Uganda, June 2008

Characteristic	Jaundice		HEV seropositivity	
	No. examined	No. (%) with jaundice	No. tested	No. (%) seropositive
Age group, y				
<2	1,352	92 (6.8)	23	7 (30.4)
2–4	2,213	370 (16.7)	49	28 (57.1)
5–9	3,361	589 (17.5)	59	36 (61.0)
10–14	2,692	504 (18.7)	70	49 (70.0)
15–44	7,155	2,402 (33.6)	189	135 (71.4)
≥45	2,186	816 (37.3)	75	48 (64.0)
Sex				
M	9,177	2,017 (22.0)	188	122 (64.9)
F	9,877	2769 (28.0)	277	180 (65.0)
Total	19,098	4789 (25.1)	469	305 (65.0)

*HEV, hepatitis E virus.

and tested by reverse transcription–PCR, 24 were found to be positive for HEV RNA. Subsequent sequence analysis showed that HEV found in all 24 specimens belonged to genotype 1. There was close resemblance of the open reading frame 2 gene of the HEV genotype 1 isolates from this and the Chad outbreak. Other causes of viral hepatitis were rare. Of 469 persons tested, only 12 (2.5%) tested positive for IgM to hepatitis A virus, 3 (0.6%) were positive for IgM to hepatitis B core antigen, and 4 (0.8%) were positive for antibody to hepatitis C virus.

Conclusions

The symptomatic HEV attack rate and HEV-related deaths observed in this epidemic were high. Given that outbreaks of hepatitis E had not been observed or reported in Uganda previously, a lack of preexisting immunity from prior exposure in this population may well have facilitated the outbreak. Nevertheless, outbreaks had been reported in neighboring countries and, although there were no sequences available from the epidemic in southern Sudan, phylogenetic-relatedness analysis showed close resemblance of the outbreak strain to the strain from the Chad outbreak. There was no clear epidemiologic link of this outbreak to the neighboring countries.

Women in this epidemic were substantially affected, and, as seen in previous epidemics, pregnant women are particularly at increased risk of death (*4*). Furthermore, our survey also showed that children (0–2 years of age) were at a higher risk of dying from hepatitis E, despite the fact they were generally asymptomatic. This finding corroborates a similar observation for a hepatitis E epidemic in the former Soviet Union in 1985–1987 (*11*). We do not know why pregnant women and young children were at increased risk for death from this infection, but there may be gender- or age-specific risk for exposure or differential susceptibility to infection (*1*,*7*).

This investigation has some limitations. For the census, the clinical diagnosis of hepatitis E was by self-report only, and the death rate data were based on verbal autopsy. Jaundice could have been overdiagnosed for young children and thus could have contributed to a skewed death rate being reported for this group.

Current understanding of HEV transmission indicates that effective prevention and control depend on ensuring a safe drinking water supply, adequate sanitation, and proper personal and environmental hygiene. However, due to the rapid transmission of HEV and the long incubation period of this disease, it is difficult to mount adequate prevention measures in a timely manner. This difficulty was evident in the long duration of the outbreak in Kitgum district. Therefore, we recommend that increased priority be given to developing a promising current hepatitis E vaccine candidate as soon as possible (*12*). Availability of vaccine is also needed in light of the high death rate of children and pregnant women. However, the safety of such a vaccine for pregnant women needs to be determined before use in this population.

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Dr Teshale is a medical epidemiologist within the Division of Viral Hepatitis at the Centers for Disease Control and Prevention. His research interests include hepatitis E virus in the industrialized and nonindustrialized world, long-term effectiveness of hepatitis B vaccine, and treatment of hepatitis C.

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Address for correspondence: Eyasu H. Teshale, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G37, Atlanta, GA 30333, USA; email: eht4@cdc.gov

Novel Human Parechovirus, Sri Lanka

Ngan Thi Kim Pham, Quang Duy Trinh, Sayaka Takanashi, Chandra Abeysekera, Asiri Abeygunawardene, Hideaki Shimizu, Pattara Khamrin, Shoko Okitsu, Masashi Mizuguchi, and Hiroshi Ushijima

Of 362 fecal samples collected from children with acute gastroenteritis in Sri Lanka during 2005–2006, 30 (8.3%) were positive for human parechovirus (HPeV) by reverse transcription–PCR. A novel HPeV, designated as HPeV10, was identified in 2 samples by sequence analysis of the viral protein 1 gene of the detected HPeVs.

Parechoviruses are small, nonenveloped, positive-sense, single-stranded RNA viruses belonging to the large family of *Picornaviridae*, a highly diverse family of important pathogens of humans and animals. The genus *Parechovirus* is composed of 2 species: *Ljungan virus*, isolated from bank voles (*1*), and *human parechovirus* (HPeV), a frequent human pathogen. The HPeV genome is ≈7.3 kb long and contains a large open reading frame coding for a single polyprotein. The polyprotein is cleaved posttranslationally into 3 structural proteins (viral protein [VP] 0, VP3, and VP1) and 7 nonstructural proteins (2A–2C and 3A–3D) (*2*,*3*).

Previous findings have shown the genetic variability of HPeVs, and the number of newly identified HPeV genotypes has been on the increase (*4*–*6*). To date there have been 9 published HPeV types assigned as types 1–8 and 14 (www.picornaviridae.com/parechovirus/hpev/hpev.htm). We identified a novel HPeV designated as HPeV10 that was detected in the stool samples of children in Sri Lanka who had acute gastroenteritis.

The Study

We used reverse transcription–PCR to screen 362 fecal samples collected from child inpatients with acute gastroenteritis at a hospital in Kandy, Sri Lanka, during September 2005 through August 2006 for HPeV. Informed consent was obtained from the mothers of all enrolled patients. The study was approved by the University of Peradeniya's Committee on Research and Ethical Review. Reverse transcription was performed by using random primer, and PCR was conducted by using primers ev22(+) and ev22(–) to amplify a 270-bp PCR product of the 5′ untranslated region (*7*).

For genotyping, samples positive for HPeV by the screening PCR were subjected to a 2-step PCR to amplify the VP1 sequence. The first PCR was done by using 2 newly developed primers, Cap-parEcho-F (5′-TCHACWTGGATGMGRAARAC-3′) and Cap-parEcho-R (5′-TCYARYTCACAYTCYTCYTC-3′), which were designed outside the VP1 region, whereas the nested PCR was performed by using the inner primer pair, VP1-parEchoF1 and VP1-parEchoR1, described by Benschop et al. (*8*). The PCR amplicons of the VP1 gene were purified and sequenced in both directions by using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA, USA). The inner primers for amplification of VP1 gene were used as sequencing primers. The sequence data were collected by an ABI Prism 310 Genetic Analyzer (Perkin Elmer-Applied Biosystems, Inc.).

Comparison analysis of the VP1 sequence was conducted between the obtained HPeV strains and reference HPeV strains of the 9 defined genotypes (HPeV1-8 and HPeV14) available in the GenBank database. The sequence data and the phylogenesis were analyzed by using BioEdit version 7.0.5 (www.mbio.ncsu.edu/BioEdit/bioedit.html). A parsimony analysis was also conducted by using MEGA version 3.1 to determine the evolutionary relationship among studied sequences (*9*). The method was performed using close-neighbor interchange with a random option and with 500 bootstrap repetitions.

Of the 362 samples tested, 30 were positive for HPeV; detection rate was 8.3%. Of these, 12 isolates were selected for amplification and sequencing of the VP1 gene. Ten of the 12 sequenced strains were of known and well-characterized genotypes (genotype 1, 7 samples; genotype 4, 3 samples). These strains were not further analyzed. Two remaining strains (LK-103 and LK-106, accession nos. GQ402515 and GQ402516, respectively) showed VP1 sequences that clustered together with none of the known 9 HPeV genotypes (HPeV1-8, 14) in the phylogenetic analysis (Figure). Nucleotide and amino acid similarities between these 2 strains were 94.4% and 99.5%, respectively (data not shown).

Identical matrix analysis of VP1 nucleotide sequences of the 2 strains from Sri Lanka and global reference strains of the 9 known genotypes available in GenBank databases was then performed. The results showed that the 2 studied strains had highest mean nucleotide and amino acid simi-

Author affiliations: University of Tokyo, Tokyo, Japan (N.T.K. Pham, Q.D. Trinh, S. Takanashi, M. Mizuguchi); University of Peradeniya, Peradeniya, Sri Lanka (C. Abeysekera, A. Abeygunawardene); Kawasaki City Institute of Health, Kanagawa, Japan (H. Shimizu); Aino Health Science Center and Aino University, Tokyo (P. Khamrin, H. Ushijima); and Aino Science Center and Aino College, Tokyo (S. Okitsu).

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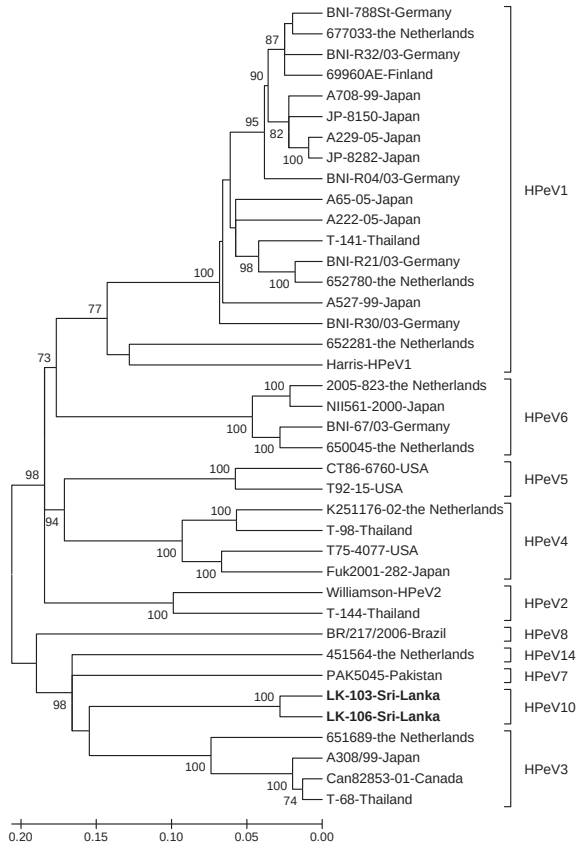

Figure. Phylogenetic tree constructed from nucleotide sequences of the structural viral protein gene of the strains studied and reference human parechovirus (HPeV) strains with 500 bootstrap repetitions. Percentage bootstrap values >70% are shown at the branch nodes. The studied HPeV strains are in **boldface**; their nucleotide sequences have been deposited in GenBank under accession nos. GQ402515 and GQ402516. Scale bar indicates nucleotide substitutions per site.

larities with HPeV3, 69.1% and 82.8%, respectively, and that the lowest mean nucleotide and amino acid similarities were found between the 2 studied strains and HPeV5, of 54.5% and 60.9%, respectively (Table). Therefore, these strains were expected to be classified into a new or previously unpublished HPeV (HPeV 9-13) genotype according to proposed criteria for assigning HPeV genotypes (*10*).

The VP1 sequences of the strains studied were submitted to the International Committee on Taxonomy of Viruses Picornavirus Study Group (www.picornastudygroup.com/types/index.html) to identify their genotype. These 2 strains were designated HPeV10 with their nucleotide and amino acid identities of 88.0% and 98.6% (strain LK-106) and 87.7% and 97.7% (strain LK-103) to the prototype BAN2004-10903 (M.S. Oberste et al., unpub. data).

The alignment of deduced amino acid sequences of the strains studied and global HPeV reference strains of HPeVs genotypes 1–8 and 14 showed that the arginine-glycine-aspartic acid (RGD) motif, which is considered to be critical for HPeV1 entry (*11*), was neither present in the strains studied nor among reference strains of HPeV3, HPeV7, HPeV8, and HPeV14 (*4–6*,*12*). Therefore, like HPeV3, HPeV7, HPeV8, and HPeV14, the lack of RGD motif in HPeV10 may imply that HPeV10 has an RGD-independent entry pathway.

Conclusions

We found HPeV in stool samples collected from hospitalized children in Sri Lanka who had acute gastroenteritis. The identified HPeV10 in this study was more closely genetically related to HPeV3 than to the remaining published HPeVs. Together with the unpublished findings of Oberste et al., this study provides basic data for future research into HPeV10. In addition, when taken together with other previous findings, our findings suggest that HPeV should be included in the spectrum of viruses for which routine screening is conducted among children with acute gastroenteritis.

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Table. Mean percentage nucleotide similarities between HPeV genotypes*

HPeV genotype	1	2	3	4	5	6	7	8	10	14
1										
2	64.1									
3	58.0	59.0								
4	63. 9	63.2	59.5							
5	61.6	60.7	53.5	65.8						
6	64.7	60.1	61.0	61.8	60.8					
7	57.3	59.9	63.8	58.7	56.0	54.1				
8	61.6	61.7	61.2	62.3	57.7	59.1	62.1			
10	60.5	56.8	69.1	60.9	54.5	55.9	66.0	63.6		
14	59.8	58.4	65.9	58.4	56.2	56.6	66.2	62.5	68.8	

*HPeV, human parechovirus.

Ms Pham is a physician and a PhD candidate at Tokyo University, Japan. Her current research interest focuses on less-explored viral pathogens of acute gastroenteritis in humans.

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Address for correspondence: Hiroshi Ushijima, Aino Health Science Center, Aino University, 2-17-3 Shibuya, Shibuya-ku, Tokyo 150-0002, Japan; email: ushijima-hiroshi@jcom.home.ne.jp

Broiler Chickens as Source of Human Fluoroquinolone-Resistant *Escherichia coli*, Iceland

Thorunn R. Thorsteinsdottir, Gunnsteinn Haraldsson, Vala Fridriksdottir, Karl G. Kristinsson, and Eggert Gunnarsson

To investigate feed as a source for fluoroquinolone-resistant *Escherichia coli* in broiler chickens, we compared antimicrobial drug–resistant *E. coli* from broiler feed and broilers with ciprofloxacin-resistant human clinical isolates by using pulsed-field gel electrophoresis. Feed was implicated as a source for ciprofloxacin-resistant broiler-derived *E. coli* and broilers as a source for ciprofloxacin-resistant human-derived *E. coli*.

In a previous study, we found a relatively high prevalence of antimicrobial and especially quinolone resistance among *Escherichia coli* isolates from broiler chickens and broiler meat (*1*), despite no known antimicrobial drug selection pressure in chicken farming in Iceland and biosecurity measures to prevent transmission of infectious agents into farms. Broiler houses are cleaned and disinfected after broiler flocks are transported to slaughter. Therefore, resistant bacteria are unlikely to persist in the broiler houses. However, animal feed can be contaminated with antimicrobial drug–resistant *E. coli* (*2*).

The high prevalence of quinolone-resistant *E. coli* isolates obtained from broilers and broiler meat coincides with an increasing prevalence of fluoroquinolone resistance among human clinical *E. coli* isolates in Iceland. This increase correlated with increased use of fluoroquinolones in clinical settings (*3*).

We examined whether the prevalence of resistant *E. coli* strains in broilers had changed since our previous study and whether broiler feed could be a source for the resistant strains. Furthermore, we compared the genotypes of ciprofloxacin-resistant broiler, broiler meat, and broiler feed *E. coli* isolates with ciprofloxacin-resistant human clinical *E. coli* isolates.

Author affiliations: University of Iceland, Reykjavík, Iceland (T.R. Thorsteinsdottir, G. Harladsson, V. Fridriksdottir, K.G. Kristinsson, E. Gunnarsson); and Landspitali University Hospital, Reykjavík (G. Haraldsson, K.G. Kristinsson)

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The Study

The sampling period for this study was May through November 2008. Pooled cecal samples (20 ceca from each flock) were taken from the 30 flocks slaughtered at all 3 broiler slaughterhouses in Iceland in June 2008. Ceca were stomachered in phosphate-buffered saline, spread on MacConkey agar with and without enrofloxacin (0.25 mg/L), and incubated overnight. Feed was sampled from feed stalls at 18 farms (of which 14 had participated in the previous study) and from 2 feed mills; the feed was suspended in buffered peptone water, mixed, incubated overnight, and spread on MacConkey agar as described above. One colony from each agar plate was selected for susceptibility testing as described in our previous study (*1*). Because isolates were collected from the 18 largest broiler farms (of the 27 farms operating in Iceland), all the broiler slaughterhouses, and the only 2 feed mills operating in Iceland, we believe this study provides a representative sample.

We selected all 34 available human *E. coli* isolates recovered from routine clinical specimens (mostly urine and blood) at the main clinical microbiology/reference laboratory in Iceland (Landspitali University Hospital) during 2006–2007, which had similar susceptibility patterns to the strains previously isolated from broilers (*1*) (ampicillin-tetracycline-sulfamethoxazole/trimethoprim-ciprofloxacin or ciprofloxacin alone). Only 1 isolate was chosen from each patient.

We performed susceptibility testing using a microbroth dilution method (VetMIC; National Veterinary Institute, Uppsala, Sweden). MICs were determined for ampicillin, cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, nalidixic acid, kanamycin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim. Cutoff values were those used in the monitoring programs in Sweden and Norway (*4*,*5*). Strains resistant to ≥3 classes of antimicrobial agents were considered multiresistant.

We compared all *E. coli* broiler ceca and feed isolates resistant to ≥1 antimicrobial agents and the 34 ciprofloxacin-resistant human *E. coli* isolates with resistant *E. coli* isolates from the previous study (2005–2007) (*1*) by pulsed-field gel electrophoresis (PFGE) using a slightly modified method of Ribot et al. (*6*). Comparison of PFGE patterns was made by visual inspection and BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). For cluster analysis, the Dice coefficient for band matching (band-position tolerance 1.5%) was used to generate an unweighted pair group method with arithmetic averages dendrogram. Isolates from the previous and present study that did not yield a satisfactory banding pattern by PFGE were genotyped by randomly amplified polymorphic DNA

(RAPD) analysis as described (*7*). Reaction products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Patterns were considered different when the profiles differed by at least 1 band. Similarity among RAPD patterns was compared as described for PFGE. Clusters for PFGE and composite RAPD profiles were defined as ≥2 isolates with ≥80% similarity. Prevalence values were compared by using the Fisher exact test.

Of the 40 broiler isolates, 20 were resistant to ≥1 of the antimicrobial drugs tested (Table); only 1 was multidrug resistant (resistant to streptomycin, tetracyclin, sulfamethoxazole, and trimethoprim). Ciprofloxacin and nalidixic acid were always cross-resistant. Compared with the previous sampling, the prevalence of resistance increased significantly for ciprofloxacin and nalidixic acid (from 18.2% to 42.5%; $p<0.0001$) but decreased significantly for ampicillin (from 18.2% to 0.0%; $p = 0.002$) and sulfamethoxazole (from 19.1% to 5.0%; $p = 0.0398$). This finding suggests that quinolone resistance was not transferred with resistance to the other antimicrobial agents and that it was selected for by other factors. Of the 22 *E. coli* isolates obtained from feed, 7 (32%) were resistant to ciprofloxacin and nalidixic acid, and all were susceptible to the other antimicrobial agents tested. Although no *E. coli* were isolated from the 2 feed mill samples, other *Enterobacteriaceae* grew on the agar plates, which could have overgrown existing *E. coli* strains, if any, demonstrating that the feed was not sterile.

The 27 resistant broiler and feed isolates were compared with 76 resistant isolates analyzed in our previous study (*1*) along with the 34 ciprofloxacin-resistant human *E. coli* isolates. Of 137 broiler, broiler meat, feed and human isolates, 110 (80%) yielded interpretable, reproducible PFGE patterns. We detected 92 profiles, of which 81 (88%) were represented by a single isolate. Isolates of different origin were intermixed forming 26 clusters, of which 12 were seen in the previous study. Of the 14 new clusters, 10 contained isolates of different origins (Figure). Human isolates clustered with broiler (2005–2006), broiler meat, broiler (2008), and feed isolates in 6 clusters (Figure). This supports previous findings of chickens and their products as a possible source of antimicrobial drug–resistant *E. coli* in humans (*8*,*9*). With the extensive genomic diversity of *E. coli* and the discriminative power of PFGE typing, finding indistinguishable isolates of different origin collected over several years is unlikely, except from a large collection (*8*,*10*). Therefore, finding human isolates closely related (≥80 similarity) to broiler, broiler meat, and feed isolates suggests an epidemiologic link between the populations. Additionally, we found closely related isolates from feed and broiler (samples from 2008 and 2005–2006) from geographically distant farms, supporting previous findings that antimicrobial drug–resistant *E. coli* could be introduced into the farm environment through broiler feed (*2*).

The isolates that did not give interpretable PFGE patterns (1 broiler [2005–2006] and 4 broiler meat [2006–2007] isolates, 11 broiler [2008] and feed [2008] isolates, and 11 ciprofloxacin-resistant human isolates) were subjected to RAPD analysis. All isolates yielded interpretable patterns displaying 26 distinct profiles; all but 1 unique profile represented a single isolate. At 80% similarity, 1 cluster was of mixed origin, containing 6 isolates from feed and broilers (2008).

Conclusions

Prevalence of fluoroquinolone-resistant *E. coli* remains moderately high in broilers, but resistance to other antimicrobial drugs is decreasing. Fluoroquinolone-resistant *E. coli* isolated from broiler feed implicates feed as the source

Table. Antimicrobial drug resistance among *Escherichia coli* isolates collected from broiler ceca and feed during 2008 compared with isolates collected from ceca and meat during 2005–2007 (*1*), Iceland*

	Resistant strains, no. (%)			
	2005–2007		2008	
Antimicrobial drug	Ceca, n = 110	Meat, n = 75	Ceca, n = 40	Feed, n = 22
Ampicillin	20 (18.2)	12 (16.0)	0	0
Cefotaxime	0	0	0	0
Ceftiofur	0	0	0	0
Chloramphenicol	0	0	0	0
Ciprofloxacin	20 (18.2)	27 (36.0)	17 (42.5)	7 (31.8)
Enrofloxacin	16 (14.5)	25 (33.3)	NT	NT
Florfenicol	1 (0.9)	0	0	0
Gentamicin	2 (1.8)	1 (1.3)	0	0
Nalidixic acid	20 (18.2)	27 (36.0)	17 (42.5)	7 (31.8)
Kanamycin	1 (0.9)	0	0	0
Streptomycin	9 (8.2)	7 (9.3)	2 (5.0)	0
Sulfamethoxazole	21 (19.1)	11 (14.7)	2 (5.0)	0
Tetracycline	15 (13.6)	8 (10.7)	1 (2.5)	0
Trimethoprim	16 (14.5)	10 (13.3)	1 (2.5)	0

*NT, not tested. Resistance breakpoints (μg/mL): ampicillin, ≥16; cefotaxime, ≥0.5; ceftiofur, ≥2; chloramphenicol, ≥32; ciprofloxacin, ≥0.12; enrofloxacin, ≥0.5; florfenicol, ≥32; gentamicin, ≥8; nalidixic acid, ≥32; kanamycin, ≥16; streptomycin, ≥64; sulfamethoxazole, ≥512; tetracycline, ≥16; trimethoprim, ≥4.

