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March 2012

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March 2012

On the Cover

Paulus Potter (c. 1625–1654)
***God Appearing to Abraham at Sichem* (1642) (detail)**
Oil on canvas
(100.4 cm x 130.8 cm)
From the collection of Dr. Gordon Gilbert, St. Petersburg, Florida, USA.
Photo by Ray Bassett

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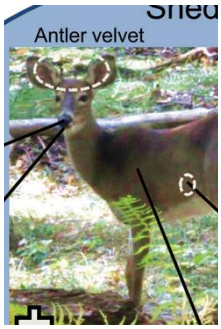

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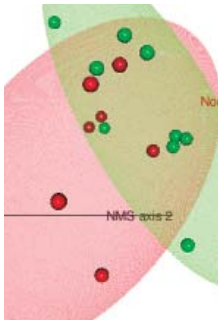

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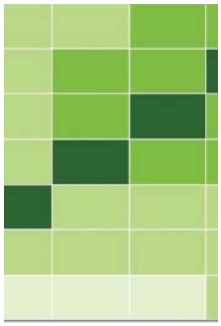

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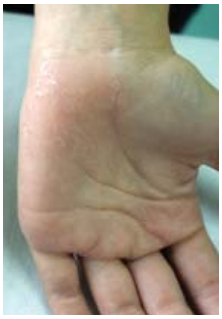

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Occurrence, Transmission, and Zoonotic Potential of Chronic Wasting Disease

Samuel E. Saunders,[1] Shannon L. Bartelt-Hunt, and Jason C. Bartz

Chronic wasting disease (CWD) is a fatal, transmissible prion disease that affects captive and free-ranging deer, elk, and moose. Although the zoonotic potential of CWD is considered low, identification of multiple CWD strains and the potential for agent evolution upon serial passage hinders a definitive conclusion. Surveillance for CWD in free-ranging populations has documented a continual geographic spread of the disease throughout North America. CWD prions are shed from clinically and preclinically affected hosts, and CWD transmission is mediated at least in part by the environment, perhaps by soil. Much remains unknown, including the sites and mechanisms of prion uptake in the naive host. There are no therapeutics or effective eradication measures for CWD-endemic populations. Continued surveillance and research of CWD and its effects on cervid ecosystems is vital for controlling the long-term consequences of this emerging disease.

Chronic wasting disease (CWD) is an inevitably fatal, infectious neurodegenerative prion disease naturally affecting North American mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), elk (wapiti, *Cervus canadensis*), and moose (*Alces alces*) (*1*,*2*). Other prion diseases, or transmissible spongiform encephalopathies, include bovine spongiform encephalopathy (BSE), scrapie in sheep and goats, and Creutzfeldt-Jakob disease (CJD) in humans (*3*). CWD was identified in the late 1960s and recognized as a spongiform encephalopathy by Williams in 1980 (*1*).

Clinical signs of CWD include weight loss and behavioral changes such as altered stance, pacing, excessive salivation, and hyperexcitability that progress over weeks or months (*1*). The infectious agent of CWD is the abnormally folded prion protein (the prion) designated PrP^{Sc}, which is distinguished from the normal cellular prion protein (PrP^{c}) by its resistance to proteolysis, propensity for aggregation, and insolubility in detergents (*4*). Misfolded prion (PrP^{Sc}) can initiate conversion of PrP^{c} to PrP^{Sc} and replicate through a yet unknown mechanism. The exact role that PrP^{Sc} plays in prion disease remains unclear, but PrP^{Sc} is known to accumulate in the central nervous system (CNS) (*1*).

CWD continues to emerge and spread in free-ranging and captive cervids throughout the United States and Canada. Effective therapeutics or and management practices for animal populations in areas to which CWD is endemic do not currently exist. Long-term effects of CWD on cervid ecosystems remain unclear, but the potential for economic consequences is serious because of the role cervids play in the hunting, tourism, and agricultural industries. Moreover, the zoonotic potential of CWD is uncertain, and exposure to CWD-contaminated meat and material will only increase as the disease continues to spread and the incidence increases in areas to which CWD is endemic. We discuss current CWD prevalence and distribution and broadly review surveillance efforts to date. We also present a detailed conceptual model for transmission of the CWD agent and provide an update on CWD interspecies transmission, strains, and zoonotic potential. In addition, we suggest key research needs that may offer hope of slowing or halting the continued emergence of CWD.