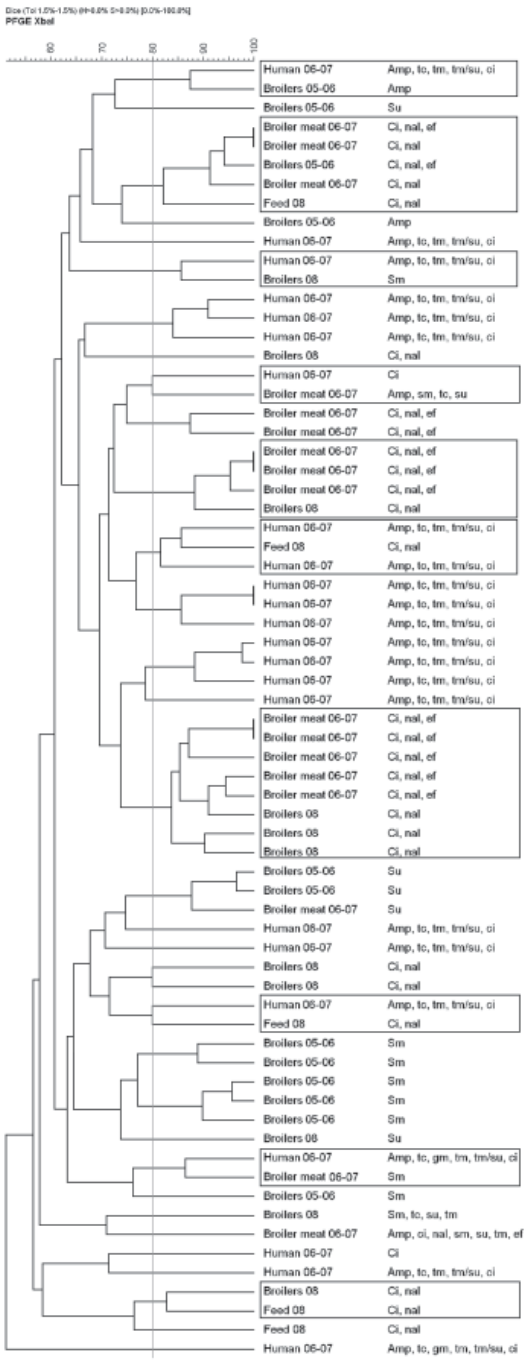

Figure. Dendrogram based on unweighted pair group method with arithmetic averages cluster analysis of pulsed-field gel electrophoresis (PFGE) patterns of the 20 broiler (2008), 7 feed (2008), and 34 human ciprofloxacin-resistant *Escherichia coli* isolates along with 29 of the most closely related broiler (2005–2006) and broiler meat (2006–2007) isolates from an earlier study (*1*), Iceland. Boxes indicate clusters (isolates with ≥80% similarity by Dice coefficient similarity analysis) of isolates of different origins not seen in the previous study. Amp, ampicillin; ci, ciprofloxacin; gm, gentamicin; nal, nalidixic acid; sm, streptomycin; su, sulfamethoxazole; tc, tetracycline; tm, trimethoprim; tm/su, trimethoprim/sulfamethoxazole.

of resistant strains into farms. Resistant isolates from feed, broilers, broiler meat, and humans were closely related, demonstrating that poultry and their food products can be a source of resistant *E. coli* in humans.

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Ms Thorsteinsdottir is a biologist and a PhD fellow at the Institute for Experimental Pathology, University of Iceland, Keldur. Her primary research interest is antimicrobial drug resistance of bacteria, particularly in relation to zoonoses.

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Address for correspondence: Thorunn R. Thorsteinsdottir, Institute for Experimental Pathology, University of Iceland, Keldur v/Vesturlandsveg, 112 Reykjavík, Iceland; email: thoruth@hi.is

Human Listeriosis Caused by *Listeria ivanovii*

Christelle Guillet, Olivier Join-Lambert, Alban Le Monnier, Alexandre Leclercq, Frédéric Mechaï, Marie-France Mamzer-Bruneel, Magdalena K. Bielecka, Mariela Scortti, Olivier Disson, Patrick Berche, José Vazquez-Boland,[1] Olivier Lortholary,[1] and Marc Lecuit

Two species of *Listeria* are pathogenic; *L. monocytogenes* infects humans and animals, and *L. ivanovii* has been considered to infect ruminants only. We report *L. ivanovii*–associated gastroenteritis and bacteremia in a man. This isolate was indistinguishable from prototypic ruminant strains. *L. ivanovii* is thus an enteric opportunistic human pathogen.

The genus *Listeria* contains 2 pathogenic species, *L. monocytogenes* and *L. ivanovii* (*1*). They both invade host cells, replicate in the cytosol after phagosomal escape, and spread from cell to cell by polymerizing actin. These mechanisms correlate with the presence in each species of genetic determinants called the *inlAB* internalization locus, the LIPI-1 intracellular survival pathogenicity island, and the *hpt* intracellular growth locus (*2*). However, each species appears to infect different hosts: *L. monocytogenes* infects humans and ruminants, whereas *L. ivanovii* is thought to infect ruminants only (*2*). *L. ivanovii* have been previously isolated, although rarely, from infected humans, indicating pathogenic potential for humans (Table). We report a case of *L. ivanovii* infection in a man with a kidney transplant. The ecology of *L. ivanovii* suggests that the rarity of human listeriosis due to this species reflects not only host tropism factors but also the rare occurrence of this species in the environment, compared with *L. monocytogenes*.

Author affiliations: Université Paris Descartes, Paris, France (C. Guillet, O. Join-Lambert, F. Mechaï, M.-F. Mamzer-Bruneel, P. Berche, O. Lortholary, M. Lecuit); Hôpital Necker–Enfants Malades, Paris (C. Guillet, O. Join-Lambert, F. Mechaï, M.-F. Mamzer-Bruneel, P. Berche, O. Lortholary, M. Lecuit); Institut Pasteur, Paris (A. Le Monnier, A. Leclercq, O. Disson, M. Lecuit); University of Edinburgh, Edinburgh, Scotland, UK (M. K. Bielecka, M. Scortti, J. Vazquez-Boland); Universidad Complutense de Madrid, Madrid, Spain (M. Scortti); Institut National de la Santé et de la Recherche Médicale, Paris (O. Disson, M. Lecuit); and Universidad de Léon, Léon, Spain (J. Vazquez-Boland)

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The Case

In January 2007, a 55-year-old man was hospitalized in Paris, France, with a 3-week history of nonbloody diarrhea, vomiting, dehydration, and low-grade fever. Medical history included renal transplantation for chronic renal failure and chronic hepatitis C. Immunosuppressive regimen included mycophenolate mofetil, tacrolimus, and prednisone. At the time of admission, his temperature was 37.8°C and he had moderate and painless abdominal distension. Laboratory values were 5.9×10^9/L leukocytes, 0.4×10^9/L lymphocytes, 9.7 g/dL hemoglobin, 137,000/mL platelets, 470 μmol/L creatinine, and <5 mg/L serum C-reactive protein. Liver tests were within normal limits except γ-glutamyltranferase, which was increased (244 U/L; reference <50 U/L).

Blood cultures yielded coryneform gram-positive rods with intensely β-hemolytic colonies; catalase and esculin hydrolysis test results were positive, consistent with *Listeria* spp. (*1*). Because listeriosis was suspected, intravenous amoxicillin and gentamicin therapy was initiated. Cerebrospinal fluid showed no abnormalities by direct examination or culture. Semiquantitative aerobic fecal culture showed the same coryneform gram-positive rods (10^6 CFU/g). The API Coryne biochemical test (bioMérieux, Marcy l'Étoile, France) identified blood and fecal isolates as *Listeria* spp. Fecal specimens were negative for *Salmonella*, *Shigella*, *Yersinia*, and *Campylobacter* spp. After 7 days, intravenous treatment was switched to oral amoxicillin for 2 weeks. The patient's condition rapidly improved, and control fecal cultures were negative.

The 3 isolates from blood and 1 from feces were referred to the French National Reference Centre for *Listeria* (Institut Pasteur, Paris, France). All were identified as *L. ivanovii* subsp. *ivanovii* and belonged to *L. ivanovii*–specific serovar 5. They showed identical profiles by pulsed-field gel electrophoresis (Figure, panel A). Agar diffusion test results were as expected for *Listeria* spp.: susceptible to amoxicillin and gentamicin; resistant to third-generation cephalosporins, clindamycin, and aztreonam (*2*). Contrary to *L. monocytogenes*, which is naturally resistant to fosfomycin in vitro (*9*), all isolates were susceptible to fosfomycin in vitro, as previously reported (*2*).

The isolates were compared with prototypic *L. ivanovii* strains from sheep (American Type Culture Collection 19119 type strain, Ivan Ivanov, 1955, PAM 19, Australia) and goats (PAM 55, Spain). We determined the activation status of the virulence gene regulator PrfA. For *L. monocytogenes,* the PrfA-regulated factors are mainly expressed in vivo, but for *L. ivanovii*, they are constitutively overexpressed in vitro (*2,11*). Some of these virulence factors have easily detectable phenotypes, such as hemolysis on

[1]These authors contributed equally to this article.

Table. Reported human cases of *Listeria ivanovii* infection

Clinical condition	Sex	Underlying condition	Year reported (reference)
Unknown*	Unknown*	Unknown*	1971 (*3*)
Uterine discharge	F	Pregnancy	1985 (*4*)
Mesenteric adenitis	Unknown	Unknown	1985 (*4*)
Stillbirth	F	Pregnancy	1990 (*5*)
Bacteremia	M	AIDS, lymphoma	1994 (*6*)
Bacteremia	M	Substance abuse	1994 (*7*)
Bacteremia	M	Hepatic carcinoma	2006 (*8*)
Gastroenteritis, bacteremia	M	Immunosuppression†	2007 (this study)

*Published as *Listeria monocytogenes* serovar 5 (pre-1986 designation of *L. ivanovii*).
†Polycystic kidney disease (which led to chronic renal failure and renal transplantation), chronic hepatitis C.

blood agar, PlcB phospholipase activity on egg yolk agar, and Hpt hexose phosphate transporter activity in acidification test (*2*,*12*). All isolates were phenotypically identical; they produced broad halos of hemolysis and lecithinase reactions and had positive glucose-1-phosphate acidification test results, reflecting the constitutive activation of the PrfA virulence regulon.

PCR mapping was used to test for *L. ivanovii*–specific pathogenicity island LIPI-2 (*13*). LIPI-2 comprises 10 internalin genes and the sphingomyelinase gene *smcL* and is perfectly conserved within *L. ivanovii,* including the distantly related subspecies *londoniensis* (*13*). All intragenic and intergenic PCRs gave identical results for all strains. The phenotypic marker for LIPI-2, *smcL*-encoded sphingomyelinase, was assessed by the synergistic hemolysis (CAMP-like) test (*13*) and was found in all strains (Figure, panel B).

Finally, we performed invasion assays with Madin-Darby bovine kidney (MDBK) cells and HeLa cells (human). Confirming previous observations (*13*), all *L. ivanovii* strains were hyperinvasive in MDBK cells and less invasive in HeLa cells compared with *L. monocytogenes* (Figure, panel C). Invasion assays expressing human E-cadherin or not did not show substantial differences, suggesting that *L. ivanovii* InlA does not interact with E-cadherin, in contrast to *L. monocytogenes* InlA (*6*) (data not shown). The 4 patient isolates showed slightly lower invasion capacity in MDBK cells than did isolates from ruminants but were still hyperinvasive relative to *L. monocytogenes*.

Conclusions

We found 3 other well-documented cases of *L. ivanovii*–associated human infection (Table) 1 febrile diarrhea (*7*) and 2 bacteremia cases (*8,10*). The infections were associated with AIDS, metastatic carcinoma, or substance abuse; 2 patients were >60 years of age. Thus, as for *L. monocytogenes* (*1*), human *L. ivanovii* infection is associated with immunodeficiency, underlying debilitating conditions, or advanced age. In at least 3 other instances, bacteria were found in human samples, 2 in fetoplacental tissue and lochia and 1 in a mesenteric lymph node (*4,5*) (Table). The pathologic changes associated with *L. ivanovii* in humans appear similar to those in ruminants, i.e., fetoplacental infections and septicemia (often accompanied by enteritis). Typically, meningoencephalitis is not caused by *L. ivanovii* in ruminants, whereas it is a hallmark of *L. monocytogenes* infection in ruminants and humans (*1*). Lack of central nervous system involvement could be a general characteristic of *L. ivanovii* infection regardless of host species. The specific pathogenic features of *L. ivanovii* may be caused by sequence differences in virulence genes shared with *L. monocytogenes* or by differences in the gene content of these 2 species (*1*,*6*).

These human cases raise questions about the supposed specificity of *L. ivanovii* for ruminants. Although the rare occurrence of *L. ivanovii* infections in humans (*3*) could result from lower pathogenicity for humans, it may reflect ecologic characteristics of the species. *L. ivanovii* is isolated only occasionally from animals or environmental sources (*2*,*4*,*5*), suggesting a limited distribution in nature, including in food. Therefore, the few human cases of *L. ivanovii* infection reported might correspond to what would be proportionally expected for a species with such sporadic occurrence.

That gastroenteritis preceded bacteremia and that the same isolates were found in the feces strongly indicate a foodborne infection in the patient reported here and that *L. ivanovii* causes gastroenteritis in humans, as reported for *L. monocytogenes* (*14*). Days before onset of gastroenteritis, the patient had eaten artisanal goat cheese made from raw milk. Unfortunately, no cheese sample was available for bacteriologic investigation. Although the portal of entry of *L. ivanovii* has not been formally established, *L. ivanovii* infection in ruminants is associated with eating spoiled silage or hay, as happens with *L. monocytogenes*, suggesting foodborne origin. *L. ivanovii* has been isolated from food, including goat milk (*15*).

Simultaneous detection of *L. ivanovii* in the feces and blood of a human, together with previous association between *L. ivanovii* and human mesenteric adenitis (*5*), suggests that these bacteria can cross the intestinal barrier in humans, cause gastroenteritis, and disseminate into the bloodstream. Although *L. monocytogenes* are by far the leading cause of human listeriosis, our report shows that

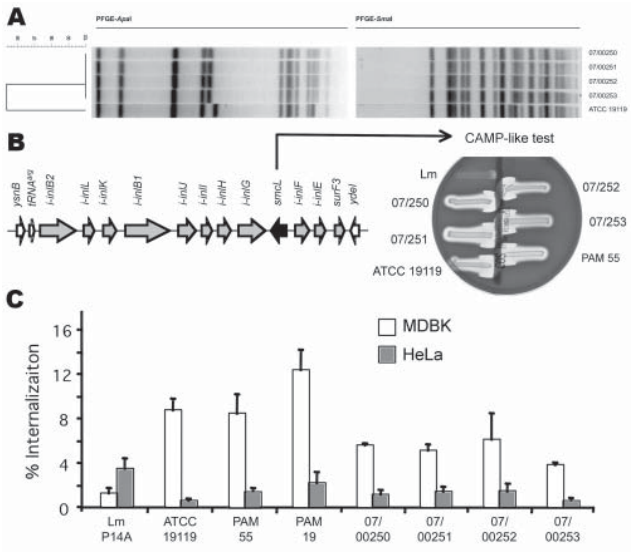

Figure. Characterization of the *Listeria ivanovii* subsp. *ivanovii* isolates from a 55-year-old man with gastroenteritis and bacteremia. A) The 4 isolates, 07/00250, 07/00251, and 07/00252 from blood, and 07/00253 from feces, were analyzed by pulsed-field gel electrophoresis (PFGE) with *Apa*I and *Sma*I restriction enzymes (*9*). The *L. ivanovii* subsp. *ivanovii* type strain American Type Culture Collection (ATCC) 19119 was used as control. Profiles were compared according to band positions by using the Dice coefficient and were clustered by using unweighted pair–group method averages. Criterion of dissimilarity = 1 band difference (maximum position tolerance 1.5%). B) *L. ivanovii*–specific virulence locus LIPI-2 and its phenotypic marker (sphingomyelinase production as shown by a CAMP-like test with an indicator strain of *Rhodococcus equi* on sheep blood agar). Left, genetic structure of LIPI-2. Arrowheads indicate positions of the oligonucleotide primers used in the 19 intragenic and intergenic PCRs to map the locus in the isolates; arrows represent genes (those belonging to LIPI-2 are gray, the sphingomyelinase gene is black, and flanking genes from the core listerial genome are white) (*10*). Right, typical shovel-shaped synergistic hemolysis reactions caused by *L. ivanovii* sphingomyelinase in the presence of *R. equi* cholesterol oxidase compared with the negative reaction given by *L. monocytogenes* (Lm). C) Invasion (gentamicin protection) assays in bovine (Madin-Darby bovine kidney) and human (HeLa) epithelial cells. The human isolates were compared with ruminant isolates ATCC 19119, PAM 55, and PAM 19 and with the *L. monocytogenes* strain P14A. Error bars indicate SEM of at least 2 duplicate experiments.

L. ivanovii can also cause bacteremia in immunocompromised, debilitated patients.

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Dr Guillet is a clinical microbiologist in the Necker Hospital, Paris Descartes University, in Paris. She has particular interests in bacterial infections in immunodeficient patients and in pediatric infectious diseases.

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Address for correspondance: Marc Lecuit, Centre d'Infectiologie Necker-Pasteur, Service des Maladies Infectieuses et Tropicales, Hôpital Necker–Enfants Malades, 149 rue de Sèvres, 75015 Paris, France; email: marc.lecuit@pasteur.fr

Acute Encephalopathy Associated with Influenza A Infection in Adults

Nelson Lee, Chun Kwok Wong, Paul K.S. Chan, Niklas Lindegardh, Nicholas J. White, Frederick G. Hayden, Edward H.C. Wong, Ka Shing Wong, Clive S. Cockram, Joseph J.Y. Sung, and David S.C. Hui

We report acute encephalopathy associated with influenza A infection in 3 adults. We detected high cerebrospinal fluid (CSF) and plasma concentrations of CXCL8/IL-8 and CCL2/MCP-1 (CSF/plasma ratios >3), and interleukin-6, CXCL10/IP-10, but no evidence of viral neuroinvasion. Patients recovered without sequelae. Hyperactivated cytokine response may play a role in pathogenesis.

Influenza-associated acute encephalopathy has been described in children, and results in a high frequency of neurologic sequelae and death. Altered consciousness, disorientation, and seizures occur within a few days after the onset of fever and respiratory symptoms (*1–3*). In some patients, symptoms are transient but in others rapid progression to necrotizing encephalitis, deep coma, and death may occur (*1–3*). Cases in adults are infrequently reported and remain poorly characterized, although the more complex clinical scenarios in adults may have hindered case recognition (*1,4–6*). The pathogenesis is unclear, but a hyperactivated cytokine response, rather than viral invasion, is believed responsible in most childhood cases (*1–5*). We describe 3 cases of acute encephalopathy associated with influenza A infection in adults. The clinical, virologic, immunologic findings (cytokines in plasma and cerebrospinal fluid [CSF]), and CSF penetration of oseltamivir for these cases are reported.

Author affiliations: The Chinese University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China (N. Lee, C.K. Wong, P.K.S. Chan, E. Wong, K.S. Wong, C.S. Cockram, J.J.Y. Sung, D.S.C. Hui); Mahidol University, Bangkok, Thailand (N. Lindegardh, N.J. White); University of Oxford, Oxford, UK (N. Lindegardh, N.J. White); and University of Virginia School of Medicine, Charlottesville, Virginia, USA (F.G. Hayden)

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The Study

At Prince of Wales Hospital, Hong Kong (*7*), from January 2007 through August 2008, influenza infection was diagnosed for >460 hospitalized adult patients for whom acute febrile respiratory illnesses had been diagnosed. Nasopharyngeal aspiration and immunofluorescence assays (IFA) were used for rapid diagnosis of influenza A and B infection, confirmed by virus isolation. Thirteen (2.8%) patients had signs of confusion or altered consciousness, together with fever and respiratory symptoms (mean ± SD age 77.7 ± 8.8 years). We studied 3 patients from whom CSF was obtained for analysis, and who fulfilled the definition of influenza-associated acute encephalopathy (altered mental status >24 hours within 5 days of influenza onset and without alternative explanation) (*1,2,4–6*).

Nasopharyngeal aspirates were subjected to IFA, virus isolation, and subsequent subtyping (*7*). CSF specimens were subjected to virus isolation using MDCK cells, and reverse transcription–PCR to detect influenza virus RNA by using H1/H3 subtype-specific primers. Herpes simplex virus, herpes zoster virus, and enterovirus DNA/RNA was detected using PCRs (online Technical Appendix, available from www.cdc.gov/EID/content/16/1/139-Techapp.pdf).

CSF and plasma samples collected on the same day were analyzed simultaneously for the concentrations of 11 cytokines/chemokines by bead-based multiplex flow cytometry. Their assay methods and plasma reference ranges (established from >100 healthy persons) have been described (online Technical Appendix) (*7*). In CSF, in patients without central nervous system (CNS) disease/infection, cytokines/chemokines are either undetectable (e.g., interleukin-6 [IL-6], CXCL8/IL-8, CXCL10/IP-10, CXCL9/MIG) or present at low levels (e.g., CCL2/MCP-1) (*8–10*).

Concentrations of oseltamivir phosphate (OP) and its biologically active metabolite oseltamivir carboxylate (OC) were measured in CSF and plasma taken simultaneously from 1 patient who received concurrent treatment, using tandem mass spectrometry (*11*). The assay methods have been described (online Technical Appendix).

The clinical and virologic findings are summarized in Table 1. All case-patients were elderly (72–86 years of age), but none were known to have neuropsychiatric illness, dementia, or to be taking psychotropic medication. None had received updated influenza vaccination (*6*). Confusion and altered consciousness developed in patients 1 and 2 one to 2 days after the onset of fever and cough. These patients had no meningismus, focal neurologic deficit, hypotension, respiratory distress, or metabolic disturbances. Brain computed tomography (CT) scans showed no acute cerebral lesion. CSF analyses showed no bacterial or viral pathogen or pleocytosis. Oseltamivir was given to patient 2 only when influenza A was later confirmed

Table 1. Clinical and laboratory findings in 3 patients with acute encephalopathy associated with influenza infection, Prince of Wales Hospital, Hong Kong*

Clinical and laboratory findings	Patient 1	Patient 2	Patient 3
Age, y/sex	76/M	86/F	72/M
Concurrent illnesses	Ischemic heart disease	Diabetes mellitus, hypertension	COPD
Influenza vaccination within 6 mo	None	None	None
Symptoms on examination	Fever >38°C, cough, disorientation, incoherent speech, mental dullness	Fever >38°C, cough, delirious, impaired consciousness, did not follow verbal command	Fever >38°C, cough, disorientation, agitation, incoherent speech, involuntary 4-limb tremor
Focal neurologic sign or meningism	Absent	Absent	Absent
Chest radiograph, consolidation	Absent	Absent	Absent
Antiviral (oseltamvir)	None	Given	Given
Outcome (duration of encephalopathy)	Recovered (2–3 d)	Recovered (3–4 d)	Recovered (6–7 d)
Brain CT scan (noncontrast)	Normal	Old ischemic changes; known small, calcified meningioma	Normal
Virus isolated from NPA	Seasonal (H1N1) 2008	Subtype H3N2	Subtype H3N2
CSF testing results			
Opening pressure, cm H_2O	11	9	14
Cell count (x 10^6/L)	1	–	0
Glucose, mmol/L	4.2	7.4	3.7
Protein, g/L	0.46	0.47	0.16
Virus isolated	None	None	None
RT-PCR for H3 and H1 influenza virus	Negative	Negative	Negative
Bacterial culture	Negative	Negative	Negative
Others	HSV, HZV, and enterovirus PCR negative	HSV, HZV, and enterovirus PCR negative	HSV PCR negative

*COPD, chronic obstructive pulmonary disease; CT, computed tomographic scan; NPA, nasopharyngeal aspirate; CSF, cerebrospinal fluid; RT-PCR, reverse transcription–PCR; HSV, herpes simples virus; HZV, herpes zoster virus. In all cases, there was no hypoglycemia, and liver and renal function test results were normal. C-reactive protein level was elevated in all cases. For patient 3, an electroencephalogram was performed and showed generalized slowing of background consistent with moderate encephalopathic change (similar to that observed in septic encephalopathy) (*1,6*). Findings are consistent with previous reports on adult cases of influenza-associated encephalopathy: patients are all unvaccinated, pleocytosis and cerebral imaging abnormalities (even with magnetic resonance imaging) are usually absent, and symptoms are generally self-limiting (*1,6*). Most reports have mentioned influenza A as a cause of encephalopathy, and more commonly subtype H3N2 (*1–6*).

by nasopharnygeal aspirate/IFA; patient 1 did not receive antiviral treatment. Both patients recovered in the next 2 days. Patient 3 had fever, severe chronic obstructive pulmonary disease exacerbation requiring noninvasive ventilatory support, complicated by acute coronary syndrome. He was given oseltamivir, 75 mg 2×/day, after influenza A infection was confirmed. Agitation and confusion developed in the patient on day 3–4 of illness (onset after the third dose of oseltamivir), despite resolution of the patient's respiratory failure. These symptoms were followed by involuntary, tremulous movements involving all 4 limbs, while at rest and during movement. Brain CT scan was normal. Electroencephalogram showed generalized slowing. Oseltamivir was stopped after the ninth dose, but tremor persisted. CSF analyses showed no pathogen or pleocytosis. The patient's symptoms resolved in the next 3–4 days without sequelae.

Despite apparently normal CSF findings, high concentrations of cytokines/chemokines were detected in the CSF and plasma specimens of all patients (Table 2). Plasma concentrations of IL-6, CXCL8/IL-8, CXCL10/IP-10, CCL2/MCP-1, and CXCL9/MIG were elevated at median values of 2.0, 2.8, 11.9, 3.7, and 2.1× the upper limits of their respective reference ranges (comparable to or higher than that observed in other hospitalized influenza patients) (Table 2) (*7*). Other cytokines were not elevated (*4,7*). In their CSF, IL-6, CXCL8/IL-8, CXCL10/IP-10, and CCL2/MCP-1 were consistently detected, and were elevated at median values of 2.6, 15.0, 3.4, and 20.0 × the upper limits of their respective plasma reference ranges. The CSF/plasma concentration ratios of CXCL8/IL-8 and CCL2/MCP-1 were >3 (median CSF/plasma ratio 5.4 and 8.0, respectively).

Simultaneous CSF and plasma OC and OP concentrations were determined for patient 3, as symptoms progressed at 18 h after oseltamivir. The concentrations (mean ± SD) of OC in duplicate CSF and plasma samples were 18.3 ± 0.9 ng/mL and 143.8 ± 3.3 ng/mL, respectively; the CSF/plasma concentration ratio was 12%–13%. The OP plasma concentration was 1.05 ± 0.03 ng/mL; it was not detectable in the CSF.

Conclusions

We report 3 adults with acute encephalopathy (altered consciousness, confusion) associated with influenza. High CSF and blood cytokine/chemokine (CXCL8/IL-8, CCL2/MCP-1, IL-6, CXCL10/IP-10) levels were detected. No ev-

Table 2. Cytokine and chemokine concentrations in CSF and plasma samples from 3 patients with acute encephalopathy associated with influenza A infection, Prince of Wales Hospital, Hong Kong*

Cytokine or chemokine	Reference range, pg/mL	CSF/plasma cytokine concentration, pg/mL (ratio)		
		Patient 1	Patient 2	Patient 3
IL-6†	<3.1	8.0/6.3 (1.3)	11.6/35.1 (0.3)	2.2/5.9 (0.4)
CXCL8/IL-8‡	<5.0	84.0/15.5 (5.4)	74.8/13.8 (5.4)	21.9/6.3 (3.5)
CXCL10/IP-10†	202–1,480	15,374/102,019 (0.2)	5,101/17,594 (0.3)	1,371/1,550 (0.9)
CCL2/MCP-1‡	< 10-57	996/82 (12.1)	1,287/336 (3.8)	–
CXCL9/MIG	48–482	11,58/14,001 (0.1)	70/333 (0.2)	145/1,019 (0.1)
IFN-γ	<15.6	UD/14.4	4.7/10.1	0.4/2.0
IL-12p70	<7.8	1.5/UD	1.3/UD	UD/UD
TNF-α	<10.0	1.7/1.4	UD/1.2	UD/UD
IL-10	<7.8	2.5/2.2	UD/7.3	UD/1.7
IL-1β	<3.9	UD/UD	UD/3.7	UD/UD
CCL5/RANTES	4,382–18,783	4/2,507	14/1,609	1.3/814

*CSF, cerebrospinal fluid; –, test not done due to inadequate sample; UD, undetectable (i.e., below the detection limit of the cytokine/chemokine assay). Cytokines: Interleukin (IL)–1β, IL-6, IL-10, IL-12p70, tumor necrosis factor α (TNF-α). Chemokines: CXCL8/IL-8, monokine induced by interferon-γ (IFN-γ) (CXCL9/MIG), IFN-γ–inducible protein-10 (CXCL10/IP-10), monocyte chemoattractant protein–1 (CCL2/MCP-1), and regulated upon activation normal T cell–expressed and secreted (CCL5/RANTES). The plasma reference ranges are established from >100 healthy adults. The assay sensitivities of IL-1β, IL-6, IL-10, IL-12p70, TNF-α, IL8, MIG, IP-10, MCP-1, RANTES, and IFN-γ are 2.5, 3.3, 3.7, 1.9, 7.2, 0.2, 2.5, 2.8, 2.7, 1.0, and 7.1 pg/mL, respectively. Coefficients of variation are all <10%. In an earlier study involving 39 adult influenza patients hospitalized with cardio-respiratory complications (*7*), the median (interquartile range) plasma concentrations of IL-6, IL-8, IP-10, MCP-1, and MIG were 10.6 (4.2–18.4), 5.4 (2.5–8.7), 7,043.0 (4,025.1–1,2381.1), 76.5 (49.5-97.0), and 992.1 (499.1–1,992.3) pg/mL, respectively. In CSF, in subjects without neurologic disease/infection, these cytokines/chemokines are either undetectable or present at low levels (*8–10*). In a pediatrics influenza cohort, CSF cytokine levels were substantially higher in encephalopathy cases when compared to those with febrile seizure; CSF/plasma concentration was <1 (*8*).
†CSF cytokine concentrations above plasma reference ranges.
‡CSF/plasma cytokine concentration ratio consistently >3 (3.5–12.1), in addition to CSF cytokine concentrations being above the plasma reference ranges. For IFN-γ, IL-12p70, TNF-α, IL-10, IL-1β and RANTES, because of their low/undetectable levels, the CSF/plasma ratios were not calculated. CSF specimens from patients 1 and 2 were collected at the peak of symptoms, and before antiviral treatment (if given); CSF from patient 3 was collected when persistent tremor developed 18 hours after the ninth dose of oseltamivir; the drug was stopped afterward.

idence of direct viral neuroinvasion was found. All patients recovered rapidly without sequelae (*1,6*).

Our findings agree with studies of influenza-associated encephalopathy in children. Influenza virus is rarely detected in the CSF, and pleocytosis is often absent (*1,2,4–6*). High levels of cytokines (e.g., IL-6, soluble tumor necrosis factor receptor 1) can be consistently found in CSF/blood specimens, correlating with disease severity and outcomes (hyperactivated cytokine response is absent in febrile seizure associated with influenza) (*2–4,8*). We found a broader range of cytokines/chemokines being activated (*7*); for certain cytokines (CXCL8/IL-8, CCL2/MCP-1), the CSF concentrations were 3× those in plasma. IL-6, CXCL8/IL-8, CCL2/MCP-1 and CXCL10/IP-10 have been shown to play pathogenic roles in CNS viral infections, cerebral injury, and acute brain syndrome in susceptible patients (*7,9,10,12*). The high CSF/plasma ratios suggest that for some cytokines, activation within the CNS might have occurred along with respiratory-tract and systemic productions (cytokines are not detected in CSF normally; (Table 2) (*4,7–10,12*). Resident macrophages/monocytes, astrocytes, microglial and endothelial cells in the CNS are shown to release cytokines/chemokines when stimulated by viral/influenza infection; activation mechanisms without involving overt CNS invasion have been suggested (*1,4,9,12–14*). Cytokines may cause direct neurotoxic effects, cerebral metabolism changes, or breakdown of the blood-brain-barrier (endothelial injury) to produce symptoms (*1–4,8,12–14*). Whether early viral suppression by antivirals can lead to attenuation of these cytokine responses and better outcomes warrants further study (*7*).

We measured oseltamivir concentrations because of the concerns over its neuropsychiatric side-effects in children and adolescents. However, only the active metabolite (OC) was detected in the CSF of patient 3; the CSF/plasma concentration ratio was 12%–13% (18.3/143.8 ng/mL) at 18-hours postdose. This degree of CSF penetration is similar to that observed among healthy patients, with a Cmax CSF/plasma concentration ratio of 3.5% (at ≈8 hours), and a ratio of ≈10% at 18 hours (concentration-time profiles for plasma/CSF differ). Assuming a similar ratio, the CSF OP concentration would have fallen below the assay's detection limit (0.25 ng/mL) by 18 hours (*11,15*). The low CSF drug-penetration, together with high cytokines in CSF and symptom progression despite drug withdrawal suggest that the manifestations of patient 3 may have been disease-related. Symptoms developed in patients 1 and 2 without antiviral exposure. Further investigations on the CNS effects of oseltamivir in the clinical setting are needed..

Our study is limited by the small patient number and the lack of feasibility in obtaining CSF for study/comparison in influenza patients without neurologic symptoms. Further studies on the clinical spectrum of influenza encephalopathy and encephalitis in adults (*1,6*) and their pathogenesis are indicated. In conclusion, acute encephalopathy may occur in adults with influenza. Exuberant

cytokine/chemokine response may play an important role in its pathogenesis.

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Dr Lee is an associate professor in infectious diseases at The Chinese University of Hong Kong. His primary research interest is severe emerging infections, including severe acute respiratory syndrome and influenza.

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Address for correspondence: Nelson Lee, Division of Infectious Diseases, Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, 9/F Clinical Sciences Building, Department of Medicine and Therapeutics, Shatin, Hong Kong, People's Republic of China; email: leelsn@cuhk.edu.hk

Enterotoxigenic *Escherichia coli* Multilocus Sequence Types in Guatemala and Mexico

Matilda Nicklasson, John Klena, Claudia Rodas, August Louis Bourgeois, Olga Torres, Ann-Mari Svennerholm, and Åsa Sjöling

The genetic backgrounds of 24 enterotoxigenic *Escherichia coli* (ETEC) strains from Mexico and Guatemala expressing heat-stable toxin (ST) and coli surface antigen 6 (CS6) were analyzed. US travelers to these countries and resident children in Guatemala were infected by ETEC strains of sequence type 398, expressing STp and carrying genetically identical CS6 sequences.

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of acute watery diarrhea among children and adults in the developing world, causing ≈400 million diarrheal episodes and 380,000 deaths in children <5 years of age every year (*1*). The diarrhea is mediated by 1 or 2 plasmid-encoded enterotoxins; the heat-stable toxin (ST) and/or the heat-labile toxin (LT) (*1*). Approximately one third of all ETEC strains isolated globally reportedly produce ST only, one third produce LT and ST, and one third produce LT only (*2*). Two genotypes of ST exist in ETEC strains infecting humans: STp and STh (*3*). Colonization of the small intestine is mediated by adhesion to the epithelial cells by colonization factors (CFs), and one of the most common CFs detected on clinical ETEC isolates from different parts of the world is the plasmid-encoded coli surface antigen 6 (CS6) (*4*).

In a recent vaccine trial conducted in Mexico and Guatemala involving adult US travelers, ST-only strains expressing only CS6 and no other CF predominated among those infected with CF-positive ETEC; this toxin-CF profile (ST/CS6) was present in 35% of diarrheal cases (*5*). In other geographic regions, e.g., Egypt and Bangladesh, studies on childhood diarrhea have reported ST/CS6 frequencies of 6.6% and 19%, respectively (*6*,*7*). We conducted this study to investigate whether adult travelers in Mexico and Guatemala are infected with certain circulating ST/CS6 ETEC strains and to determine whether ETEC strains with the same *E. coli* clonal background may be infecting resident children in the same areas.

Author affiliations: University of Gothenburg, Gothenburg, Sweden (M. Nicklasson, C. Rodas, A.-M. Svennerholm, Å. Sjöling); United States Naval Medical Research Unit 3, Cairo, Egypt (J. Klena); Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA (A.L. Bourgeois); and Instituto de Nutrición de Centro América y Panamá, Guatemala City, Guatemala (O. Torres)

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The Study

Seventeen clinical ST/CS6 isolates collected from adult US travelers, who were either visiting various locations in Guatemala or remaining at least 14 days in Antigua in Guatemala, or in Cuernavaca, Mexico, were included in the study. Isolates were collected from 1998 to 2001 during ETEC vaccine trials (*5*,*8*) or in 2002 and 2003 during a study of antimicrobial drug treatment (*9*). During June 2001–October 2003, clinical stool specimens were collected from children living in Santa María de Jesús in Guatemala as part of an ongoing childhood ETEC study (O. Torres, unpub. data). Seven CS6 isolates obtained from that study, collected in the summer of 2002, were included in the present study. Clinical isolates from both children and adults were shipped to Sweden, where toxin and CF profiles were confirmed as described (*10*). All strains in the study expressed STp, except for strain E874, which expressed STh.

Isolates were analyzed by multilocus sequence typing (MLST) by using the *E. coli* MLST scheme (http://mlst.ucc.ie/mlst/dbs/Ecoli), which is based on sequencing of internal regions of the 7 housekeeping genes *adk, fumC, gyrB, icd, mdh, purA,* and *recA* (*11*). PCR was performed as described but by using the same annealing temperature (54°C) for all genes.

Seven MLST sequence types were observed among the 24 ST/CS6 ETEC isolates (Table). The most common were MLST sequence type 398 (ST-398) (n = 10 [all from Guatemala]), sequence type 182 (n = 6 [4 from Guatemala, 2 from Mexico]), and sequence type 278 (n = 4 [2 each from Guatemala and Mexico]). Three novel MLST sequence types (all from Guatemala) were identified and, upon submission to the *E. coli* MLST database, were designated as MLST sequence types 712, 726, and 727. Sequence type 726 clustered closely with the sequence type 182 isolates (Figure); these 2 sequence types are single locus variants, differing only in *mdh* (mdh-6 v mdh-1). Sequence type 727 is a single locus variant of sequence type 278, differing only in *gyrB* (gyrB-1 v gyrB-33). Sequence type 712 is a single locus variant of sequence type 398, differing only in *fumC* (fumC-23 v fumC-7).

The single STh/CS6 strain in the study, E874 from a child, was the only representative for MLST sequence type 443. This strain did not cluster closely with any of the other

Table. Comparison of ETEC isolates and clinical and demographic information collected during study of US travelers to Guatemala and Mexico and resident children from Guatemala*

Isolate†	Toxin/CF profile	Geographic origin	Date of collection	Severity of diarrhea‡	Age/sex	MLST sequence type
Travelers						
E617	STp/CS6	Antigua, Guatemala	2000 Jun 19	Mild	24 y/M	182
E830	STp/CS6	Traveling in Guatemala	2002 Sep 23	Moderate–severe	30 y/M	182
E539	STp/CS6	Cuernavaca, Mexico	2000 Jul 13	Moderate–severe	36 y/F	182
E576	STp/CS6	Cuernavaca, Mexico	2000 Jul 15	Moderate–severe	50 y/M	182
E494	STp/CS6	Antigua, Guatemala	1999 Jul 3	Moderate–severe	37 y/F	182
E695	STp/CS6	Antigua, Guatemala	2001 Jan 15	Moderate–severe	44 y/M	182
E396	STp/CS6	Antigua, Guatemala	1998 Jul 30	Moderate–severe	49 y/M	726
E848	STp/CS6	Traveling in Guatemala	2003 Apr 28	Moderate–severe	27 y/M	727
E368	STp/CS6	Cuernavaca, Mexico	1999 Aug 11	Asymptomatic§	30 y/F	278
E416	STp/CS6	Antigua, Guatemala	1999 May 30	Moderate–severe	25 y/M	278
E521	STp/CS6	Cuernavaca, Mexico	2001 Apr 8	Moderate–severe	51 y/F	278
E837	STp/CS6	Traveling in Guatemala	2002 Sep 23	Moderate-severe	30 y/M	278
E844¶	STp/CS6	Traveling in Guatemala	2003 Apr 28	Moderate–severe	29 y/F	712
E382	STp/CS6	Antigua, Guatemala	1998 Jul 1	Moderate–severe	21 y/F	398
E447	STp/CS6	Antigua, Guatemala	1999 Jun 21	Moderate–severe	46 y/F	398
E670	STp/CS6	Antigua, Guatemala	2000 Jul 17	Moderate–severe	27 y/F	398
E850¶	STp/CS6	Traveling in Guatemala	2003 May 8	Asymptomatic	29 y/F	398
Children						
E856	STp/CS6	SMJ, Guatemala	2002 Jun 21	Persistent moderate–severe	10 mo/M	398
E861	STp/CS6	SMJ, Guatemala	2002 Jul 4	Persistent moderate–severe	34 mo/M	398
E870	STp/CS6	SMJ, Guatemala	2002 Jul 26	Moderate	11 mo/M	398
E871	STp/CS6	SMJ, Guatemala	2002 Aug 1	Moderate	22 mo/F	398
E872	STp/CS6	SMJ, Guatemala	2002 Aug 7	Moderate	12 mo/F	398
E879	STp/CS6	SMJ, Guatemala	2002 Aug 23	Moderate	26 mo/F	398
E874	STh/CS6	SMJ, Guatemala	2002 Aug 19	Moderate	18 mo/F	443

*ETEC, enterotoxigenic *Escherichia coli*; CF, colonization factor; MLST, multilocus sequence typing; SMJ, Santa María de Jesús.
†World Health Organization Collaborating Centre for Research on Enterotoxigenic *Escherichia coli* strain collection number.
‡Classification of diarrheal disease severity in travelers was based on the study case definition of travelers' diarrhea with an associated gastrointestinal symptom rated as mild, moderate, or severe (*5*). Moderate symptoms interfered with daily activity, and severe symptoms prevented normal daily activity. Disease classification in children was based on the number of diarrheal stools in a 24-hour period (3/24 h, mild; 4–5/24 h, moderate; >6/24 h, severe). Diarrhea accompanied by vomiting was classified as severe; diarrhea lasting >14 days was classified as persistent.
§Not travelers' diarrhea.
¶Strains E844 and E850 were isolated from the same person participating in the antimicrobial drug treatment study. Strain E850 was isolated after completion of a course of antimicrobial drugs.

isolates by MLST (Figure). The 6 remaining isolates from the childhood study (all STp/CS6) clustered into sequence type 398. Three isolates from adult travelers collected in Antigua in Guatemala during 1998, 1999, and 2000 (E382, E447, and E670) and 1 collected from a traveler in Guatemala in 2003 (E850) also clustered into sequence type 398. The remaining adult travelers were infected by ETEC strains that clustered mainly into sequence types 182 (n = 6) and 278 (n = 4) (Table; Figure); these sequence types were found in Mexico and Guatemala during 1999–2002. Two of the strains, E830 (sequence type 182) and E837 (sequence type 278), were isolated from the same person on the same day, highlighting the possibility for several pathogens to simultaneously cause diarrhea.

A 540-bp internal region of the CS6 operon (*cssABCD*) encompassing the distal part of *cssB*, an intergenic untranslated region, and the proximal part of *cssC*, contains sequence differences in LT-only strains (*14*). Sequencing of the same region (performed as described [*14*]) in the 24 ST-only isolates in this study showed that all STp/CS6 isolates had identical CS6 sequences to the STp/CS6 ETEC strain GB124 (GenBank accession no. DQ538390, nt 815–1354) (*14*), isolated in Guinea Bissau. On the other hand, the only STh/CS6 isolate in the study, E874, had a different CS6 sequence with 7 nonsynonymous and 4 synonymous nucleotide substitutions compared with the other strains. The CS6 sequence of E874 was most similar to the CS6 sequence of the STh+LT–positive strain E10703 (GenBank accession no. U4844) (*15*), with only 2 adjacent nucleotide differences, resulting in a Q to R substitution at nt 139 of *cssC* in strain E874.

Conclusions

US travelers to Mexico and Guatemala were infected by strains of 6 different MLST sequence types, showing that the high prevalence of ST/CS6 among travelers in this region is not due to infection by a single circulating ST/CS6 ETEC strain. Results also showed that adult travelers and

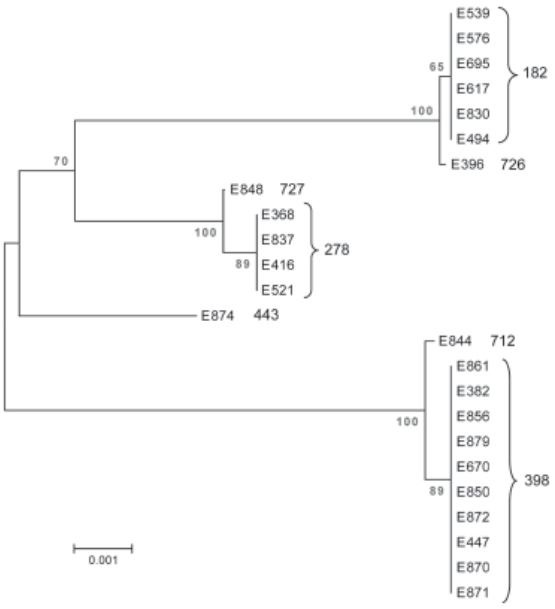

Figure. Dendrogram of the 24 enterotoxigenic *Escherichia coli* strains from Guatemala and Mexico included in the study, showing multilocus sequence types. Sequences were assembled with BioEdit and aligned using ClustalX within BioEdit (*12*). The dendogram represents the relationship of a concatenation of the sequences from each strain and was constructed by using MEGA 3.1 (*13*). Phylogenetic reconstructions were created by using the neighbor-joining method with the Kimura 2-parameter substitution model, using 1,000 bootstrap replicates. A similar arrangement of the strains was indicated by eBURST version 2 analysis (http://eburst.mlst.net). Scale bar indicates dissimilarity, where 0 is completely identical and 1 is completely dissimilar.

resident children may be infected by ETEC strains with the same *E. coli* genetic background because ST/CS6 strains of MLST sequence type 398 infected both adult travelers and resident children in Guatemala. This MLST sequence type circulated in Guatemala for at least 6 years, indicating the presence of persistent MLST sequence types.

All STp/CS6 strains in the study had identical sequences in a region of the CS6 operon previously found to vary greatly in LT-only strains (*14*), even though the STp/CS6 strains represented 6 different MLST sequence types and were collected over 6 years and at different sampling sites in Guatemala and Mexico. This sequence was identical to the corresponding CS6 sequence in an STp/CS6 strain from Guinea-Bissau (*14*) but differed from the available sequences of ST/LT and LT-only CS6 strains (GenBank accession nos. UO4844, UO4846). This finding may indicate that plasmids carrying the genes for STp and CS6 are genetically conserved and have spread across the world because identical sequences were found in northern Latin America and in West Africa. Further studies have therefore been initiated to explore the genetics and epidemiology of ETEC strains expressing CS6 and STp in a global perspective.

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Dr Nicklasson is a molecular microbiologist working at the World Health Organization Collaborating Centre for Research on Enterotoxigenic *Escherichia coli* (ETEC), which is located at the Institute of Biomedicine, Department of Microbiology and Immunology, University of Gothenburg. She recently held a research position at the Naval Medical Research Unit 3 in Cairo. Her main research interests are virulence gene expression and transmission pathways of ETEC.

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Address for correspondence: Matilda Nicklasson, Institute of Biomedicine, Department of Microbiology and Immunology, University of Gothenburg, Box 435, 405 30 Göteborg, Sweden; email: matilda.nicklasson@microbio.gu.se

Laboratory Surge Capacity and Pandemic Influenza

Martin I. Meltzer, K. Mills McNeill, and Joseph D. Miller

In this issue, Crawford et al. describe their experiences running a clinical diagnostic laboratory during the first 3 weeks of the influenza A pandemic (H1N1) 2009 outbreak (*1*). During the early weeks of the outbreak, their laboratory, which serves 15 hospitals and affiliated physician practices in the greater New York City metropolitan area, experienced an ≈8× increase in respiratory virus testing, reaching a maximum of about 900 samples processed in 1 day.

As part of their outbreak response, the laboratory increased weekly work hours by ≈60% and doubled weekend work hours. Physical laboratory space was also rapidly expanded. Equally important to the response plan were 2 decisions to alter testing protocols: cultures were screened 1 time rather than 3, and the use of the Luminex xTAG Respiratory Virus Panel assay (Luminex Molecular Diagnostics, Toronto, Ontario, Canada) was prioritized for testing specimens from hospitalized patients.

The missions of clinical laboratories and public health laboratories (PHL) differ markedly. Clinical laboratories have the primary (almost sole) responsibility of testing samples to aid clinical decision-making. Although PHLs also test samples to aid clinical decisions, functions like surveillance, strain identification, and tracking of drug resistance are arguably their main priorities. Clinical laboratories often have resources available that allow for rapid expansion, but PHLs typically work on fixed budgets that have little flexibility despite unpredictable changes in demand for services.