Prevalence and Surveillance

Originally recognized only in southeastern Wyoming and northeastern Colorado, USA, CWD was reported in

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Canada in 1996 and Wisconsin in 2001 and continues to be identified in new geographic locations (Figure 1, panel A). CWD has been identified in free-ranging cervids in 15 US states and 2 Canadian provinces and in ≈100 captive herds in 15 states and provinces and in South Korea (Figure 1, panel B). Except in South Korea, CWD has not been detected outside North America. In most locations reporting CWD cases in free-ranging animals, the disease continues to emerge in wider geographic areas, and prevalence appears to be increasing in many disease-endemic areas. Areas of Wyoming now have an apparent CWD prevalence of near 50% in mule deer, and prevalence in areas of Colorado and Wisconsin is ≤15% in deer. However, prevalence in many areas remains between 0% and 5% according to reports and data obtained from state and provincial wildlife agencies. Prevalence in elk is lower than in deer but reaches 10% in parts of Wyoming. Known risk factors for CWD include sex and age, and adult male deer show the highest prevalence (*5*). Polymorphisms in the PrP (*PRNP*) gene appear to influence susceptibility in deer and elk (*2*,*6*,*7*), but remain less understood than the strong genetic influences for scrapie.

CWD surveillance programs are now in place in almost all US states and Canadian provinces (Figure 2, panel A). More than 1,060,000 free-ranging cervids have reportedly been tested for CWD (Figure 2, panel B) and ≈6,000 cases have been identified (Figure 2, panel C) according to data from state and provincial wildlife agencies. Following years of limited surveillance in select states and provinces, a nationwide surveillance effort was initiated for the 2002–2003 season, which greatly increased the number of states and provinces performing testing, animals tested, and cases identified (Figure 2). Initial surveillance in most states was generally designed to detect ≥1 positive animal at a 95% confidence level if the population disease prevalence was ≥1%, although this goal has not always been achieved.

Many states have now shifted to more targeted surveillance of known disease-endemic areas, areas bordering states reporting cases, or areas surrounding facilities for captive cervids. Samples tested are typically from animals killed by hunters, animals clinically suspected of having CWD, animals killed by vehicles, and targeted sharpshooter kills. Testing of captive cervids is routine in most states and provinces, but varies considerably in scope from mandatory testing of all dead animals to voluntary herd certification programs or mandatory testing of only animals suspected of dying of CWD. A detailed analysis of state and provincial CWD surveillance regimens and disease prevalence is beyond the scope of this report. However, such an analysis would be valuable, not only to evaluate and improve surveillance strategies across the continent (and world) but also to provide insights into spatial and temporal disease dynamics.