Author affiliations: Associate Editor, Emerging Infectious Diseases journal (M.I. Meltzer, K.M. McNeill); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (M.I. Meltzer, J.D. Miller); and Centers for Disease Control and Prevention, Kampala, Uganda (K.M. McNeill)

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In their article, Crawford et al. (*1*) discuss many lessons they learned that have universal application for all laboratories engaged in influenza surge response planning. First and foremost was that they had an established plan to deal with such an emergency. Equally important, the laboratory leadership understood the plan and how to adapt it to the specific situation at hand. The leadership also was willing to prioritize testing and triage the flow of samples. The laboratory's ability to adapt rapidly was limited most notably by the number of suitably trained and experienced staff who could be brought in to provide surge capacity assistance. To be useful, emergency plans must be more than mere documents; they must be rooted in an adequate assessment of capacity and a realistic understanding of the degree to which capacity can be increased rapidly.

The Centers for Disease Control and Prevention has developed a software tool called FluLabSurge (http://www.cdc.gov/flu/tools/flulabsurge), which is designed to assist laboratory directors in planning for a surge in demand for testing. Each laboratory has unique operating characteristics. However, by using FluLabSurge, we determined that the availability of suitably trained laboratory staff is probably the factor that most affects the ability of PHLs to rapidly expand capacity. Thus, public health officials must quickly impose appropriate triage systems at the beginning of public health events, such as an influenza pandemic, to ensure that existing PHL capacity is used effectively and wisely.

Perhaps the most important lessons in the article by Crawford et al. are 1) the need to continually communicate to all clients and stakeholders the need for triaging the flow of clinical samples and 2) the need to explain how testing priorities may change over the course of a pandemic. Such enhanced communication, which clearly explains the limitations of existing laboratory capacity, may help build a constituency that will aid future expansions of PHL capacity.

Dr Meltzer is a Senior Health Economist and a Distinguished Consultant in the Division of Emerging Infections and Surveillance Services at CDC. His research interests include the modeling of potential responses to smallpox as a bioterrorist weapon, examining the economics of vaccinating restaurant food handlers against hepatitis A, and assessing the response to pandemic influenza.

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Address for correspondence: Martin I. Meltzer, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D59, Atlanta, GA 30333, USA; email: mmeltzer@cdc.gov

Fatal Case of Pneumonia Associated with Pandemic (H1N1) 2009 in HIV-Positive Patient

To the Editor: Pandemic (H1N1) 2009 virus first appeared in March 2009 in Mexico. In June 2009, a pandemic was declared by the World Health Organization (*1*). Influenza A virus (H1N1) caused a pandemic in 1918–1919; estimated deaths were ≈100 million worldwide (*2*). Symptoms of pandemic (H1N1) 2009 are similar to those of seasonal influenza (fever, cough, sore throat, body aches, headache, chills, and fatigue) (*3*). Pandemic (H1N1) 2009 should be considered in the differential diagnosis of patients with acute febrile respiratory illness who have been in contact with persons with confirmed influenza or reside in areas where influenza has been reported (*2*).

Although most cases of pandemic (H1N1) 2009 in the United States have been mild, 2%–5% of infected persons have required hospitalization (*2*). Immunosuppressed persons, the elderly, persons with underlying lung or cardiac disease, pregnant women, persons with diabetes, obese persons, and children <5 years of age are at increased risk for this disease (*4*).

We report pneumonia associated with pandemic (H1N1) 2009, which resulted in respiratory and renal failure and death, in a 39-year-old HIV-positive woman. She had type 1 diabetes and a diagnosis of AIDS 7 years ago and had received highly active antiretroviral therapy. She also had an ill child at home with an influenza-like illness.

Her medical history included pleuropericardial *Nocardia* spp. infection, recurrent pleural effusions requiring thoracentesis, and hepatomegaly of unknown cause. Her most recent CD4 cell count was 166 cells/μL with undetectable viral load 1 month before admission. Medications prescribed included combivir, efavirenz, and trimethoprim/sulfamethoxazole but she was noncompliant. She had received the 2008–09 seasonal influenza vaccine and pneumococcal vaccine.

The patient was admitted to Winthrop-University Hospital (Mineola, NY, USA) on June 5, 2009, for community-acquired pneumonia. She received empiric moxifloxacin and atovaquone. Because of concern for persistent *Nocardia* spp. infection, she was also treated with doxycycline. The result of a rapid influenza test (QuickVue; Quidel, San Diego, CA, USA) was negative for a nasal swab specimen on day 1 of hospitalization. Over the next 48 hours, her clinical status deteriorated, and she experienced worsening hypotension and respiratory distress.

On admission, she had fever (101°F) for 3 days, pulse rate of 109 beats/min, blood pressure of 86/52 mm Hg, respiratory rate of 22 breaths/min (oxygen saturation of 88% on room air), generalized weakness, nonproductive cough, and increasing shortness of breath. She was alert and oriented. Physical examination showed decreased breath sounds at bases, hepatomegaly, and bilateral edema in the lower extremities. Laboratory tests showed 3,000 leukocytes/mm^3 (93% neutrophils, 2% bands, and 3% lymphocytes), hemoglobin level of 12.7 g/dL, and 118,000 platelets/mm^3. Other laboratory values were blood urea nitrogen 66 mg/dL, creatinine 2.9 mg/dL, creatinine phosphokinase 2,276 IU/L, and lactic acid 3.6 mmol/L (anion gap 13). A chest radiograph showed moderate pleural effusion in the right lung and retrocardiac air space disease. Test results for influenza virus, respiratory fluorescent antibodies (D3 Ultra DFA Respiratory Virus Screening and Infectious Disease Kit; Diagnostic Hybrids, Inc., Athens, OH, USA), and virus culture were negative.

The patient was transferred to the intensive care unit and required intubation, pressor support, and continuous venovenous hemofiltration for fluid removal. Empiric oseltamivir (150 mg 2×/d) was started on hospital day 3; moxifloxicin was discontinued, and meropenem was given for pneumonia (*5*). Thoracentesis showed transudative fluid negative for acid-fast bacilli, bacteria, and fungi.

Results of blood cultures and urine analysis for *Legionella* spp. antigen were negative. Repeat chest radiography showed a right-sided pneumothorax and worsening bilateral airspace disease. A chest tube was inserted in the right lung, and bronchoscopy was performed on hospital day 5. Results of bronchoalveolar lavage were negative for *Pneumocystis jiroveci*, virus inclusions, fungi, acid-fast bacilli, bacteria, and mycobacteria. However, clusters of filamentous organisms were seen. On hospital day 5, results of a second rapid influenza test, respiratory fluorescent antibody test, and nasopharyngeal virus culture were negative. Diagnosis was based on a positive result for pandemic (H1N1) 2009 by real-time reverse transcription–PCR (RT-PCR) for a nasopharyngeal swab specimen (New York State Department of Health). Despite empiric treatment with oseltamivir, the patient died on June 15, 2009 (day 11 of hospitalization).

Symptoms of pandemic (H1N1) 2009 in HIV-infected persons are not known. However, these persons have a higher risk for complications. In previous seasonal influenza outbreaks, HIV-infected persons had more severe infections and increased hospitalization and mortality rates (*6*).

Although a diagnosis of pandemic (H1N1) 2009 was first considered for our patient because of her ill child, she was not initially treated with oseltamivir because of the negative influenza test result and concern for opportunistic infections. Only the result of an RT-PCR for pandemic (H1N1) 2009

was positive. No other pathogens were detected in her blood, urine, sputum, bronchoalveolar lavage, or thoracenthesis fluid.

Empiric treatment in patients with pandemic (H1N1) 2009 should be considered in those seeking treatment for influenza-like symptoms, especially in the setting of sick contacts with respiratory illnesses. Rapid influenza tests, respiratory fluorescent antibody tests, and viral cultures may not provide a diagnosis. An RT-PCR for pandemic (H1N1) 2009 may be needed to provide a diagnosis.

Natalie C. Klein, Azfar Chak, Marilyn Chengot, Diane H. Johnson, and Burke A. Cunha

Author affiliations: Winthrop-University Hospital, Mineola, New York, USA; and State University of New York School of Medicine, Stony Brook, New York, USA

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Address for correspondence: Burke A. Cunha, Infectious Disease Division, Winthrop-University Hospital, Mineola, NY 11501, USA.

Human Herpesvirus 8 in Healthy Blood Donors, Argentina

To the Editor: Human herpesvirus 8 (HHV-8), or Kaposi sarcoma–associated herpesvirus, is associated with malignant disorders such as Kaposi sarcoma, primary effusion lymphoma, and multicentric Castleman disease. Although HHV-8 does not necessarily cause life-threatening infection in healthy persons, it causes more severe infection in those who are immunocompromised, such as organ recipients and HIV-infected persons.

HHV-8 has been found in a number of clinical specimens (blood, saliva, and semen) from persons with HHV-8 related diseases (*1*,*2*). Identification of infectious virus in lymphocytes from a healthy blood donor and evidence that HHV-8 might be transmitted by blood has raised concern about the safety of the blood supply (*3*,*4*). Few studies have detected viral DNA in blood samples of blood donors from areas with low HHV-8 prevalence (*5*–*7*). During January 2000 and December 2002, the Virology Department, National Institute of Infectious Diseases, Administración Nacional de Laboratorios e Institutos de Salud, "Dr C G. Malbrán" conducted an HHV-8 serosurvey of 6 blood banks from 5 South American regions and found overall seroprevalence to be 3.7% (range 1.9%–6.7%). The 6.7% seroprevalence from a blood bank in Buenos Aires city was substantially higher than that of other blood banks (*8*).

From July 2004 through January 2005, to look for the virus in blood and saliva, we conducted the study reported here, an HHV-8 survey at the same blood bank. A total of 577 volunteer blood donors (431 men and 146 women), mean age 39 years (range 17–76 years), were enrolled at the Hemotherapy Service, Hospital of Infectious Diseases "Francisco Javier Muñiz." The protocol was approved by the Teaching and Research Committee.

Serum and whole blood were collected from all 577 donors, and paired blood–saliva samples were obtained from 394. Serum samples were routinely tested for hepatitis B and C viruses, HIV, human T-lymphotropic viruses I and II, *Treponema pallidum, Brucella* spp., and *Trypanosoma cruzi*; results were used to determine associations between HHV-8 and these agents. Specimens were stored at –20°C until serologic and molecular investigation at the Virology Department, National Institute of Infectious Diseases.

Serologic screening for HHV-8 infection was performed by indirect immunofluorescence assay by using lytically induced cells; serum samples were diluted 1:40 (*8*). Then 45 blood and 39 paired blood–saliva samples from HHV-8-seroreactive donors were investigated for viral genome by open reading frame 26 nested PCR. DNA was purified from 0.3 mL of whole blood by using FlexiGene DNA Kit (QIAGEN, Gmbh, Hilden, Germany); concentrations and quality were measured with a UV spectrophotometer, and 1 μg was used for PCR. The QIAamp DNA Mini Kit (QIAGEN, Gmbh,) was used to obtain DNA from 0.2-mL saliva samples. Crude pellets were resuspended in 20μL of Tris EDTA, pH 8, then 5 μL were added to the PCR. Quality of DNA isolated from negative PCR samples was tested by amplifying the human housekeeping gene β-globin. In addition, inhibitors were investigated by add-

ing the minimum viral DNA amount detected by our nested PCR, previously assessed by 10-fold serial dilutions of DNA from body cavity–based lymphoma 1 cells. The results are expressed as percentages, 95% confidence intervals (CIs), and proportions (positive/total). When necessary, the associations between variables were tested by means of χ^2 or logistic regression. Significance was defined as $p<0.05$. Data were analyzed by using the Epidat 3.0 program, available from www.paho.org.

Positive immunofluorescence assay results were obtained for 45 (7.79%) of the 577 blood donors; seroprevalence was independent of gender ($p = 0.8$) and increased with age (odds ratio 1.04, 95% CI 1.01–1.07, $p = 0.028$). No association was found between HHV-8 and seroreactivity to the infectious agents tested ($p = 0.3438$). HHV-8 DNA was found in 3 seroreactive blood donors: 1 in saliva only and 2 in blood and saliva. Of the 45 HHV-8 seropositive samples, 38 were nonreactive to any infectious agents tested in the blood bank. One donor was seroreactive for hepatitis B.

In summary, we found HHV-8 in blood and saliva of blood donors even in an area where the virus is not endemic. Seroprevalence for HHV-8 was similar to that previously reported (*8*). Also, low viral loads might be undetectable by PCR but high enough to cause an infection with usual volumes of blood used in transfusions (*9*), especially when the hemoderivatives are given to immunocompromised recipients. This study was done in a blood bank from a hospital for infectious diseases in which the recipient population consisted of numerous HIV patients (*10*). It is a concern that these patients could have received blood infected with HHV-8. The fact that saliva samples were also positive is consistent with previously reported findings (*1*,*2*) and might indicate that the virus is active at a site from which samples are easier to obtain and in which the virus easier to detect than the bloodstream. This study provides further evidence that blood transfusion carries a potential risk for HHV-8 infection, even in areas where its prevalence is low.

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Celeste L. Pérez, Mónica I. Tous, Norma Zala, and Sofía Camino

Author affiliations: Administración Nacional de Laboratorios e Institutos de Salud, Buenos Aires, Argentina (C.L. Perez, M.I. Tous); and Hospital de Enfermedades Infecciosas "Francisco J Muñiz," Buenos Aires (N. Zala, S. Camino)

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Address for correspondence: Celeste L. Pérez, Tissue Culture Service, Virology Department, National Institute of Infectious Diseases, ANLIS "Dr. Carlos G Malbrán," Av Velez Sársfield 563-C1281 AFF, Buenos Aires, Argentina; email: cperez@anlis.gov.ar

Real-Time PCR for Diagnosis of Oculoglandular Tularemia

To the Editor: Oculoglandular tularemia accounts for 3%–5% of all diagnosed tularemia cases (*1*). We report the diagnosis of this disease in 2 patients in France by real-time PCR.

Patient A, a 43-year-old woman, was referred in October 2006 to the infectious disease department of Auch Hospital (Auch, France). She had a fever (39°C) and severe conjunctivitis of the right eye that had evolved over 2 weeks despite administration of amoxicillin/clavulanate. The patient lived in a rural area endemic for tularemia and had regular contact with dogs and ring doves. She remembered harvesting mushrooms in a nearby forest a few days before onset of clinical symptoms. Physical examination showed a hyperemic and painful right conjunctiva, enlarged (0.5–1.5 cm in diameter) and tender preauricular and submandibular lymph nodes, and cellulitis of the right hemiface. Her condition rapidly improved after she received doxycycline and gentamicin.

Patient B, a 42-year-old woman, was referred in October 2008 to the infectious disease department of Dijon University Hospital (Dijon, France) for intermittent fever (38.5°C) and swollen left-sided pretragal and cervical lymph nodes, which had evolved for 3 weeks despite administration of amoxicillin, followed by pristinamycin and prednisone, and ciprofloxacin for 7 days. The patient remembered being scratched on the left hand by her dog several weeks earlier; the scratch healed spontaneously. She had recently walked in a nearby forest that was endemic for tularemia. Physical examination showed enlarged (2–3 cm in diameter), tender lymph nodes and bilateral conjunctivitis. Her condition improved after doxycycline therapy, but the pretragal lymph nodes were removed surgically in late November 2008 because of suppuration and necrosis. Ofloxacin was administered until January 2009 because of persistence of inflammation in cervical lymph nodes and suppuration with skin fistulization in the pretragal region.

Diagnostic investigations (Table) conducted at Grenoble University Hospital included serologic tests (microagglutination and indirect immunofluorescent antibody assay by using locally prepared *Francisella tularensis* subsp. *holarctica* antigen), culture, and 2 real-time PCRs. These PCRs were specific for insertion sequence *ISFtu2* or the Tul4 protein–encoding gene of *Francisella* sp. and used previously described primers, probes, an amplification protocol (*2*), and a LightCycler 2.0 apparatus (Roche, Meylan, France). We tested 5 μL of DNA extracted from clinical samples by using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). Three negative controls (DNA-free water) and 1 positive control (DNA extracted from the *F. tularensis* subsp. *holarctica* LVS strain) were used for each PCR.

Seroconversion was found between acute-phase and convalescent-phase serum samples from both patients. A conjunctival cotton swab sample from patient A and pretragal lymph node suppuration and biopsy samples from patient B were positive for *F. tularensis* by both real-time PCRs. A *Francisella* sp. strain was isolated from the conjunctival discharge from patient A at Auch Hospital and Grenoble Hospital laboratories. Cultures were grown in a BioSafety Level 3 laboratory at Grenoble University Hospital because results of both PCRs were positive. Cultures of specimens from patient B were negative.

Table. Characteristics of the 2 patients in the study and test results for tularemia, France*

Characteristic	Patient A	Patient B
Age, y/sex	43/F	42/F
Blood leukocyte count at admission, cells/mm^3	21,600	4,000–10,000
Blood granulocyte count at admission, cells/mm^3	17,900	2,000–8,000
C-reactive protein level at admission, mg/L	51	39
Serologic test results for *Francisella tularensis*		
First serum sample, d	6	16
Microagglutination titer	<20	<20
Immunofluorescent IgM titer	<20	<20
Immunofluorescent IgG titer	<20	<20
Second serum sample, d	90	39
Microagglutination titer	80	160
Immunofluorescent IgM titer	160	320
Immunofluorescent IgG titer	160	320
Real-time PCR result for *ISFtu2* and *tul4*, sample (cycle threshold for each test, respectively)	Positive, conjunctival discharge (32.4 and 34.9)	Positive, pretragal lymph node (22.3 and 24.5)
Francisella sp. culture, sample	Positive, conjunctival discharge	Negative
Francisella subsp. identification	subsp. *holarctica*	subsp. *holarctica*

*Ig, immunoglobulin.

Both patients were infected with an *F. tularensis* subsp. *holarctica* strain. Infection was identified by PCR amplification and sequencing of the 16S rRNA gene (fD1 and rP2 primers) and the intergenic spacer region (FTitsFw 5′-ACCACGGAGTGATTCATGACTG-3′ and FTitsRv 5′-TCTCAATTGATTTCTCTTCCTAAGG-3′ primers) from the strain isolated from patient A and directly from the lymph node biopsy specimen from patient B.

Conjunctival inoculation of *F. tularensis* usually occurs by contact when a contaminated finger comes into contact with the eyes, e.g., after handling of an infected animal or tick (*3,4*), but the source of infection often remains undetermined, as for our 2 patients. Symptoms are not specific and correspond to Parinaud oculoglandular syndrome (*1*). Reported complications include keratitis, occasional corneal perforation, and lymph node suppuration; tonsillitis, cellulitis in nearby skin tissue, retinitis, erythema nodosum, and progression to systemic disease occur less frequently (*3*–*7*). A specific microbiologic diagnosis is needed for appropriate treatment because many microorganisms can cause Parinaud oculoglandular syndrome and clinical symptoms are not specific (*1,8*).

Fluoroquinolones are now considered first-line treatment for tularemia; β-lactam antimicrobial agents are not effective (*9*). Oculoglandular tularemia is a painful disease with a short incubation period (3–5 days), and results of serologic tests of acute-phase samples are often negative (*1,9*). Isolation of *F. tularensis* is difficult and hazardous to laboratory personnel (*1,9*). PCR-based techniques may enable a more rapid diagnosis (*1,9,10*). Heating clinical samples before testing prevents laboratory-acquired infections.

We report the use of real-time PCR for detection of *F. tularensis* from a conjunctival swab specimen. Many clinical laboratories are now equipped with this technology. Transport conditions of clinical samples (4°C, no transport medium, 24–48 h) are not restrictive. When compared with PCR, real-time PCR does not require post-PCR processing, enabling a faster turn-around time.

Oculoglandular tularemia is a rare but underestimated disease. Real-time PCR detection of *F. tularensis* DNA from conjunctival swab suspensions now provides a rapid, noninvasive, sensitive, and specific diagnosis of oculoglandular tularemia. This assay enables early establishment of specific antimicrobial drug therapy and poses no risk of infection for laboratory staff.

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Max Maurin, Bernard Castan, Nathalie Roch, Brieuc Gestin, Isabelle Pelloux, Alexandra Mailles, Christophe Chiquet, and Pascal Chavanet

Author affiliations: Grenoble University Hospital, Grenoble, France (M. Maurin, B. Gestin, I. Pelloux, C. Chiquet); Joseph Fourier University, Grenoble (M. Maurin, B. Gestin, C. Chiquet); Auch Hospital, Auch, France (B. Castan); Dijon University Hospital, Dijon, France. (N. Roch, P. Chavanet); and French Institute for Public Health Surveillance, Saint Maurice, France (A. Mailles)

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Address for correspondence: Max Maurin, Laboratoire de Bactériologie, Centre Hospitalier Universitaire de Grenoble, BP 217, 38043 Grenoble CEDEX 9, France; email: mmaurin@chu-grenoble.fr

Increase in Serotype 6C Pneumococcal Carriage, United Kingdom

To the Editor: *Streptococcus pneumoniae* is a major human pathogen. In 2007, Park et al. identified a novel serotype, 6C (*1*), which emerged from serotype 6A. A study of children in the Netherlands who had not previously received a pneumococcal vaccine found low prevalence of this newly identified serotype before the heptavalent pneumococcal conjugate vaccine Prevnar/Prevenar (PCV7) (Wyeth, Taplow, UK) was introduced (*2*). Studies have shown cross-protection between vaccine serotype 6B and vaccine-related serotype 6A. However, PCV7 elicits no cross-protection against serotype 6C.

The potential exists for the emergence of nonvaccine serotypes or novel clones. These serotypes and clones may be better adapted to colonize the nasopharynx, evade the human immune response, and cause disease. A recent study showed an increase in prevalence of serotype 6C pneumococci in children and a corresponding decrease in serotype 6A after introduction of PCV7 (*3*). We studied the underlying genetic basis for expansion of serotype 6C. Initial data from an ongoing study of pneumococcal carriage are presented.

This study was reviewed and approved by the Southampton and South West Hampshire Research Ethics Committee (B) (reference 06/Q1704/105). A total of 697 nasopharyngeal swab specimens were collected from unselected (not selected by a method) children ≤4 years of age in the pediatric outpatient department of a large teaching hospital in the United Kingdom. Samples were obtained during October 2006–March 2007, during implementation of PCV7 in the infant immunization schedule of the United Kingdom. During October 2007–March 2008, a total of 202 pneumococci were isolated. All pneumococci were characterized by serotype and genotype.

In the first year of this study, we identified 3 (3.1%) serotype 6C pneumococci belonging to 3 sequence types (STs): ST65, ST1714, and ST1692 (online Appendix Figure, available from www.cdc.gov/EID/content/16/1/154-appF.htm). ST1714 and ST 1692 shared a common clonal complex. Only ST 65 was shared between serotype 6C and serotype 6A. In the second year, we identified 14 (13.6%) serotype 6C pneumococci belonging to 6 STs (online Appendix Figure). Two of these STs, of the same ST, were from siblings. Three of them (ST1692 [n = 8], ST1714 [n = 2], and ST395 [n = 1]) were members of a common clonal complex with a predicted founder of ST395. Each of the remaining 3 STs (ST398, ST1862, and ST3460) was isolated only once. One serotype 6A isolate of ST1692 was also observed.

No serotype 6C ST65 was observed in the second year. We isolated more serotype 6C pneumococci in year 2 than in year 1 ($p \leq 0.01$), which was explained mostly by a large increase in ST1692 ($p \leq 0.03$) (online Appendix Figure). A recent study by Nunes et al. reported serotype 6C ST1692 within a clonal complex that also included ST395 and ST1714 (*4*), and we identified the same clonal complex in year 2 of our study.

Our study showed a large increase in ST1692 in serotype 6C pneumococci during the implementation of PCV7 and an increase in serotype 6C. Depending on the extent of cross-protection between vaccine-related serotypes, introduction of conjugate vaccines could induce clearance or emergence of vaccine-related serotypes. This introduction could also contribute to their substitution with novel or existing serotypes that are better adapted to the ecologic niche. However, our data may only be relevant to carried pneumococci and not reflected in pneumococcal disease epidemiology. Nevertheless, the increase in serotype 6C pneumococci in the United Kingdom, which is supported by a similar observation in the United States (*3*), highlights the potential for emergence of serotypes not included in the current study and newly developed pneumococcal conjugate vaccines.

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Anna S. Tocheva, Johanna M.C. Jefferies, Myron Christodoulides, Saul N. Faust, and Stuart C. Clarke

Author affiliations: University of Southampton School of Medicine, Southampton, UK (A.S. Tocheva, J.M.C. Jefferies, M. Christodoulides, S.C. Clarke); and University of Southampton Wellcome Trust Clinical Research Facility, Southampton (S.N. Faust)

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Address for correspondence: Stuart C. Clarke, Molecular Microbiology and Infection, Sir Henry Wellcome Laboratories, South Block, Southampton General Hospital, Southampton SO16 6YD, UK; email: s.c.clarke@southampton.ac.uk

Oseltamivir- and Amantadine-Resistant Influenza Virus A (H1N1)

To the Editor: We previously reported detection of double resistance to oseltamivir and amantadine of influenza virus A (H1N1) in Hong Kong during the first half of 2008 (*1*). Three different strains of A/Hong Kong/2652/2006-like (clade 2C) viruses that carried the S31N mutation in the matrix (M2) gene associated with amantadine resistance acquired a neuraminidase (NA) gene with CAT→TAT change at position 274 through either reassortment with an oseltamivir-resistant A/Brisbane/59/2007-like (clade 2B) virus or spontaneous H274Y mutation in the NA gene. A clade 2C strain resistant to both oseltamivir and amantadine also was detected in Cambodia in 2007 (*2*).

From July 2008 through December 2008, we continued to monitor antiviral susceptibility of all influenza A (H1N1) viruses in our laboratory, using previously described methods (*1*). Resistance to oseltamivir increased from 16.9% in July to 97.8% in December (Table). Sequencing of the hemagglutinin (HA) gene showed that, beginning in October, A/Brisbane/59/2007-like clade 2B virus had overtaken A/Hong Kong/2652/2006-like clade 2C virus to become the predominating circulating influenza A virus (H1N1) in Hong Kong. Of 916 isolates, 6 (0.7%), isolated from July through September 2008, were resistant to both amantadine and oseltamivir. Genetic analysis showed that 5 were similar to those we described previously, 4 were A/Hong Kong/2652/2006-like clade 2C viruses with spontaneous H274Y mutation in the NA gene, and 1 was a clade 2C virus but acquired a clade 2B NA gene carrying the H274Y mutation. The sixth double-resistant virus was an A/Brisbane/59/2007-like clade 2B virus with a spontaneous S31N mutation in the M2 gene. No epidemiologic link was detectable between these viruses. From October through December 2008, no double-resistant viruses were detected.

From January through June 2009, A/Brisbane/59/2007-like clade 2B virus continued to be the predominating strain. Of the total 1,537 influenza virus A (H1N1) isolates tested during the period, 1,509 (98.2%) were resistant to oseltamivir. Of the 1,509 oseltamivir-resistant isolates tested from April through June 2009, 50 (3.3%) also were resistant to amantadine (Table). Nucleotide sequencing of the HA, NA, and M2 genes was performed on all 50 oseltamivir- and amantadine-resistant viruses. All were A/Brisbane/59/2007-like clade 2B viruses that had acquired an M2 gene carrying the S31N mutation by reassortment with an amantadine-resistant A/Hong Kong/2652/2006-like clade 2C virus. Nucleotide sequencing of the other 5 internal genes (nonstructural, nucleoprotein, polymerase acidic, polymerase basic 1, and polymerase basic 2 proteins) was performed on 2 double-resistant strains isolated in April and on 3 isolated in June. Sequence comparison showed that 1 virus in April, in addition to acquiring an M2 gene, acquired a nonstructural protein gene from an A/Hong Kong/2652/2006-like clade 2C virus. All the viruses were susceptible to zanamivir and were not associated with unusual severity of disease.

Along with pandemic (H1N1) 2009, seasonal influenza viruses continue to circulate in Hong Kong (*3*). An alarming proportion of the circulating seasonal influenza A virus

Table. Prevalence of oseltamivir-resistant and amantadine- and oseltamivir-resistant influenza A virus (H1N1) detected in Hong Kong, with clade designations, July 2008–June 2009

Date detected	No. isolates tested	No. (%) isolates oseltamivir resistant	No. (%) isolates oseltamivir and amantadine resistant	Predominating influenza A virus (H1N1) clade, no. identified/total no. sequenced (%)
2008 Jul	462	78 (16.9)	4 (5.1)	Clade 2C, 104/182 (57.1)
2008 Aug	313	45 (14.4)	1 (2.2)	Clade 2C, 51/95 (53.7)
2008 Sep	61	21 (34.4)	1 (4.8)	Clade 2B, 20/39 (51.3)
2008 Oct	19	13 (68.4)	0	Clade 2B, 13/16 (81.3)
2008 Nov	16	15 (93.8)	0	Clade 2B, 16/16 (100)
2008 Dec	45	44 (97.8)	0	Clade 2B, 41/41 (100)
2009 Jan	327	313 (95.7)	0	Clade 2B, 90/104 (86.5)
2009 Feb	769	755 (98.2)	0	Clade 2B, 138/153 (90.2)
2009 Mar	279	279 (100)	0	Clade 2B, 61/61 (100)
2009 Apr	63	63 (100)	2 (3.2)	Clade 2B, 18/18 (100)
2009 May	22	22 (100)	2 (9.1)	Clade 2B, 7/7 (100)
2009 Jun	77	77 (100)	46 (59.7)	Clade 2B, 55/55 (100)

(H1N1) became resistant to both oseltamivir and amantadine in a short span of 1 month. Oseltamivir-resistant A/Brisbane/59/2007-like clade 2B virus that had reassorted with A/Hong Kong/2652/2006-like clade 2C virus had apparently spread in the community and to other regions of the world. The possibility of reassortment with pandemic (H1N1) 2009 virus is a major concern. Resistance to oseltamivir of pandemic (H1N1) 2009 virus will compromise its use in treatment and render the billion-dose stockpile useless. Although the recently detected oseltamivir-resistant pandemic (H1N1) 2009 virus in Hong Kong was not a reassortant virus (*4*,*5*), we will continue to closely monitor antiviral drug resistance among circulating viruses, including pandemic (H1N1) 2009 virus and seasonal influenza virus A (H1N1), as well as influenza A (H3N2) viruses, to track how antiviral resistance evolves.

Peter K.C. Cheng, Amanda P.C. To, Tommy W.C. Leung, Peter C.K. Leung, Connie W.C. Lee, and Wilina W.L. Lim

Author affiliation: Department of Health, Hong Kong, People's Republic of China

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Address for correspondence: Wilina W.L. Lim, Public Health Laboratory Centre, 382 Nam Cheong St, Shek Kip Mei, Kowloon, Hong Kong Special Administrative Region, People's Republic of China; email: wllim@pacific.net.hk

Pandemic (H1N1) 2009 Reinfection, Chile

To the Editor: Since March 2009, influenza A pandemic (H1N1) 2009 has spread worldwide (*1*), and in South America, Chile was 1 of the countries most affected by the pandemic, with 21.4 cases among every 1,000 persons. Treatment guidelines in Chile recommended antiviral drug treatment with oseltamivir or zanamivir for 5 days for all patients with confirmed or suspected virus subtype H1N1 infection (*2*). In persons with seasonal influenza, specific antibody responses reach peak titers by 4 weeks after infection and confer protection against the infecting strain and closely related strains (*3*). Reinfection is rarely seen in nonpandemic influenza A. We report on 3 patients with confirmed influenza A pandemic (H1N1) 2009 reinfection after successful treatment with oseltamivir.

Patient 1, a healthy 14-year-old girl, had fever, sore throat, and nasal congestion on clinical examination. Pandemic (H1N1) 2009 infection was diagnosed by viral culture and confirmed by PCR specific for subtype H1N1 (LightMix Kit Inf A swine H1; TIB MOLBIOL, Berlin, Germany, for Roche Diagnostic, Indianapolis, IN, Light Cycler 2.0 instrument). The patient received oseltamivir, and symptoms resolved 48 hours after treatment. Twenty days later, fever, muscle aches, and vomiting developed in the patient. Influenza A virus was isolated by viral culture. The patient received a preliminary diagnosis of seasonal influenza and was treated with amantadine. She recovered from the infection before PCR results confirmed it was caused by pandemic (H1N1) 2009 virus.

Patient 2, a 62-year-old woman, experienced a high fever, cough, and nasal congestion during a prolonged hospitalization for bowel resection after intestinal ischemia. Pandemic (H1N1) 2009 was confirmed by PCR and viral culture. Oseltamivir was administered 5 days after the onset of symptoms, and the symptoms resolved within the following 5 days. The patient had a new episode of fever, productive cough, and bronchial obstruction 2 weeks later while still hospitalized. Culture results were positive for influenza, and PCR results were positive for pandemic (H1N1) 2009. The patient was again treated with oseltamivir, and PCR results were negative for influenza after 48 hours of antiviral treatment.

Patient 3, a previously healthy 38-year-old man, underwent mitral and aortic valve replacement while hospitalized for acute mitral and aortic endocarditis due to *Staphylococcus aureus*. Eleven days after surgery, he had a sore throat, nasal congestion, cough, and low-grade fever. PCR test results were positive for pandemic (H1N1) 2009. The patient received oseltamivir, and respiratory symptoms resolved within 5 days. He was discharged from the hospital but was

readmitted 18 days later with nasal congestion, cough, and high fever. PCR results were again positive for pandemic (H1N1) 2009, and the patient was successfully treated with oseltamivir.

Patient 2 and probably patient 3 acquired their infections while hospitalized, suggesting potential nosocomial transmission. No other respiratory viruses were found in any of these patients. The viral isolates were all tested (LightMix for detection of influenza A virus oseltamivir resistance [H274Y]; TIB MOLBIOL) for possible resistance to oseltamivir, but none had the resistance-implicated H274Y mutation in the neuraminidase gene

Shedding of seasonal influenza A virus ceases within 5–7 days during natural infection and during infections treated with neuraminidase inhibitors (*4*). Although clearing of virus after the first infection was not documented in the 3 patients described here, it is unlikely that virus persisted between the 2 episodes of influenza since each of the patients fully recovered after specific antiviral drug treatment. However, we cannot rule out that patient 2 may have never cleared the virus due to her immune suppression.

As described by mathematical modeling (*5*), the 3 patients described were susceptible to reinfection with pandemic (H1N1) 2009 due to the high rate of community infection and to their incomplete immunologic protection within the period of reexposure. During the current pandemic of influenza subtype H1N1, healthcare workers and patients should be aware that symptomatic reinfection might occur after a first episode of infection.

Carlos M. Perez, Marcela Ferres, and Jaime A. Labarca

Author affiliations: Pontificia Universidad Catolica de Chile, Santiago, Chile

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Address for correspondence: Carlos Miguel Perez, Pontificia Universidad Catolica de Chile – Medicine, Marcoleta 357, Santiago 00000, Chile; email: cape@med.puc.cl

Skin Lesion Caused by ST398 and ST1 MRSA, Spain[1]

To the Editor: Human infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type 398 (ST398) have been emerging in recent years in Europe (*1,2*). Pigs represent a common reservoir of MRSA ST398, and working with these animals may constitute a risk factor for MRSA carriage and possible infection (*2–4*). We report a case of human infection caused by MRSA ST398 in Spain.

A 12-year-old girl living close to a pig farm, where her father and mother worked, sought treatment for a skin lesion on her chin. Two types of MRSA were recovered from the lesion, and it resolved after topical treatment with mupirocin over 10 days. MRSA isolates recovered were characterized by multilocus sequence typing (MLST) and by staphylococcal cassette chromosome (SCC) *mec*, *spa*, and *agr* typing as described (*3*). The presence of genes encoding Panton-Valentine leukocidin (PVL) (*lukF/lukS*), toxic shock syndrome toxin-1 (*tsst1*), and exfoliative-toxin A and B (*eta*, *etb*) was investigated by PCR (*2,3*). Antimicrobial susceptibility tests were carried out by using the VITEK-2 system (bioMérieux, Marcy l'Etoile, France), and *mecA, ermA*, *ermB*, *ermC*, *mrsA*, *tetK, tetL, tetM*, *ant(4')-Ia*, *aph(3')-III* , and *aph(2')-aac(6')* resistance genes were studied by PCR (*5*). *dfrK* gene detection was performed by using primers designed from the sequence FM207105 included in GenBank (*dfr*K-F 5′-GAGAATCCCAGAGGATTGGG; *dfr*K-R, 5′-CAAGAAGCTTTTCGCTCATAAA), and the obtained amplicons were sequenced for confirmation. Mutations in quinolone targets were determined by sequence analysis of *grlA* and *gyrA* genes (*6*). In addition, MRSA isolates were typed by pulsed-field gel electrophoresis (PFGE) (www.harmony-microbe.net/microtyping.htm).

One of the clinical MRSA isolates recovered from the lesion and typed as ST398-*spa*-t011 showed resistance to tetracycline, macrolides, and lincosamides and harbored 5 antimicrobial resistance genes. The second MRSA strain was typed as ST1-*spa*-t127 and showed a multiresistance phenotype with 11 resistance genes, as well as the Ser80Phe and Ser84Leu amino acid changes in GrlA and GyrA proteins, respectively.

To establish the MRSA nasal colonization status of the patient and of her relatives (father, mother, and brother, all of whom had contact with

[1]This study was presented as a poster at the 19th European Congress of Clinical Microbiology and Infectious Diseases, Helsinki, Finland, 2009.

animals) and to elucidate the possible origin of these strains, we analyzed nasal swabs for MRSA recovery. Nasal samples were plated in oxacillin-resistant *S. aureus* agar media (ORSA, Termofisher, UK), colistin nalidixic acid agar media (bioMérieux), and blood agar media (Oxoid); colonies suggestive of *S. aureus* were initially selected, further identified, and characterized for bacterial typing and antimicrobial resistance mechanisms. Samples from all relatives and the patient showed that all 4 persons were MRSA nasal carriers presenting the following genetic lineages: patient girl, ST398-t108; mother, ST398-t108 and ST1-t127; father, ST398-t108; and brother, ST398-t011 (2 variants) (Table). MRSA t127 and t011 variants detected in the skin lesion of the patient were not found in her nasal sample but were found in the nasal samples of her mother and brother. Another study has been initiated to analyze the presence of MRSA in nasal swabs of swine on the farm.

The ST1-t127 strains obtained from the skin lesion of the girl (C1569) and from the mother's nasal sample (C1578) showed the same multiresistance phenotype and genotype (Table) and an indistinguishable PFGE pattern. This clonal type seems to be associated with community-acquired MRSA isolates circulating in Europe (*7*). The animal origin of this MRSA type and its possible transmission from horses and cows to humans has been suggested in previous reports (*8,9*).

In addition, all family members were colonized by MRSA ST398. All recovered nasal MRSA ST398 strains showed resistance to β-lactams, macrolides, lincosamide, and tetracycline, and 3 strains also showed diminished susceptibility to quinolones. Strain ST398 C1576, recovered from the brother, showed a multiresistance phenotype (Table).

The ST398 strains of our study were classified in the *spa*-types t011 and t108, 2 of the most frequently described types of the clonal complex 398 (*1,4*) and were untypeable by PFGE. Most MRSA ST398 strains in our study showed a resistance phenotype that included tetracycline, macrolides, and lincosamides. Tetracycline resistance is a common trait of ST398 and suggests that its use in veterinary medicine may have been implicated in selection of this resistance. The first reports, describing ST398 strains, indicated β-lactams and tetracycline as the unique resistance markers, which were susceptible to other antimicrobial agents. Nevertheless, reports of ST398 showing resistance to other antimicrobial drugs are increasing. One of the ST398 strains from the brother showed an uncommon multiresistance phenotype. Moreover, all the MRSA strains of this study were negative for all tested toxin genes, included PVL, although some ST398 PVL–positive strains have been occasionally described (*10*).

We report a skin lesion on the daughter of a pig farmer in Spain associated with ST398-t011 and ST1-t127 MRSA strains. The results obtained suggest the specific animal origin of these strains and subsequent transference among family members. Of special interest is the multiresistance phenotype of the clinical and nasal

Table. Characteristics of the 9 MRSA strains recovered in Spain from a patient's lesion and from nasal samples obtained from patient's family members*

Strain	Origin of sample	SCC*mec* type	MLST	*spa* type	*agr*	Antimicrobial resistance phenotype	Resistance genes detected	Amino acid change in: GrlA	Amino acid change in: GyrA
C1570	Patient/ skin lesion	V	ST398	t011	I	OXA, FOX, TET, ERY, CLI, TEL	*mecA, tetK, ermA, ermC, msrA*	NP	NP
C1569	Patient/ skin lesion	II	ST1	t127	III	OXA, FOX, TET, ERY, CLI, TEL, GEN, TOB, KAN, CIP, LEV, SXT	*mecA, tetL, tetK, ermA, ermB, ermC, msrA, aph(2')-acc(6'), ant(4´)-Ia, aph (3'), dfrK*	S80F	S84L
C1571	Patient/ nasal swab	V	ST398	t108	I	OXA, FOX, TET, ERY, CLI, TEL, CIP†, LEV†	*mecA, tetK, tetL, tetM, ermA, ermC, msrA*	Wild	Wild
C1577	Mother/ nasal swab	V	ST398	t108	I	OXA, FOX, TET, ERY, CLI, TEL, CIP†, LEV†	*mecA, tetK, tetM, ermA, ermC, msrA*	Wild	Wild
C1578	Mother/ nasal swab	II	ST1	t127	III	OXA, FOX, TET, ERY, CLI, TEL, GEN, TOB, KAN, CIP, LEV, SXT	*mecA, tetL, tetK, ermA, ermB, ermC, msrA, aph(2')-acc(6'), ant(4´)-Ia, aph (3'), dfrK*	S80F	S84L
C1574	Brother/ nasal swab	V	ST398	t011	I	OXA, FOX, TET, ERY, CLI, TEL	*mecA, tetK, ermA, ermC, msrA*	NP	NP
C1576	Brother/ nasal swab	V	ST398	t011	I	OXA, FOX, TET, ERY, CLI, TEL, GEN, TOB, KAN, SXT	*mecA, tetL, tetK, ermA, ermB, ermC, msrA, aph(2')-acc(6'), ant(4´)-Ia, aph (3'), dfrK*	NP	NP
C1572	Father/ nasal swab	V	ST398	t108	I	OXA, FOX, TET, ERY, CLI, TEL, CIP†, LEV†	*mecA, tetK, tetL, tetM, ermA, ermC, msrA*	Wild	Wild

*MRSA, methicillin resistant *Staphylococcus aureus*; SCC, staphylococcal cassette chromosome; MLST, multilocus sequence typing; ST, sequence type; OXA, oxacillin; FOX, cefoxitin; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; TEL, telithromycin; NP, not performed; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; CIP, ciprofloxacin; LEV, levofloxacin; SXT, sulfamethoxazole-trimethoprim.
†Intermediate category for the indicated antimicrobial drug.

ST1-t127 MRSA clinical strains and of 1 nasal strain belonging to ST398 lineage. Nasal colonization by different ST398 genetic lineages and by other lineages of MRSA as ST1-t127 seems to be frequent in persons living in close proximity to farm animals. Dissemination of MRSA ST398 (and probably also MRSA ST1) in humans who have contact with farm animals, is an emerging problem in Spain.

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Carmen Aspiroz, Carmen Lozano, Ana Vindel, Juan J. Lasarte, Myriam Zarazaga, and Carmen Torres

Author affiliations: Hospital Royo Villanova, Zaragoza, Spain (C. Aspiroz); Universidad de La Rioja, Logroño, Spain (C. Lozano, M. Zarazaga, C. Torres); Centro Nacional Microbiología, Madrid, Spain (A. Vindel); and Centro Salud San Mateo de Gállego, Zaragoza (J.J. Lasarte)

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Address for correspondence: Carmen Torres, Area Bioquímica y Biología Molecular, Universidad de La Rioja, Madre de Dios 51, 26006 Logrono, Spain; email: carmen.torres@unirioja.es

Identification of a Rotavirus G12 Strain, Indonesia

To the Editor: Group A rotaviruses are the most common etiologic agents of acute gastroenteritis in infants and young children, each year resulting in ≈100 million diarrhea episodes and 600,000 deaths worldwide (*1*). The genome of rotavirus comprises 11 segments of double-stranded RNA, which encode 6 structural viral proteins (VPs) and 6 nonstructural proteins (NSPs). Recent scientific reports have identified novel rotavirus strains, such as G12 (*2–5*), which were first described in 1987 among Filipino children with diarrhea (*6*). In Indonesia, a rotavirus study showed that a broad variety of VP7 types (G1, G2, G3, G4, G8, G9) and VP4 types (P[4], P[6], P[8], P[9], P[10], P[11]), especially G9 and P[8] and G9P[8], were the genotype combinations most frequently encountered (*7*).

From 2005 through 2008, we conducted a nationwide surveillance study among children who had diarrhea to determine etiologies among Indonesian children seeking health services for diarrhea at hospitals and health clinics. Patients were enrolled after obtaining consent from parents/guardians of those eligible in accordance with an institutional review board protocol approved by the US Naval Medical Research Unit No. 2 (NAMRU-2) and the Ethical Committee of the Indonesian National Health Research and Development Institute. Stool specimens and clinical enrollment data were collected for each eligible patient, and all collected items were transported to NAMRU-2 in Jakarta, Indonesia. In December 2007, a stool specimen was collected from a 14-day-old afebrile infant brought to Sumber Waras Hospital in West Jakarta with diarrhea, vomiting, moderate dehydration, and malnutrition. This patient was infected with the rotavi-

rus G12 strain, was hospitalized for 6 days, and was discharged after recovering fully. Bacterial cultures and ova/parasite evaluations were negative for enteric pathogens.

Rotavirus was detected in this specimen and genotyped by multiplex, seminested reverse transcription–PCR (RT-PCR) targeting the VP4 and VP7 genes (*8,9*). The specimen was typed as P[4]P[6] but was G-nontypeable. Primers to detect G12 were then used for RT-PCR and identified the proper G12 amplicon size (*2,3*). By use of published primers (*9*), sequencing of the VP7 gene segment confirmed the presumptive G12 genotype. Sequencing reactions were performed by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on the Applied Biosystems 3130xl sequencer. Sequence analysis was done by using Sequencher 4.8 version (Gene Codes Corporation, Inc., Ann Arbor, MI, USA). Nucleotide sequences were submitted to GenBank for a BLASTN search (http://blast.ncbi.nlm.nih.gov) on the National Center for Biological Information website. We then created alignments of nucleotides and deduced amino acid sequences and compared them with a selection of reference strains from the GenBank database. Genetic relationships among G12 were determined by using PAUP version 4.0 beta 10 software (http://paup.csit.fsu.edu). A phylogenetic tree was constructed on the basis of nucleotides 1–971 of the VP7 gene by using the neighbor-joining method and applying the Kimura 2-parameter method with 1,000 bootstrap replicates of the neighbor-joining model.

The BLASTN search of the VP7 989 nucleotide sequence of the putative G12 Indonesian rotavirus (Indo SWJ0806) showed 98% similarity with published VP7 sequences of rotavirus G12 strains from Japan (CP727; GenBank accession no.AB125852), Argentina (Arg721; GenBank accession no. EU496255), and Thailand (T152; GenBank accession no. AB071404). The Indonesia G12 clustered into the lineage II composed of rotavirus G12 reference strains from Japan, Argentina, South Korea, and Thailand (Figure). Lineage II is a minority cluster when compared with lineage III, which consists of rotavirus G12 from the United States (US6588, Se585), Saudi Arabia (MD844), India (13B2), Bangladesh (RV161), and other Thailand strains (MS051) (*4*). The nucleotide sequence divergence between lineage II and lineage III ranges from 2.6% to 3.2%. Analysis of the deduced amino acid sequence alignment on the neutralization epitopes that code for the antigenic regions A, B, and C show high conservation of the most immunodominant sites (data not shown). Antigenic regions A, B, C, D, E, and F of Indonesia SWJ0806 show 100% amino acid similarity to Japan G12 strains; K12 and CP727 (*9*). The amino acid residue at position 142 of the antigenic region B has characterized lineage I and II (Val) and lineage III (Leu).

Phylogenetic analysis showed that the virus clusters into lineage II and that the deduced amino acid sequence is highly conserved compared with other reported rotavirus G12 strains identified. The combination of the P[6] genotype in this rotavirus strain suggests the possibility of a zoonotic transmission (*10*). Continued surveillance for rotavirus is an essential component of a country's public health infrastructure and diarrhea prevention programs. Rotavirus genotyping from the data obtained provides necessary information for vaccine development and identification of novel and emerging rotavirus strains.

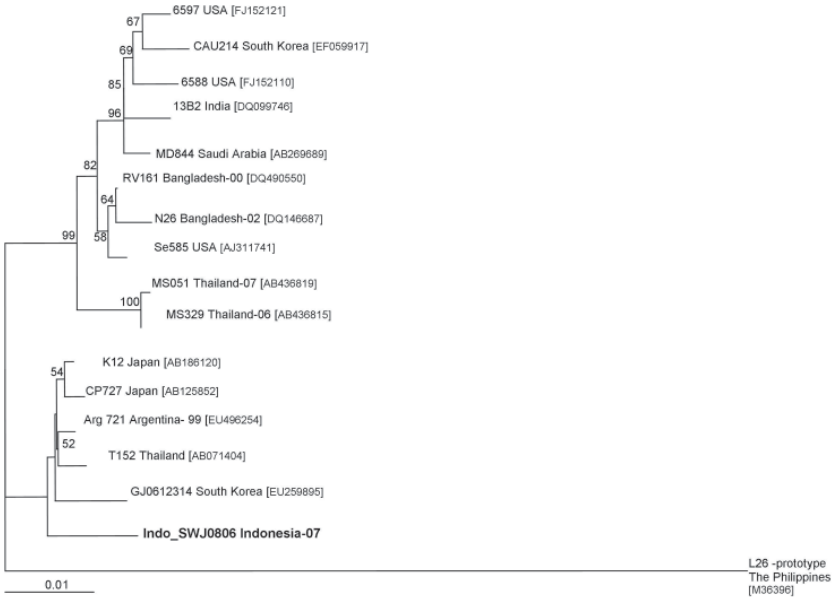

Figure. Phylogenetic analysis of the viral protein (VP) 7 genotype G12 rotavirus of Indonesia, with reference strains downloaded from GenBank. The GenBank accession numbers of each strain appear next to the strain. The multiple alignment was constructed by using ClustalX version 1.81 (www.clustal.org). The phylogenetic tree was based on the 971 nt sequence of the VP7 gene and constructed by using the neighbor-joining method and applying the Kimura 2-parameter method with 1,000 bootstrap replicates of the neighbor-joining model. The isolate identified in this study is shown in **boldface**. Bootstrap values <50% are not shown. Scale bar indicates nucleotide substitutions per site.

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Wahyu N. Wulan, Erlin Listiyaningsih, Kiki M.K. Samsi, Magdarina D. Agtini, Matthew R. Kasper, and Shannon D. Putnam

Author affiliations: US Naval Medical Research Unit No. 2, Jakarta, Indonesia (W.N. Wulan, E. Listiyaningsih, M.R. Kasper, S.D. Putnam); Sumber Waras Hospital, Jakarta (K.M.K. Samsi); and Indonesian Ministry of Health, Jakarta (M.D. Agtini)

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Address for correspondence: Matthew R. Kasper, US Embassy Jakarta UNIT 8132 NAMRU-2, FPO AP 96520; email: kasper@namrutwo.org

Age-based Human Influenza A Virus (H5N1) Infection Patterns, Egypt

To the Editor: In April 2009, a representative of the World Health Organization in Cairo voiced concern about the changing age-based pattern of human influenza A virus (H5N1) infection in Egypt (*1*). From March 2006 through July 2009, a total of 83 persons in Egypt were confirmed to have human influenza A (H5N1); the patients' ages ranged from >1 year to 75 years (*2*). However, from December 2008 through July 2009 in Egypt, 28 of 32 human infections were in children ≤8 years of age.

The frequency of human influenza A virus (H5N1) infections parallels the pattern for seasonal influenza. Thus, for analytical purposes, virus subtype H5N1 infections in Egypt can be grouped into 12-month periods, beginning with August of 1 year and ending in July of the following year. The results for 1-way analysis of variance indicate that the age at time of virus subtype H5N1 infection in Egypt differs significantly among these 4 periods (Kruskal–Wallis test statistic = 20.732, $p<0.0004$).

Further analysis shows that persons infected from August 1, 2008 through July 31, 2009, were much younger than those infected in the preceding 12-month period (Mann-Whitney U test statistic = 328.500, $p<0.001$). The median age of the 12 confirmed case-patients from August 1, 2007, through July 31, 2008, was 23.5 years, but the median age of the 33 confirmed case-patients from August 1, 2008, through July 31, 2009, was 3.0 years. The Table shows the distribution of case-patients by age group, the median age of each group, and the case-fatality ratio (CFR) for the 4 seasonal 12-month periods.

This recent rise of subtype H5N1 influenza cases among children represents a major change in the pattern of human influenza A virus (H5N1) infections in Egypt compared with the pattern for earlier influenza seasons. Confirmation reports by the World Health Organization generally indicate associations with dead and sick poultry for these recent cases among children. The cultural patterns and customs of poultry husbandry have not changed in Egypt since the first human cases of influenza A (H5N1) were confirmed in 2006; thus, it is not clear why more children have been infected since December 2008. One explanation may be the increased recognition of the clinical signs of nonfatal influenza A (H5N1) among children and increased confirmation by laboratory testing. The lack of influenza A virus (H5N1) infection among the infected children's parents and caregivers suggests that the virus is still not easily transmissible among humans in Egypt.

Not only has there been a recent increase in infections of influenza A (H5N1) among children, but there has also been a recent decline in deaths

Table. Age groups, median ages, and case-fatality ratios for influenza A (H5N1) case-patients, by influenza season, Egypt*

Influenza season, August–July	No. case-patients by age group, y						Total no. case-patients	Median age, y	Case-fatality ratio
	1–8	9–20	21–30	31–40	41–74	≥75			
2005–06	4	4	3	2	0	1	14	18.0	42.8
2006–07	13	5	4	2	0	0	24	6.5	37.5
2007–08	3	2	5	1	1	0	12	23.5	58.3
2008–09	28	1	1	3	0	0	33	3.0	15.1
Total	48	12	13	8	1	1	83	—	—

*Age at date of clinical sign onset; data from the World Health Organization (*2*).

among confirmed infected persons. From 2006 through 2008, the annual CFR for influenza A (H5N1) in Egypt ranged from 36% to 55% (*3*). Since January 1, 2009, the CFR in Egypt has been 11%. The recent increases in infections among children coupled with a decrease in the CFR in the most recent 12-month period suggests that the strain of influenza A virus (H5N1) now circulating in Egypt may be becoming less virulent as it continues to spread among young children, a segment of the population that is highly vulnerable to influenza infections (*4*,*5*).

Alan Schroedl

Author affiliation: Anthrometrix Corporation, Salt Lake City, Utah, USA

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Address for correspondence: Alan Schroedl, Anthrometrix Corporation, 2359 Dayspring Lane, Salt Lake City, UT 84124, USA; email: aschroedl@anthrometrixcorp.com

Imported Chikungunya Virus Infection

To the Editor: Chikungunya is a disease caused by an arboviral alphavirus transmitted to humans by *Aedes* mosquitoes (*Aedes aegypti*, *Ae. albopictus*). Symptoms include fever, myalgia, rash, and joint pain (which can last for several months) (*1*). During the 2005–2006 epidemics on Reunion Island, clinical manifestations such as severe hepatitis, severe maternal and fetal disease, and meningoencephalitis not described previously were observed (*2*). Occurring in an immunologically uninfected population, this outbreak spread quickly, infecting approximately one third of the population (266,000 of 775,000 inhabitants) (*2*). The case-fatality rate on Reunion Island was estimated to be 1/1,000 cases, with excess deaths observed mainly among persons ≥75 years of age (*3*).

Chikungunya disease is endemic to western, central, eastern, and southern Africa; on Indian Ocean and west Pacific Ocean islands; and in Southeast Asia (*1*). Before 2005–2006, no outbreak of this disease had been described on islands in the Indian Ocean (Comoros, Mayotte, Madagascar, Reunion Island, Mauritius, and Seychelles). Since the epidemic on Reunion Island, many imported cases caused by this arbovirus have been reported elsewhere in areas where the disease is not endemic, particularly in Europe and the United States.

The main competent vector of chikungunya virus, a mosquito, *Ae. albopictus*, is indigenous to Southeast Asia and some islands of the western Pacific and Indian Ocean. The mosquito spread to the eastern Pacific, the Americas, Central Africa (Nigeria, Cameroon, Equatorial Guinea and Gabon), Europe, and the Middle East (*4*,*5*). Entomologic studies have shown that *Ae. albopictus* mosquitoes can now be found in the southeastern part of the United States, Mexico, Central and South America, the Caribbean, the Middle East, Japan, and southern Europe (Spain, Italy, Bosnia-Herzegovina, Croatia, France, Greece, the Netherlands, Serbia and Montenegro, Slovenia, Switzerland, and Albania) (*4*,*6*). This mosquito has also been intercepted in Australia's seaports and is now established in northern Queensland (*7*).

Ae. aegypti mosquitoes are indigenous to Africa and disseminated around the tropical and subtropical regions. The southeastern United States, the Middle East, Southeast Asia, Pacific and Indian islands, and northern Australia are also infested by this mosquito. In continental Europe, it

has been documented in southern regions but today seems to no longer to be present there (*8*).

Climate change, increasing globalization, and ease of travel could favor the continuing spread of mosquitoes to nonindigenous habitats, expanding the number of regions in the world where local transmission of vector-borne disease could occur. In these countries where competent vectors are present, patients coming from disease-endemic areas at an early stage of infection may import the virus and be responsible for locally acquired mosquito-transmitted cases of chikungunya. The risk for local transmission in these countries is not simply theoretical, as shown by the epidemic of chikungunya in the county of Emilie-Romagna, Italy, in which 205 cases were identified between July 4 and September 27, 2007 (*9*). In the United States, such secondary transmission of vector-borne disease has also been observed with malaria (*10*).

To determine regions of the world at risk for an epidemic of chikungunya virus, we first listed the imported chikungunya cases (i.e., cases diagnosed in nonendemic areas) reported around the world. A literature review was undertaken on Medline by Pubmed and websites provided by the World Health Organization, Eurosurveillance, European Center for Disease Prevention and Control, Health Protection Agency (United Kingdom), Institut de Veille Sanitaire (France), and the Centers for Disease Control and Prevention (United States) were searched for information on imported chikungunya cases. Data were then mapped and compared with the known and theoretical geographic distributions of *Ae. albopictus* and *Ae. aegypti* mosquitoes around the world (online Appendix Figure, available from www.cdc.gov/EID/content/16/1/162-appF.htm) (*4*–*8*). This figure shows that imported cases were reported in many countries where mosquito vectors for chikungunya virus are well established.

These facts underscore the need for clinicians to consider the possibility of chikungunya disease in patients who experience acute unexplained fever with joint pain and live in regions where mosquito vectors are established. The presence of imported cases and well-established vectors also confirms the need for an active surveillance system; early detection of unexpected new diseases by physicians will enable the timely implementation of suitable control measures that can interrupt the transmission chain.

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Man-Koumba Soumahoro, Didier Fontenille, Clément Turbelin, Camille Pelat, Anders Boyd, Antoine Flahault, and Thomas Hanslik

Author affiliations: Université Pierre et Marie Curie, Paris, France (M.-K. Soumahoro, C. Turbelin, C. Pelat, A. Boyd); Institut National de la Santé et de la Recherche Médicale, Paris (M.-K. Soumahoro, C. Turbelin, C. Pelat, A. Boyd, A. Flahault, T. Hanslik); Research Institute for Development, Montpellier, France (D. Fontenille); French School of Public Health, Rennes, France (A. Flahault); Hôpital Ambroise Paré, Boulogne Billancourt, France (T. Hanslik); and Assistance Publique Hôpitaux de Paris, Paris (T. Hanslik)

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Address for correspondence: Man-Koumba Soumahoro, Institut national de la santé et de la recherche médicale, Unité Mixte de Recherche en Santé 707, Faculté de Médecine Pierre et Marie Curie, Site Saint-Antoine/Porte 808, 27 rue Chaligny, 75571 Paris CEDEX 12, France; email: soumahor@u707.jussieu.fr

Distinct Molecular Signature of Bovine Spongiform Encephalopathy Prion in Pigs

To the Editor: In a recent article in Emerging Infectious Diseases, Espinosa et al. (*1*) investigated the porcine transmission barrier to infection with bovine and ovine transmissible spongiform encephalopathies (TSEs) in transgenic mice expressing the porcine prion protein. Bovine spongiform encepatholopathy of the classical type (BSE) derived from cattle and sheep, as well as atypical scrapie, transmitted to these mice, although with different efficiencies. Whereas sheep BSE showed a 100% attack rate, cattle BSE and atypical scrapie showed a higher transmission barrier in the first passage. Unexpectedly, the electrophoretic profile of the proteinase K–resistant prion protein (PrP^{res}) in Western immunoblot (WB) analysis of all 3 TSEs shifted toward a common signature upon transmission. This was a 3-band pattern with a predominant monoglycosylated PrP^{res} moiety and, therefore, clearly differed from those of the BSE and atypical scrapie inocula. The authors speculated that the porcine cellular prion protein (PrP^{c}) might allow only for few options as it changes its conformation to the disease-associated prion protein. However, whether this effect is attributable to the porcine PrP^{c} transgene or to the genetic background of the mouse model remains unknown.

To our knowledge, BSE has been successfully transmitted to pigs in 1 study, but WB data were not reported (*2*). We had access to central nervous system tissues of 1 of these animals (kindly provided by the Veterinary Laboratories Agency TSE Archive, Weybridge, UK) and aimed at assessing whether a similar effect occurs when cattle BSE affects pigs. Our results show a PrP^{res} signature in BSE-infected pigs similar to that described for the porcine PrP^{c} transgenic mice and clearly different from that in cattle (Figure). These findings support the finding by Espinosa et al. that the molecular shift most likely was due to intrinsic properties of the porcine PrP^{c}. Therefore, in this respect the mouse model appears to reflect the situation in the pig.

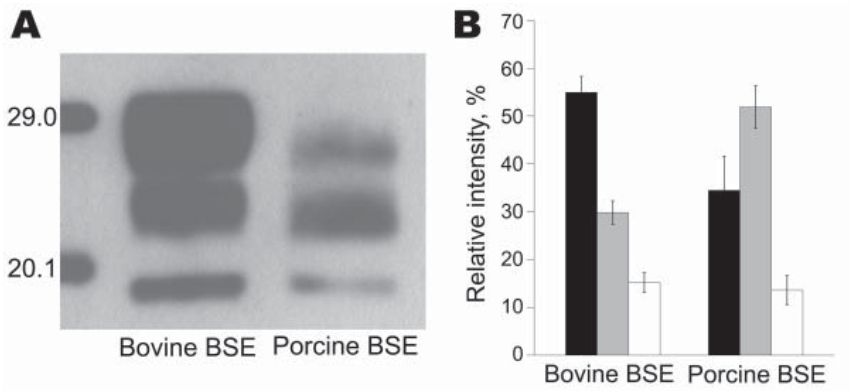

Figure. Molecular signature of bovine spongiform encephalopathy (BSE) in pigs. A) Comparative Western immunoblot analysis of the proteinase K–resistant core fragment (PrP^{res}) of the pathologic prion protein in BSE in cattle and in an experimentally BSE-infected pig using the monoclonal antibody 6H4 (Prionics, Schlieren, Switzerland). B) Average relative intensities of the diglycosylated (black bars), monoglycosylated (gray bars), and unglycosylated (white bars) PrP^{res} moieties as determined by the Quantity One software package (Bio-Rad, Rheinach, Switzerland). Data are based on 4 independent runs, and error bars indicate SD. Note the different extent of PrP^{res} glycosylation in bovine and porcine BSE. By contrast, the molecular masses of the unglycosylated PrP^{res} were similar and scored 18.89 kDa (SD ± 0.28 kDa) and 18.90 kDa (SD ± 0.42 kDa) in bovine and porcine BSE, respectively. Molecular masses of the standards are indicated on the left in panel A.

BSE prions are considered to transmit to other species, such as exotic ruminants, cats, macaques, humans, sheep, and goats, without any obvious alterations of the molecular phenotype (*3,4*). Our study provides evidence that the molecular phenotype of classical BSE also may shift upon genuine interspecies transmission. Attempts to discriminate BSE from other prion diseases in humans and animals often rely at first on the analysis of the PrP^{res} signature in WB. Consequently, the situation described in our study complicates the interpretation of such disease surveillance data to assess public health risks for animal TSEs. Whether this applies to other TSEs and species remains to be addressed.

Torsten Seuberlich and Andreas Zurbriggen

Author affiliation: University of Berne, Berne, Switzerland

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Address for correspondence: Torsten Seuberlich, NeuroCentre, Reference Laboratory for TSE in Animals, University of Berne, Bremgartenstrasse 109a, CH-3001 Berne, Switzerland; email: torsten.seuberlich@itn.unibe.ch

Parvovirus 4 in Blood Donors, France

To the Editor: In the past few years, several novel parvoviruses have been identified, including human parvovirus B19–related strains V9 and A6, and bocavirus. In 2005, parvovirus 4 (PARV4), a new putative member of the family *Parvoviridae*, was identified in the plasma of a patient in North America who had an acute virus infection (*1*). This virus had limited sequence homology with parvovirus B19 (<30% aa similarity) despite a conserved genomic organization showing 2 large nonoverlapping open reading frames (ORF). Phylogenetic studies performed with near-complete sequences have proposed that human PARV4 can be described by ≥3 genogroups (*2*).

Little information is available about the natural history of the virus, and its clinical role remains unknown. Initial studies showed that PARV4 was present in the blood of febrile patients, intravenous drug users, and persons positive for hepatitis C virus or HIV at prevalences of 6%–30% (*3*–*5*). Two recent studies also showed the virus in cohorts of kidney transplant patients (*6,7*). PARV4 has also been identified in persons without apparent pathologic changes, such as blood donors (1.0%–3.95%), and in blood products negative for parvovirus B19 DNA (*4*–*6*). Virus DNA also was found in bone marrow and various tissues or organs, suggesting possible dispersion of the virus in diverse biologic locations (*8*).

To provide new insights into the dispersion of PARV4 in healthy persons, we assessed the frequency of PARV4 viremia in a cohort of blood donors by using a dual real-time assay. A total of 304 volunteer blood donors (154 men; mean age of all volunteers 40 years) living in southeastern France entered the study. The sex ratio (men:women: 1.03) and age distribution (19–65 years) of the cohort were considered representative of the population of blood donors at that time. Blood samples were collected in vacuum tubes (Vacutainer, SST, Becton Dickinson, Meylan, France) and centrifuged, and 1-mL plasma aliquots were stored at −80°C until use. Nucleic acids were extracted as described previously (*7*) and tested for PARV4 genomic DNA immediately after extraction to avoid freezing/thawing cycles.

Five microliters of the eluted material served for PARV4 DNA detection using real-time PCR TaqMan methods (StepOne Plus, Applied Biosystems, Courtaboeuf, France) and primers (ORF2) compatible with the detection of the 3 virus genogroups described at this time (*2,4,7*). Two probes were tested in separate amplification assays: PARV4-O (5′-FAM-TGTTCAACTTTCTCAGGTCCTACCGCCC-TAMRA-3′) (*4,7*) and PARV4-N (5′-FAM-TCCTACYGCCCSCTCCTCCTTCTT-TAMRA-3′). The second primer was designed after identification and sequencing of several in-house real-time PCR products (GenBank accession nos. FJ883557–61), and more particularly those detectable on agarose gels but showing negative signal with PARV4-O TaqMan assay because of mismatches identified on the probe recognition site (Figure). Amplification reactions were performed as described previously (*7*). Both TaqMan assays were estimated to detect as few as 10 copies of PARV4 DNA per reaction using dilutions of a synthetic template (*7*).

Fifteen (4.9%) blood donors gave positive signal with probe PARV4-O; 62 (20.4%) were positive with probe PARV4-N; 6 (2.0%) samples were positive in both assays. Overall prevalence for PARV4 DNA was 24.0% in the blood donors tested.

PARV4 origin of randomly selected PCR products from PARV4-O and PARV4-N TaqMan assays (5 each) was confirmed after cloning and M13 sequencing. Point mutations located on the PARV4-O recognition site were retrieved in amplicons originating from samples positive with PARV4-N assay (Figure). However, subsequent molecular studies aiming to characterize virus strains were not feasible because of the low titer of PARV4 DNA (<500 copies/mL of plasma) in positive samples. No specific correlation was identified between sex or geographic origin and PARV4 viremia, whereas analysis of the distribution of PARV4-positive

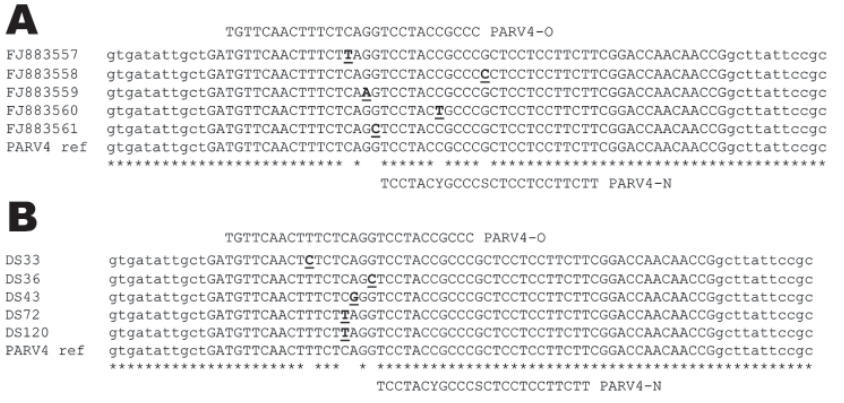

Figure. Alignment of parvovirus 4 (PARV4) sequences showing the location of the 2 probes used in the real-time experiments. A) Partial sequences used for the design of probe PARV4-N: PARV4 prototype isolate (AY622943) and in-house PCR products characterized initially (GenBank accession nos. FJ883557–61). B) Examples of point mutations located on the PARV4-O recognition site identified in amplicons originating from samples positive with PARV4-N assay. Mismatches identified in the alignments are underlined. Nucleotides shown in lowercase letters correspond to 5′/3′ ends of the real-time primers. Mismatches identified in the alignments are underlined and in **boldface**.

samples in age groups highlighted a relative homogeneity throughout the corresponding cohort.

Our study shows that PARV4 infection is readily detectable in French blood donors. Prevalence results using probe PARV4-O were comparable to those obtained in previous studies involving healthy persons originating from various countries (*4*–*6*). Conversely, the high prevalence obtained by using probe PARV4-N was unexpected because only 1 study demonstrated a higher value (45.7%) after the investigation of PARV4 DNA in bone marrow aspirates of AIDS patients from Italy (*9*).

This finding suggests a larger dispersion of PARV4 than expected initially in the general population and highlights the need for improvement in detection systems directed toward PARV4 DNA, particularly by interlaboratory collaborations, in direct connection with studies investigating PARV4 genetic diversity. These considerations are consistent with the recent description of a new PARV4 genogroup in humans and characterization of highly divergent variants in bovine and porcine species (*10*). In addition, such data raise the question of the consequent persistence of PARV4 infection in healthy persons. Future studies need to explore both dispersion and potential clinical impact of PARV4 on infected hosts.

Mhammed Touinssi, Nadège Brisbarre, Christophe Picard, Coralie Frassati, Bertrand Dussol, Rathviro Uch, Pierre Gallian, Jean-François Cantaloube, Philippe de Micco, and Philippe Biagini

Author affiliations: Université de la Méditerranée, Marseille, France (M. Touinssi, N. Brisbarre, R. Uch, P. Gallian, J.-F. Cantaloube, P. de Micco, P. Biagini); Service Immunogénétique-HLA, Etablissement Français du Sang Alpes-Méditerranée, Marseille (C. Picard, C. Frassati); and Centre de Néphrologie et de Transplantation Rénale, CHU, Marseille (B. Dussol)

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Address for correspondence: Philippe Biagini, UMR CNRS 6578 Equipe Emergence et Coévolution Virale, Etablissement Français du Sang Alpes-Méditerranée et Université de la Méditerranée, 27 Blvd Jean Moulin, 13005 Marseille, France; email: philippe.biagini@efs.sante.fr

Otomastoiditis Caused by *Mycobacterium abscessus*, the Netherlands

To the Editor: Nontuberculous mycobacteria (NTM) are increasingly recognized as human pathogens (*1*). Otomastoiditis is a rare extrapulmonary NTM disease type first described in 1976; *Mycobacterium chelonae–M. abscessus* group bacteria, which are rapidly growing NTM, are the most frequent causative agents and the disease mostly affects children (*1*–*3*). In the Netherlands, *M. chelonae–M. abscessus* group isolates have been reported from the otologic samples of an average of 2 patients annually since 2006, as compared to 6 patients in the preceding 10 years. This emergence is not a likely result of improved laboratory facilities or awareness in clinicians because liquid culture and molecular identification techniques predate the rise in notification and Dutch guidelines advise against performing cultures for chronic otorrhea.

We identified 10 patients from the national reference laboratory database with otologic samples yielding *M. chelonae–M. abscessus* group bacteria during January 1995–June 2007. We resubjected these isolates to molecular identification by *rpoB* gene sequencing (*4*) and performed a medical file review.

The *rpoB* gene sequencing showed that *M. abscessus* was the causative agent in all 10 cases; *M. abscessus* seems to have a predilection to cause otomastoiditis. Closely related *M. bolletii* and *M. massiliense* (*4*) were not found. Early reports identified *M. fortuitum* or *M. chelonae* as causative agents, which may be because the taxonomy of the rapidly growing NTM has long been debated (*2,4–7*); many of these agents may have, in fact, been *M. abscessus*. All primary isolates were found susceptible to clarithromycin and resistant to fluoroquinolones and aminoglycosides in our agar dilution method (*8*), which is not the recommended method for rapidly growing NTM (*1*). Two patients acquired clarithromycin resistance during treatment.

Clinical data are summarized in the online Appendix Table (available from http://www.cdc.gov/EID/content/16/1/167-appT.htm) and match those of previous studies (*3,5,6*). All patients had a history of ear infections and tympanostomy tube placement, previously associated with NTM disease (*7*). Nine patients had used ototopical medication, including quinolone antimicrobial agents (n = 5), steroids, aminoglycosides (n = 2), or both (n = 2). Clinical signs were nonspecific, with persistent tympanic membrane perforation, chronic painless otorrhea resistant to antimicrobial drug therapy, and hearing loss.

The fact that this disease primarily affects children, with a mean age of 6 years, may be related to age-specific environmental exposures, e.g., playing in sand pits or swimming (*9*). Two patients with *M. abscessus* otomastoiditis are siblings (patients 5 and 6); a clonal relationship between the causative bacteria is possible and should be investigated by molecular typing tools.

Primary isolates were from biopsy material (n = 5) or otorrhea fluid (n = 5) and were positive for acid-fast bacilli by direct microscopy for 9 patients. Five patients had a computed tomography (CT) scan performed, which showed fluid in the mastoid (n = 4), bone erosion of the mastoid (n = 2), and mucosal swelling (all); the online Appendix Figure (available from www.cdc.gov/EID/content/16/1/167-appF.htm) displays typical findings.

The mean interval between first symptoms and diagnosis of *M. abscessus* otomastoiditis was 155 days for (range 14–360 days). Otorrhea unresponsive to antimicrobial drug therapy should raise a clinical suspicion of NTM otomastoiditis (*3*), especially in patients with bone destruction visible on CT images. In patients with otorrhea unresponsive to antimicrobial drug therapy, routine CT scanning and *Mycobacterium* spp. cultures, preferably from tissue biopsies (*1*), may reduce diagnostic delay and prevent further damage.

Patients with *M. abscessus* otomastoiditis received drug treatment for a mean duration of 3 months (range 28–150 days) and 1.8 episodes of surgery. Five patients with *M. abscessus* otomastoiditis received clarithromycin monotherapy, 5 received multidrug therapy with fluoroquinolones (n = 3), fluoroquinolones, rifampin, and ethambutol (n = 1), or meropenem (n = 1 (online Appendix Table).

Complications of surgery comprised delayed wound healing (n = 4) and fistula formation (n = 2; online Appendix Table). Two patients underwent incus removal and later chain reconstruction surgery (patients 7 and 8). In 1 patient, the infection spread and caused culture-proven cervical lymphadenitis, a retroauricular abscess, fistula, and facial nerve palsy.

Eight patients were eventually cured, defined by symptomatic improvement and in some cases confirmed by negative cultures. Two patients were still receiving treatment at the time of data collection. Five patients had persistent conductive hearing loss after treatment (42%; range 30–80 dB; online Appendix Table).

American Thoracic Society guidelines for treatment of soft tissue and bone infections caused by *M. abscessus* advocate 4–6 months of therapy with a macrolide, an aminoglycoside and cefoxitin or a carbapenem, based on in vitro drug susceptibility test results, combined with surgical debridement when possible (*1*). Treatment regimens in this study deviated in duration and content; clarithromycin monotherapy is likely to invoke resistance (*1*) and no evidence supports fluoroquinolone use (*1*). Moreover, use of parenteral agents was limited; its reasoning was not generally captured during file review.

M. abscessus otomastoiditis is a serious, potentially emerging condition that affects children who have had previous infections, tympanostomy tubes, and ototopical antimicrobial drug or steroid use in the Netherlands. The diagnostic delay and treatment regimens warrant improvement to prevent deterioration, additional episodes of surgery, acquired drug resistance, and to prevent or limit permanent hearing loss.

Jakko van Ingen, Frank Looijmans, Piet Mirck, Richard Dekhuijzen, Martin Boeree, and Dick van Soolingen

Author affiliations: Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands (J. van Ingen, F. Looijmans, R. Dekhuijzen, M. Boeree); National Institute for Public Health and the Environment, Bilthoven, the Netherlands (J. van Ingen, D. van Soolingen); and Academic Medical Centre, Amsterdam, the Netherlands (P. Mirck)

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Address for correspondence: Jakko van Ingen, RIVM;LIS, National Mycobacteria Reference Laboratory (pb22), PO Box 1, 3720BA Bilthoven, the Netherlands; email: jakko.van.ingen@rivm.nl

Diseases Tracked by Using Google Trends, Spain

To the Editor: We read the article by Pelat et al. (*1*) with great interest and decided to explore whether this tool could be applicable for non-English and non-French speaking countries and, more specifically, for Spain. We compared the Google queries related to influenza-like illness (ILI) and chickenpox described by Pelat et al. (*1*), and constructed additional queries with symptoms and conditions frequently associated with ILI.

The weekly queries from January 2004 through February 2009 were downloaded from Google Insights for Search (*2*). We studied the correlation (Spearman ρ) of these queries with the data from the national reporting of notifiable diseases, available from the Spanish National Epidemiology Center website (*3*), assuming a maximum difference of 4 weeks.

The queries for *gripe* (Spanish for influenza) showed a maximum correlation (ρ = 0.70) 2 weeks before the declared ILI (DILI). When excluding the terms for *aviar* (avian) and *vacuna* (vaccine), the correlation peak (ρ = 0.81) was likewise observable 2 weeks before the DILI. The maximum correlation observed for symptom queries was for *tos* (Spanish for cough) 2 weeks before the DILI (ρ = 0.74); for conditions associated with influenza the correlation was for *neumonía* (Spanish for pneumonia, accented or unaccented) 2 weeks after the DILI (ρ = 0.84). The queries for *varicela* (Spanish for chickenpox) showed a maximum correlation (ρ = 0.96) 1 week after the declared illness, as observed by Pelat et al (*1*).

In conclusion, our study points out the utility of Internet queries for the surveillance of ILI and chickenpox in Spain. In the case of ILI, this information can be used as an early warning tool used complementarily to standard surveillance systems. More detailed studies are necessary regarding the usefulness and limitations of this tool in Spain, as well as in other contexts.

Antonio Valdivia[1] and Susana Monge-Corella[1]

Author affiliations: Hospital Universitario de La Princesa, Madrid, Spain (A. Valdivia); and Instituto de Salud Carlos III, Madrid (S. Monge-Corella).

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Address for correspondence: Antonio Valdivia, c/Quevedo, no.º 8, piso 1º C, Alcalá de Henares 28807 Spain; email: tonyvald@hotmail.com

[1]These authors contributed equally to this article.

Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Detection of Newly Described Astrovirus MLB1 in Stool Samples from Children

To the Editor: We read with interest the article by Finkbeiner et al. describing an epidemiologic survey of newly described astrovirus MLB1 (AstV-MLB1) conducted in the United States in 2008 (*1*). This study was an extension of recently published reports of characterization of AstV-MLB1 from a fecal sample obtained in Australia in 1999 (*2*,*3*). These studies provide evidence of a divergent group of astroviruses and their etiologic association with human disease.

However, the occurrence of a MLB1-like AstV in humans has already been documented. Walter identified a novel AstV in an 8-month-old child with diarrhea in Mexico in 1991 (*4*). In that study, Walter screened fecal samples for AstVs by using a variety of techniques. Sequencing of selected PCR products identified a unique virus that had typical AstV morphologic appearance, but was nonreactive with human AstV-specific monoclonal or polyclonal antibodies. Phylogenetic analysis of fragments of open reading frame 1a (ORF1a) and ORF2 genome regions of this virus strain (M3363) showed that it was only distantly related to other mammalian AstVs, including human AstVs (*4*). This sequence divergence from canonical human AstVs suggested that M3363 might have been transmitted from an animal reservoir (*4*,*5*).

When we reanalyzed ORF1a of M3363, we found that this strain was actually an MLB1-like AstV with >98% amino acid similarity to prototype and strains from the United States (Figure) that dated back to 1991. The fact that such closely related viruses were found in a scattered temporal and spatial pattern in children may indicate that MLB1-like AstVs represent a true human enteric virus, which was probably overlooked in the absence of adequate diagnostic reagents and protocols.

Krisztián Bányai, Edina Meleg, Paschalina Moschidou, and Vito Martella

Author affiliations: Veterinary Medical Research Institute, Budapest, Hungary (K. Bányai); University of Pécs, Pécs, Hungary (K. Bányai E. Meleg); and University of Bari, Valenzano, Italy (P. Moschidou, V. Martella)

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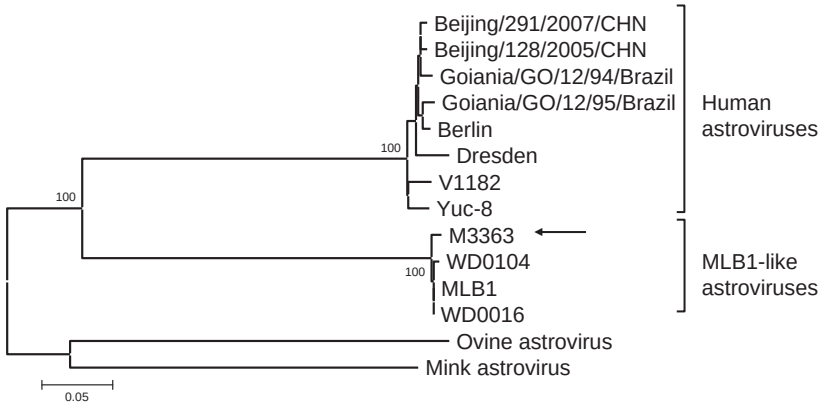

Figure. Neighbor-joining tree based on partial sequences of open reading frame 1a protein of human and animal astroviruses. Amino acid sequences of the Mexican M3363 strain (arrow) were obtained from Walter (*4*); other sequences were obtained from GenBank. Bootstrap values ≥90 are indicated. Scale bar is proportional to genetic distance and indicates nucleotide substitutions per site.

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Address for correspondence: Krisztián Bányai, Veterinary Medical Research Institute, Hungarian Academy of Sciences, Hungária Krt. 21, H-1143, Budapest, Hungary; email: bkrota@hotmail.com

In Response: We thank Bányai et al. (*1*) for drawing attention to the unpublished data of Walter (*2*), which was not part of the peer-reviewed literature at the time we described the complete genome of astrovirus MLB1 (*3*) or when we described our epidemiologic survey of stools collected in St. Louis, Missouri, USA (*4*). The results of Walter extend the known geographic range of astrovirus MLB1 to include Mexico, thus supporting our recent proposal that astrovirus MLB1 is likely to be globally widespread (*4*). We look forward to including the partial sequence generated by Walter in future analyses of astrovirus MLB1 genetic diversity. We strongly encourage Bányai et al. to submit their sequence data to any

of the publicly accessible and searchable international sequence databases such as GenBank, the European Molecular Biology Nucleotide Sequence Database, or the DNA Database of Japan so that it can be readily accessed by the scientific community.

Stacy R. Finkbeiner, Binh-Minh Le, Lori R. Holtz, Gregory A. Storch, and David Wang

Author affiliation: Washington University School of Medicine, St. Louis, Missouri, USA

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Address for correspondence: David Wang, Washington University School of Medicine, Campus Box 8230, 660 S Euclid Ave, St. Louis, MO 63110, USA; email: davewang@borcim.wustl.edu

Optimal Therapy for Multidrug-Resistant *Acinetobacter baumannii*

To the Editor: I read with interest the article by Doi et al. about a lung transplant patient presumed to have *Acinetobacter baumannii* ventilator-associated pneumonia (*1*), but some points deserve comment. *A. baumannii* is a relatively avirulent organism that frequently colonizes body fluids. For multidrug-resistant strains, antimicrobial drug selection is limited. Resolution of this patient's pulmonary infiltrates suggests that they were not caused by *A. baumannii* that persisted in respiratory secretions. Because *A. baumannii* persisted in this patient's respiratory secretions, colistin and cefepime were given. Colistin is an antimicrobial drug with low resistance potential; but when given by inhalation, it may lead to drug resistance (*2*,*3*).

Doi et al. stated that the patient's *A. baumannii* strain lacked susceptibility to all available antimicrobial drugs but that cefepime and tigecycline were intermediately susceptible (MICs 16 μg/mL and 2.0 μg/mL, respectively) (*1*). Intermediate susceptibility may also be interpreted as relatively susceptible when achievable serum or tissue concentrations exceed the MIC. The article did not mention the dosages of colistin, tigecycline, and cefepime. A 2-g dose of cefepime given intravenously results in peak serum levels of ≈163 μg/mL with a relatively low volume of distribution (0.29 L/kg), which would not be expected to eradicate *A. baumannii* in respiratory secretions. High-dose intravenous tigecycline (initial dose of 200 mg followed by 100 mg daily) has been used to treat *A. baumannii*, achieving peak concentrations of ≈3 μg/mL, which exceed the isolate's MIC of 2 μg/mL, and a high volume of distribution (8 L/kg), which would be expected to eradicate *A. baumanii* in respiratory secretions.

Optimal treatment for *A. baumannii* depends on susceptibility, pharmacokinetic principles, and site of infection. For optimal effectiveness, cefepime and tigecycline should have been given at high doses. To prevent potential resistance, antimicrobial drugs should not be given by inhalation (*3*). The alleged advantage of inhalation therapy is high local drug concentrations, but concentrations in some alveoli may be subtherapeutic (*3*). If possible, tigecycline should not be used to treat *A. baumannii* infections; however, if it is used, high doses should be given to optimize its pharmacokinetic attributes (*4*,*5*).

Burke A. Cunha

Author affiliations: Winthrop-University Hospital, Mineola, New York, USA; and State University of New York, Stony Brook, New York, USA

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Address for correspondence: Burke A. Cunha, Infectious Disease Division, Winthrop-University Hospital, Suite 432, 222 Station Plaza North, Mineola, NY 11501, USA; email: emccaffrey@winthrop.org

In Response: We welcome Burke A. Cunha's letter (*1*) but disagree with him regarding 4 issues. First, he states that colistin has a "low resistance potential" (*1*). Although colistin has had low resistance for a long time, we are concerned that development of resistance to colistin is a growing problem. Heteroresistant *Acinetobacter* isolates are readily found (*2*). Lee et al. (*3*) recently found decreases in polymyxin B susceptibility during therapy.

Second, Cunha states, "Intermediate susceptibility may also be interpreted as relatively susceptible when achievable serum or tissue concentrations exceed the MIC" (*1*). We support the concepts that break points are artificial and that pharmacodynamic optimization may enable treatment for some organisms that are not susceptible to certain antimicrobial drugs. However, consideration of more than the MIC is necessary. Cefepime is a time-dependent bactericidal drug; therefore, effectiveness depends more on the time that the concentration of drug is above the MIC than on peak serum concentrations.

Third, Cunha states, "For optimal effectiveness, cefepime and tigecycline should have been given at high doses" (*1*). Unfortunately, in the current era of antimicrobial drug resistance, there are no "shoulds." The high-dose tigecycline regimen that Cunha proposes for multidrug-resistant organisms has never, to our knowledge, been evaluated in randomized trials or even in large prospective evaluations. We all must admit that we do not know the optimal way to treat such infections and that we need rigorous evaluation of novel regimens. Anecdotal experience must not be translated into imperatives.

Fourth, Cunha states that "antimicrobial drugs should not be given by inhalation" (*1*). We agree that widespread use of aerosolized antimicrobial drugs cannot be recommended. However, aerosolized amikacin with a new-generation nebulizer is being tested in phase 2 clinical trials (*4*). As to the potential utility of aerosolized antimicrobial drugs, we prefer to keep an open mind pending the results of these trials.

David L. Paterson and Yohei Doi

Author affiliations: University of Queensland Centre for Clinical Research, Brisbane, Queensland, Australia (D.L. Paterson); and University of Pittsburgh, Pittsburgh, Pennsylvania, USA (Y. Doi)

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Address for correspondence: David L. Paterson, UQCCR Building, Royal Brisbane and Women's Hospital, Brisbane, Queensland 4029, Australia; email: david.antibiotics@gmail.com

Case Studies in Infectious Disease

Peter M. Lydyard, Michael F. Cole, John Holton, William L. Irving, Nino Porakishvili, Pradhib Venkatesan, and Katherine N. Ward

Garland Science, New York, NY, USA, 2010
ISBN: 978-0-8153-4142-0
Pages: 608; Price: US $50.00

The authors have assembled a collection of case studies about the 40 infectious diseases that cause the most illness and death worldwide. Each chapter begins with a brief case presentation. This example is followed by a section on microbiologic aspects of the organism, including the pathophysiology of infection. The host response is then described, followed by a discussion of clinical manifestations, diagnostic methods, and treatment options, including prevention. A summary highlights salient points of each section. References, suggestions for further reading, and websites for additional information are all provided. Chapters conclude with a series of questions (answers are given at the end of the book).

The book is meant for use by medical students in a microbiology course, but it can also be used by any clinician who wants a concise review of the pathogens that cause infectious diseases. The case presentations are short and not presented as conditions having an unknown cause, but rather they serve as a clinical starting point to open discussion. The microbiology sections are geared more toward the student in a microbiology course and tend to have more details than are needed by a practicing clinician. The sections on patient symptoms are generally quite good and are inclusive. The varied clinical manifestations, particularly of the tropical diseases, are presented in an easy-to-understand format. The level of detail given provides a thorough yet succinct picture of each disease. The sections on diagnosis are generally inclusive, although a few did not mention some available diagnostic options used in the United States; this may have been due to differences in the availability of some tests in the United Kingdom, where many of the authors are based. The treatment sections tend to be abbreviated and frequently do not include the length of therapy and some other details that a practicing clinician would want to know. For those needing specific therapy guidelines, another source will be necessary.

The summary sections are quite good and are an excellent quick reference source if one wants just the highlights and a brief summary about the pathogen and disease. The questions at the end tend to be multiple choice with several possible correct answers for each one; they are not structured to prepare for testing purposes (such as for a board review). The websites are helpful sources for downloadable slides as well as for further information if more details are wanted.

The only chapter that was confusing was that on coxsackie viruses. The authors kept referring to other enteroviruses. The chapter could benefit from either fewer references to other enteroviruses or renaming it to be a section on enteroviruses in general.

Case Studies in Infectious Disease is a valuable compilation of information on the most common diseases that cause illness and death worldwide. The presentation format with distinct sections makes it readable and well suited for either students just learning about the pathogens causing infectious disease or clinicians who need an update. The level of detail is well thought out and gives the reader a useful summary of each pathogen and disease state. The condensed presentations make it a good reference source for those with insufficient time to read through more detailed textbooks.

Philip S. Brachman, Jr.
Author affiliations: Atlanta ID Group, Atlanta, Georgia, USA; Emory University School of Medicine, Atlanta; and Mercer University, Atlanta

DOI: 10.3201/eid1601.091254

Address for correspondence: Philip S. Brachman, Jr, Atlanta ID Group, Piedmont Hospital, 2001 Peachtree Rd, Ste 640, Atlanta, GA 30309, USA; email: pbrac01@emory.edu

Infectious Disease: Pathogenesis, Prevention and Case Studies

Nandini Shetty, Julian W. Tang, and Julie Andrews, editors

Wiley-Blackwell, Chichester, UK, 2009
ISBN: 978-1-4051-3543-6
Pages: 664; Price US $129.95

The organizing vision of this textbook is neither a taxonomic outline of the microbiologic world nor an epidemiologic understanding of our evolving insights into epidemics. Rather it is translational, ecologic, holistic, and distinctly clinical. It is a fun and readable book that engages the imagination and retains the interest of the clinically oriented reader while conveying an understanding of the direct implications of molecular characteristics of infectious agents to the practice of medicine.

The chapters in Part 4, Infections of Global Impact, and Part 5, Emerging and Resurgent Infections, are especially likely to fire the imaginations of students in introductory clinical microbiology or infectious disease classes. The chapters in Part 1, General Principles of Infectious Diseases, will equally effectively assist infec-

tious disease professionals in mid to late career who seek an easy and enjoyable way to refresh and update their understanding of such topics as microbial structure, mechanisms of action of antimicrobial agents, and laboratory approaches to investigation and diagnosis.

That said, this is not a book for all audiences. The success with which it integrates the microbiologic world with the world of medical practice sacrifices approaches that might engage persons for whom the end point of microbiologic interest is not human disease. It is neither balanced nor comprehensive enough to function as a definitive reference. Also, chapters vary in quality. In the chapter on host defenses, for example, attempts to simplify occasionally lead to unfortunately loose statements such as "Within man, there are certain well known racial differences in disease susceptibility…," among which it identifies "Dark skinned individuals have an increased susceptibility to coccidioidomycosis." These statements confuse me.

I am aware that early 20th century surveys identified lower prevalence of hookworm disease among residents of the rural American South who are of African descent than among their neighbors of European descent despite similar living habits, environmental conditions, and levels of impoverishment. Accurately or not, these differences were attributed to racial variability in the effectiveness of skin as a barrier against larval hookworm invasion. But what can be the relevance of dark skin to an infection that invades primarily by inhalation of spores distributed in the soil of the American Southwest, Mexico, Central, and South America? In fact, susceptibility to primary coccidioidal infection is not affected by racial background. The frequency of dissemination is higher among Filipinos, Hispanics, and blacks. This frequency may reflect genetic host factors, or it may identify ethnicity or socioeconomic status as a marker for risk of environmental exposure to larger inocula. The jury is out.

Similar occasional failures to speak with precision distracted me and thus detracted from what is overall an excellent book. In contrast, the chapters on influenza, infections in the returning traveler (a tutorial in how to think like a travel medicine specialist), and emerging and resurgent infections were excellent.

Louisa Chapman

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: 10.3201/eid1601.091408

Address for correspondence: Louisa Chapman, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D75, Atlanta, GA 30333, USA; email: lchapman@cdc.gov

Some Haphazard Aphorisms for Epidemiology and Life

John M. Cowden

For more than a quarter of a century, I have accumulated from unexpected sources a ragbag of quotations relevant to the practice of epidemiology. I have even coined a few myself. This paper brings many of them together in one place and acknowledges their origins for the amusement and instruction of colleagues. My motives are not entirely altruistic: I do hope to benefit a little from the reflected glory of those I quote—and of course any publication is a bonus.

The collection is necessarily idiosyncratic and personal. It favors quotes from outside public health rather than the better known sayings of experts in the field. Because I am an epidemiologist, it is biased towards epidemiology rather than microbiology or infection control. It pretends to be neither comprehensive nor representative of anything but my own taste, and it obviously cannot include everyone's favorites. It may be flawed, but I will consider it a success if, like the British Broadcasting Corporation, it "informs, educates, and entertains."

Methods

I consulted my memory for quotations of interest to epidemiologists and health protection professionals. Where possible, I confirmed the actual words of quotations and cited their origins. I have categorized the entries subjectively by topic. I have flouted the convention that the Methods section should contain methods exclusively, and that the results and discussion should be confined to their designated sections.

My citations are haphazard. Having tried hard and failed to identify the first quote, I took the advice of W.C. Fields (1880–1946), "If at first you don't succeed, try, try again. Then quit. There's no point in being a damn fool about it." I have accordingly described my sources in as much detail as possible with minimum effort. "If a job's worth doing, it's worth doing well enough for the purpose in hand—to do it any better is wasted effort" (Anonymous). My citations, although insufficiently precise for a scientific paper, match or exceed those in most books of quotations. If a reader wants to run a quote to earth, there is enough information to get them started.

I have tried to avoid clichés (though I mention "lies, damned lies, and statistics" in a comment), the knowledge of which confers little cachet on the user, in favor of less widely known and wittier quotes. If there are any clichés, I hope I have been true to the spirit of Samuel Goldwyn (1879–1974), who declared, "We don't want old clichés. Let's have some new clichés."

Most of the quotes speak for themselves, but occasionally I have been unable to refrain from comment. I have not done so systematically.

Results

Surveillance

"Being approximately right most of the time is better than being precisely right occasionally."—Anonymous.

I first saw this saying in a paper by Tom Reilly, an Australian microbiologist. Tom directed me to his source, Richard Platt, an American epidemiologist. Richard acknowledged using it, but denied knowledge of its origin. My search thus involved 3 continents and was ultimately unsuccessful. I have made less effort with subsequent quotes.

"It is the mark of an educated mind to rest satisfied with the degree of precision which the nature of the subject admits and not to seek exactness where only an approximation is possible."—Aristotle (philosopher, 384–322 BC). "Consistency is more important than accuracy."—John M. Cowden (epidemiologist, 1953–)

Although this has the virtue of brevity, it is (aptly) not quite true. Both are important, but in surveillance absolute accuracy is unachievable (see Aristotle). Consistency can

Author affiliation: Health Protection Scotland, Glasgow, Scotland, UK

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be achieved and failure to do so is extremely damaging to credibility among the ignorant classes (especially journalists), as has been observed:

"You cannot hope to bribe or twist / Thank God the British journalist / But when you see what he will do unbribed / There's no occasion to."—Humbert Wolfe (poet, 1885–1940). "Not everything that counts can be counted, and not everything that can be counted counts.—Albert Einstein (physicist, 1879–1955)."

Good old Albert. Got himself a reputation as one of the cleverest men in history but still managed to get his girlfriend pregnant accidentally. Hence (I paraphrase):

"It just goes to show that God gave man enough blood to run his brains and his genitals, but not at the same time."—Robin Williams (actor, 1951–). "'When you are a bit older' a judge in India once told an eager young British civil servant, 'you will not quote Indian statistics with that assurance. The government is very keen on statistics—they collect them, add them, raise them to the nth power, take the cube root and prepare wonderful diagrams. But what you must never forget is that every one of those figures comes from the chowty dar, or village watchman, who just puts down what he damn pleases.'"—Josiah Charles Stamp (economist, 1880–1941, in "Some Economic Factors in Modern Life").

I found this quote, not in the original text, but in Darrell Huff's "How to Lie with Statistics," published by Victor Gollancz in 1954. I am indebted to a reviewer for drawing my attention to its source.

Outbreak Investigation

"You take a mess. You work hard. You clean it up. Life distilled. All the rest is nonsense."—Harrison Ford (actor, 1942–).

As applicable to outbreak management as it is to life. I'm sure I first saw this in an interview in a British Sunday newspaper, but an online search produced the link www.mensjournal.com/harrison-ford.

"Prejudice is a great time saver. You can form opinions without having to get the facts."—E.B. White (writer, 1899–1985). "It is incident to physicians, I am afraid, beyond all other men, to mistake subsequence for consequence."—Samuel Johnson (lexicographer, 1709–1784). "If it looks like a duck, and quacks like a duck, we have at least to consider the possibility that we have a small aquatic bird of the family Anatidae on our hands."—Douglas Adams (science fiction writer, 1952–2001). "The race is not always to the swift, nor the battle to the strong. "—Ecclesiastes 9:1. "The race is not always to the swift, nor the battle to the strong—but that's the way to bet!"—Damon Runyon (writer, 1884–1946).

Although Runyon gives this excellent advice, an echo of what I was taught as a student—"Common things are commoner"—he is wise enough to know that you should not always bet on the sure thing. Hence:

"One of these days in your travels, a guy is going to come up to you and show you a nice brand-new deck of cards on which the seal is not yet broken, and this guy is going to offer to bet you that he can make the Jack of Spades jump out of the deck and squirt cider in your ear. But, son, do not bet this man, for as sure as you are standing there, you are going to end up with an earful of cider."—Damon Runyon (in "The Idyll of Miss Sarah Brown").

And if you wonder how Runyon can hold these two conflicting points of view (i.e., you should bet on what seems most likely, but a bet on a rock-solid certainty will be wrong), see his quote in the Statistics section.

"When you have eliminated the impossible, whatever remains, however improbable, must be the truth."—Arthur Conan Doyle (author, 1859–1930, in "The Sign of Four"). "It is a capital mistake to theorize before you have all the evidence. It biases the judgment.—Arthur Conan Doyle (in "A Scandal in Bohemia")."

The latter quote is laudable but impractical advice for practicing public health professionals, who must often make judgments and act (or deliberately refrain from doing so) on the basis of available evidence.

"The man who insists on seeing with perfect clearness before he decides, never decides."—Henri-Frederic Amiel (philosopher, 1821–1881). "If you choose the most likely option and it turns out to be wrong, it was still the right choice. Presented with the same evidence, you should make the same choice again. If you're wrong again, re-evaluate your information."—John M. Cowden. "Life can only be understood backwards, but it must be lived forwards."—Soren Kierkegaard (philosopher, 1813–1855).

How many times have you intervened on the basis of a hypothesis, subsequently failed to confirm it, and been accused of being precipitate? Or alternatively, waited for the case–control study or microbiology and been accused of unnecessary caution? You can only be sure when to act in retrospect. That is almost worth quotation status by itself!

"Chance favors the prepared mind."—Louis Pasteur (chemist, 1822–1895). "You won't be surprised that diseases are innumerable—count the cooks."—Seneca (philosopher, 4 BC–65 AD). "Circumstantial evidence is not the same as weak evidence. A naked man in your wife's wardrobe is circumstantial evidence of infidelity."—John M. Cowden. "All business proceeds on beliefs, or judgments, or probabilities, and not on certainties."—C.W. Eliot (president of Harvard University, 1834–1926).

Research

"Of course we don't know what we're doing, that's why it's called research."—Albert Einstein.

I have found this in a couple of places online, but not in a primary or even reliable looking secondary source, so, as they say in Scotland, "I hae ma doots."

"The tragedy of science is the slaying of a beautiful hypothesis by an ugly fact."—T.H. Huxley (scientist, 1825–1895).

Huxley was an ardent supporter of the theory of evolution and was known as "Darwin's Bulldog." His grandson, Aldous (who died on the day Kennedy was shot), was a mystic—which just goes to show that evolution does not necessarily advance in a straight line.

"Belief is no substitute for arithmetic."—Henry Spencer (computer programmer and Internet pioneer, 19??–).

Although Henry Spencer has a Wikipedia entry, much of which seems to be in a foreign language, I have been unable, in a grueling 15-minute search, to ascertain his date of birth.

"To every complex question there is a simple answer … and it is wrong."—H.L. Mencken (writer and wit, 1880–1956). "Semmelweis was right—but he died a broken man and was buried in a pauper's grave."—John M. Cowden

The example of Ignatz Semmelweis (1818–1865) contradicts Mencken's view. Before the invention of microbiologists, he proposed and confirmed handwashing as the simple solution to the massive maternal mortality rates at the Vienna General Hospital. He proved, however, that being right does not necessarily make you successful.

"If you can't explain it simply, you don't understand it well enough."—Albert Einstein. "I have yet to see any problem, however complicated, which, when you look at it in the right way, did not become still more complicated."—P. Anderson (science fiction writer, 1926–2001). "A scrutiny so minute as to bring an object under an untrue angle of vision, is a poorer guide to a man's judgment than a sweeping glance which sees things in their true proportion."—A.W. Kinglake (historian, 1809–1891).

I found this last quote in Winston Churchill's "My Early Life" and nowhere else, so it must be obscure. Churchill attributes it only to "Kinglake," assuming his reader would know who Kinglake was. Well I did not, so I did an Internet search. He produced an 8-volume history of the Crimean War, which says a lot for his own sense of proportion.

"It's a scientist's right to re-examine his theory with each new piece of evidence."—Eli Talbert (screenwriter, 19??–, in the "CSI: Crime Scene Investigation" episode "$35K O.B.O.").

Like Sherlock Holmes, Talbert's character Gil Grissom could furnish many quotations, but this is my favorite. I should have preferred him to say "duty" rather than "right," but it did not fit the context.

"In great affairs we ought to apply ourselves less to creating chances than to profiting from those that offer."—F. de la Rochefoucauld (writer, 1613–1680).

The meaning is not immediately apparent, but I think an epidemiologist should take from this that it is better to take advantage of an outbreak and investigate it in detail, rather than design a research project from scratch. It goes well with the previous Pasteur quote. But as an anonymous Frenchman said, "That's all very well in practice, but will it work in theory?"

"Editors are often accused of fussing over trivialities. What is contained in the paper is said to be of far greater importance than the way in which it is set out. This may be true. Nevertheless carelessness and inconsistency in the preparation of a paper inevitably suggest carelessness and inconsistency in the conduct of the work itself and prejudice the reader against the author. A reasonable compromise is desirable, but accuracy and lucidity must be insisted on."—Graham Wilson (microbiologist, 1895–1987).

This is from Sir Graham's "Guidance in Preparing the Typescript of Scientific Papers." It provides excellent, unpedantic advice (note his use of a preposition with which to end a sentence). Sir Graham was director of the late lamented Public Health Laboratory Service in England. My own dog-eared copy of the guidance was a gift from the best editor ever to have had the dubious pleasure of savaging my work, Professor Norman Noah. The guidance states that requests for reprints "should be addressed to the editor, Monthly Bulletin (Section II), Central Public Health Laboratory, Colindale Avenue London NW9." Good luck. I am sure that Sir Graham and Professor Noah would have agreed with the next 2 pieces of advice:

"In composing, as a general rule, run your pen through every other word you have written; you have no idea what vigour it will give your style."—Sydney Smith (clergyman, 1771–1845). "Brevity in writing is the best insurance for its perusal."—Rudolf Virchow (pathologist and polymath, 1821–1902). "A microbiologist is someone who is happy to use a colleague's toothbrush, but not his methods."—Anonymous. "Knowledge is of two kinds: we know a subject ourselves, or we know where we can find information upon it."—Samuel Johnson.

Statistics

"To understand God's thoughts we must study statistics, for these are the measure of His purpose."—Florence Nightingale (nurse and pioneer in the use and presentation of statistics, 1820–1910). "All models are wrong, but some are useful."—George Box (statistician, 1919–)

I am indebted to one of this paper's reviewers for the previous quote.

"Cowden's first rule of statistics: statistical significance is not the same thing as practical importance. Cowden's second rule: the more complex the test required to show statistical significance, the less important to an individual the association is likely to be. Lind didn't need a p-value

to show that lime juice prevented scurvy. Cowden's third rule: the word 'significant' without the prefix 'statistical' is usually a coward's way of implying 'important' without mathematical evidence."—John M. Cowden. "People commonly use statistics like a drunk uses a lamppost: for support rather than for illumination."—Mark Twain (writer and humorist, 1835–1910).

I have seen this last quote attributed to lots of people, but to Mark Twain more often than anyone else. He may have nicked it, however, because he also used Sir Benjamin Disraeli's (1804–1881) "lies, damned lies, and statistics" in his autobiography without attribution. Maybe he anticipated the following advice:

"Plagiarize! Plagiarize! Remember why God gave you eyes!"—Tom Lehrer (mathematician and satirist, 1928–).

"I came to the conclusion long ago that all life is six to five against."—Damon Runyon. "If you wait long enough the improbable is bound to happen."—John M. Cowden. "Random is not the same as haphazard. Random is likely to be representative, haphazard is likely to be biased. Random is much more difficult to achieve."—John M. Cowden. "Most people have more than the average number of legs."—Anonymous. "The plural of anecdote is data."—Raymond Wolfinger (political scientist, 1931–).

I have found the opposite of this last quote, "The plural of anecdote is not data," attributed to numerous people, though I disagree with it. I also like the anonymous riposte "the plural of datum is not proof."

"There are three kinds of epidemiologist: those who can count and those who can't."—Anonymous (adapted by John M. Cowden).

Discussion

Winston Churchill (1874–1965) observed, "It is a good thing for an uneducated man to read books of quotations. The quotations, when engraved upon the memory, give you good thoughts." Four hundred years earlier, Michel Eyquem de Montaigne (1533–1592) said, "I quote others only the better to express myself." If I'd said that yesterday, I doubt if it would make it into a book of quotations, but Montaigne reinforces Churchill's point: quotations are often very helpful. I hope those in this collection are also amusing. Having made an effort to identify their origins, I hope that if anyone uses any of them, they reference this paper, which will have the benefit, for me, of enhancing my CV. If, however, they try to pass off a saying as their own, they will be in good company. When the painter James McNeil Whistler (1834–1903) made a witty remark in the hearing of the writer Oscar Wilde (1854–1900) and Wilde said, "I wish I'd said that," Whistler replied "You will, Oscar. You will."

Acknowledgment

Kevin Pollock is thanked for technical expertise and discussion.

Dr Cowden is a consultant epidemiologist in the Gastrointestinal/Zoonoses Section at Health Protection Scotland. His interests include the public health, epidemiologic, and microbiologic aspects of infectious intestinal disease and zoonoses.

Address for correspondence: John M. Cowden, Health Protection Scotland, Clifton House, Clifton Pl, Glasgow, Scotland G3 7LN, UK; email: john.cowden@hps.scot.nhs.uk

Erratum—Vol. 15, No. 11

Two numbers were listed incorrectly in the article Dengue Virus Serotype 4, Northeastern Peru, 2008 (B.M. Forshey et al.). The final sentence before the Conclusions should read: "This lineage is distinguished from previously reported DENV-4 genotype II strains by 3 conserved amino acid variations in the E protein: S64L, A222T, and S354A." The article has been corrected online (www.cdc.gov/eid/content/15/11/1815.htm).

Pieter Claesz (1597–1660) Still Life with Turkey Pie (1627) Oil on wood panel (75 cm × 132 cm) Rijksmuseum, Amsterdam, the Netherlands

Tasty Bits a Dutch Treat

Polyxeni Potter

"The old Dutch whalers of two or three centuries ago," wrote Herman Melville in Moby-Dick, "were high livers." The author arrived at this conclusion by transcribing what a ship with a crew of 100 carried for each month at sea in 1636 into what a fleet of 180 Dutch whalers might carry: "400,000 lbs of beef, 60,000 lbs Friesland pork, 150,000 lbs of stock fish, 550,000 lbs biscuit, 72,000 lbs soft bread, 2,800 firkins of butter … 10,800 barrels of beer." This tale of whale fishery is not unlike other accounts of legendary feasting in the Netherlands during the 17th century, the Dutch Golden Age.

Though fishing was a substantial part of the Dutch economy, it was trade with countries near and afar that converted tiny Holland into a vast empire. Described as "the best fed population in Europe," the Dutch enjoyed a high standard of living. Food that was not grown domestically was imported from all over the world, and even the poor "were supplied with fare meant to approximate to the diet of the more fortunate." Though generally avert to excess, Netherlanders celebrated the birth of a child, the New Year, the purchase of a house, the departure or return of a family member, the wedding or funeral of a friend with a sumptuous feast, tinged with the tastes of India and the Spice Islands and washed down with the best wines from Spain, Italy, and France.

Commercial prosperity created a large affluent society, demand for exotic goods and luxuries, and thriving art markets in Delft, Haarlem, The Hague and other cities. Though shaken by the iconoclastic fervor of Protestant Reformation as it spread throughout northern Europe and the loss of patronage by the Catholic Church, the art scene remained competitive to accommodate burghers seeking artwork for their large homes. Artists became specialized to meet the collectors' interests in secular subjects that showcased their newly acquired wealth through genre scenes and still life paintings.

Still life paintings, the "foot soldiers in the army of art," as they have been called for their modest ranking, found a niche as an independent form beginning in the 16th century at the same time in Italy, Spain, and northern Europe. While natural philosophers turned to investigation as a tool for learning and in Delft, Antonie van Leeuwenhoek was seeing "with great wonder…many very little living animalcules" with his microscope, artists shifted their focus from

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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mythologic subjects to landscapes, animals, and plants. Art captured the bounty of God's creation, providing not only an affirmation of wealth but also an opportunity for symbolism and moral lessons, in line with Calvinist doctrine. In the midst of abundance, the Dutch feared becoming corrupted by it, so their art often contained proverbs and other warnings against frivolity and excess.

Still lifes encompassed all sorts of inanimate objects and even some small animals or insects. Some, called banquet pieces, were lavish arrangements of food and table settings. Others portrayed breakfast offerings of herring, bread, beer, or wine. Fine artists of the period painted these, among them Pieter Claesz, a master of visual feasts. Very little is known about Claesz' life. A native of Berchem, a village near Antwerp, he likely began his studies there but later moved to the larger art community in Haarlem, where he was noticed for his innovative style. He was very prolific. His work was widely imitated, copied, and reproduced during his lifetime. His son, Nicolaes Pietersz Berchem became an important landscape artist.

Claesz painted scenes with bowls, glasses, and other objects luxuriously appointed and filled with spices, fruit, and allusions to the fleeting nature of earthly pleasures and the vanity of life. He pioneered a nearly monochromatic palette with subdued hues and achieved remarkable naturalism by focusing on how light affects a scene and how objects are perceived against each other and as reflections on highly polished surfaces. Along with still life greats Willem Heda and Clara Peeters, he created the subtle and refined banquet style identified with the Haarlem art scene.

In Still Life with Turkey Pie, on this month's cover, the muted colors alone betray Claesz' genius. They allow the light to reflect off the tumbler of white wine, the nautilus shell goblet, the plates, even the glistening olives and grapes. A glimpse of the room can be had on the pewter pitcher. The turkey, having supplied the namesake feast, also donated its light-streaked feathers and beak as decorative headdress for the raised pie.

The composition engages the senses and turns a still life into a dynamic one, a banquet in progress, even without humans. We are invited to sample. A plate and knife balance off the edge of the table within reach, a serving spoon rests atop the half-eaten mince pie. The lemon is half peeled. The tablecloth, live with folds and shadows of objects and set against a rug, bleeds to the edge. A Delft bowl rests invitingly at an angle filled with fruit and crowned with leaves and twigs. The formality is broken by bread placed directly on the cloth, along with a star fruit and nuts cracked and strewn between the plates along with slices of lemon and empty seashells.

Mince pie, flavored with currants and spices, was a treat for special occasions, as were imported lemons and olives. Salt and the pepper poured out of a rolled paper cone made a fitting complement to the luxury of oysters and white bread. The fruit was meant to whet the senses and stimulate the appetite as were the shellfish, a didactic treat, what with their empty shells hinting at the ephemeral.

The abundance and variety of food so eloquently celebrated in art of the Golden Age did not escape the attention of those responsible for its safe distribution and use. The quality of perishables was monitored, and government regulations banned the sale of rotting vegetables and fruits. Bakers were required to wash their feet with hot water and nontoxic soap before using them to knead tough rye dough. Food preservation techniques, among them smoking, drying, and pickling, were widely practiced. And against a common misconception, spices were used to enhance the flavor of food, not to cover its taste.

Despite van Leeuwenhoek's observation in the mouth of an old man of "an unbelievably great company of living animalcules," or bacteria, no connection had yet been made between them, or the other microorganisms he discovered, and human disease. Yet, food, then as now, was linked to disease, often unfairly. In 1655, blue plums and black cherries, blamed for the plague because of their close resemblance to buboes, were temporarily banned, and pineapple was thought by some to cause "gastric ailments from the Orient."

Centuries later, the abundance of food in some parts of the world rivals that of the Dutch Golden Age. But neither the distribution nor the safety of the global food supply has become foolproof. While microbiology has revolutionized the way we perceive food safety, movement of people and goods has compromised it. Now as in the 17th century, consumers in affluent societies sit at sumptuous banquets to a mythical array of goods, bar none. Yet, sweet or sour, rare or local, organic, bioengineered, opulent, or simply served, food carries warnings, no longer about frivolity and excess but about disease risk. When van Leeuwenhoek's animalcules are pathogenic, each meal becomes a gamble. Infection, the great equalizer, has turned banquet to Dutch treat.

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Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D61, Atlanta, GA 30333, USA; email: PMP1@cdc.gov

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Effects of Circulating Human Coronaviruses

Tropheryma whipplei in Patients with Pneumonia

Imported Methicillin-Resistant *Staphylococcus aureus*, Sweden

Community-associated *Clostridium difficile* Infection, North Carolina, USA

Increased Resistance in Tuberculosis Despite Treatment Adherence

Household Responses to Pandemic (H1N1) 2009–related School Closures, Australia

Airborne Tularemia Outbreak, Germany

School-based Mass Influenza Vaccination Program

Association between *Mycobacterium tuberculosis* Strains and Phenotypes

Employment and Compliance with Pandemic Influenza Mitigation Recommendations

Cost-effectiveness of Pharmaceutical-based Pandemic Mitigation Strategies

Domestic Animals and Epidemiology of Visceral Leishmaniasis, Nepal

Epidemiology of *Cryptococcus gattii*, British Columbia, Canada, 1999–2007

Human Hendra Virus Encephalitis Associated with Equine Outbreak, Australia, 2008

Cause of Epidemic among Native Americans of New England, 1616–1619

Mammalian Ancestry of Pandemic (H1N1) 2009

Clonal Distribution of Invasive Pneumococci, Czech Republic, 1996–2003

White-Nose Syndrome Fungus in Bat, France

Pandemic (H1N1) 2009, Buenos Aires, Argentina

Complete list of articles in the February issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 19–21, 2010
2nd International Berlin Bat Meeting: Bat Biology and Infectious Diseases
Berlin, Germany
http://www.izw-berlin.de

March 18–22, 2010
Fifth Decennial: International Conference on Healthcare-Associated Infections 2010
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.decennial2010.com

March 24–26, 2010
16th ISHEID (International Symposium on HIV & Emerging Infectious Diseases)
Marseille, France
http://www.isheid.com

July 11–14, 2010
International Conference on Emerging Infectious Diseases 2010
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.iceid.org

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Article Title

Public Health Threat of New, Reemerging, and Neglected Zoonoses in the Industrialized World

CME Questions

1. Which of the following infections is correctly matched with its animal host?

A. Q fever → fish, domestic animals, birds, and ticks
B. Rift Valley fever → pigeons
C. Leishmaniasis → chimpanzees
D. *Bartonella henselae* → dogs

2. Which of the following organisms is most likely to mutate?

A. Yeasts
B. Fungi
C. Bacteria
D. Viruses

3. Which of the following statements about emerging zoonotic infections is most accurate?

A. Water sports can expose humans to brucellosis
B. Importation of animals for sport does not affect rates of infection
C. The greatest risk for human rabies comes from dogs
D. Of all bushmeat, nonhuman primates afford the lowest risk for infection

4. Which of the following statements about rickettsial disease is most accurate?

A. Increased tourism has not influenced the prevalence of rickettsial disease
B. Neuropathy is evident within 3 months of infection
C. Spotted fevers are an emerging zoonosis
D. Rates of Lyme borreliosis in humans have fallen

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES®

Zoonoses

December 2009

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

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Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also 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. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief 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 be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief 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 a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

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