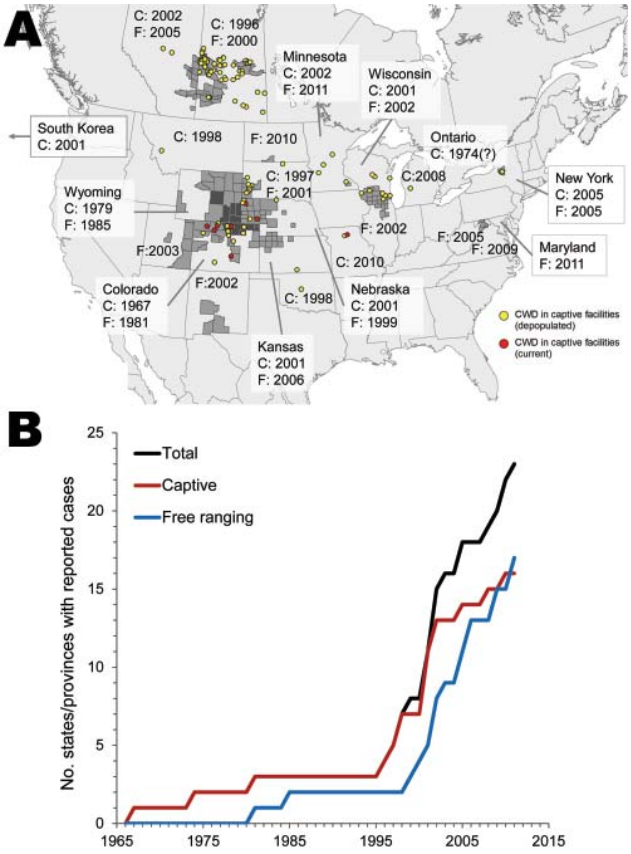

Figure 1. US states and Canadian provinces reporting chronic wasting disease (CWD) cases. A) Year or season CWD was first identified/confirmed in captive (C) or free-ranging (F) cervids. Underlying map shows geographic distribution of CWD (from the US Geological Survey National Wildlife Health Center, updated October 2011, www.nwhc.usgs.gov/disease_information/chronic_wasting_disease/). Light gray shading, current CWD in free-ranging populations; dark gray shading, known distribution of CWD in free-ranging populations before 2000. All locations are approximations based on best available information. B) Cumulative totals of states and provinces that have reported CWD cases in captive or free-ranging cervids. Totals also include South Korea (2001, captive). Many states have reported captive cases for only 1 or 2 years.

Long-term effects of CWD on cervid populations and ecosystems remain unclear as the disease continues to spread and prevalence increases. In captive herds, CWD might persist at high levels and lead to complete herd destruction in the absence of human culling. Epidemiologic modeling suggests the disease could have severe effects on free-ranging deer populations, depending on hunting policies and environmental persistence (*8*,*9*). CWD has been associated with large decreases in free-ranging mule deer populations in an area of high CWD prevalence (Boulder, Colorado, USA) (*5*). In addition, CWD-infected deer are selectively preyed upon by mountain lions (*5*), and may also be more vulnerable to vehicle collisions (*10*).

Long-term effects of the disease may vary considerably geographically, not only because of local hunting policies, predator populations, and human density (e.g., vehicular collisions) but also because of local environmental factors such as soil type (*11*) and local cervid population factors, such as genetics and movement patterns (S.E. Saunders, unpub. data).

Transmission and Role of the Environment

Horizontal Transmission and Agent Shedding

Horizontal transmission of the agent causing CWD is a major mechanism of natural transmission (Figure 3), and maternal transmission is not necessary for disease transmission (*1*). Oral inoculation is an effective route of CWD agent transmission (*1*). Oral lesions facilitate CWD agent transmission in transgenic mice expressing cervid PrP^c (*12*). Nasal inoculation is also an effective route of transmission in transgenic mice expressing cervid PrP^c (*13*). However, nasal infection and the effect of oral lesions on infection have not yet been evaluated for cervids. Overall, the natural routes and mechanisms of CWD prion uptake are incompletely described.

The CWD agent is shed from infected hosts in urine, feces, saliva, blood, and antler velvet (Figure 3) and can occur in preclinical and clinically affected animals (*14*). CWD prions are also present nearly ubiquitously throughout a diseased host, including skeletal muscle; cardiac muscle; fat; a wide range of glands, organs, and peripheral nervous tissue; and in the highest concentrations in the CNS (*2*,*15*). Thus, CWD prions will enter the environment through shedding from diseased and deaths animals (carcasses). Although quantification of infectious CWD titers in excreta and tissue is challenging, the total titer shed from an infected animal during its lifespan may be approximately equal to the total titer contained in an infected carcass (*16*).

Indirect Environmental Transmission

Environmental transmission of the CWD agent was reported in studies demonstrating that an infected deer carcass left in a pasture for 2 years could transmit the agent to immunologically naive deer (*17*). Exposure of naive deer to pasture previously inhabited by an infected deer also led to CWD transmission, as did cohabitation of naive and infected deer (*17*). Naive deer exposed to water, feed buckets, and bedding used by CWD-infected deer contracted the disease (*18*).

Epidemiologic modeling suggests that indirect environmental routes of CWD transmission also play a major role in transmission (*8*). Environmental transmission of scrapie is well documented, and scrapie prions may remain infectious after years in the environment (*19*,*20*; S.E. Saunders, unpub. data). Nevertheless, environmental transmission of scrapie may be less efficient than transmission by direct contact (*19*). Conversely, the relative efficiency of CWD transmission by direct contact versus indirect, environmental routes remains unclear, but evidence suggests environmental transmission may be a major mechanism (*8*). The proportion of transmission by direct versus indirect routes may vary not only between captive and free-ranging cervid populations, but also among cervid species and free-ranging habitats and ecosystems.

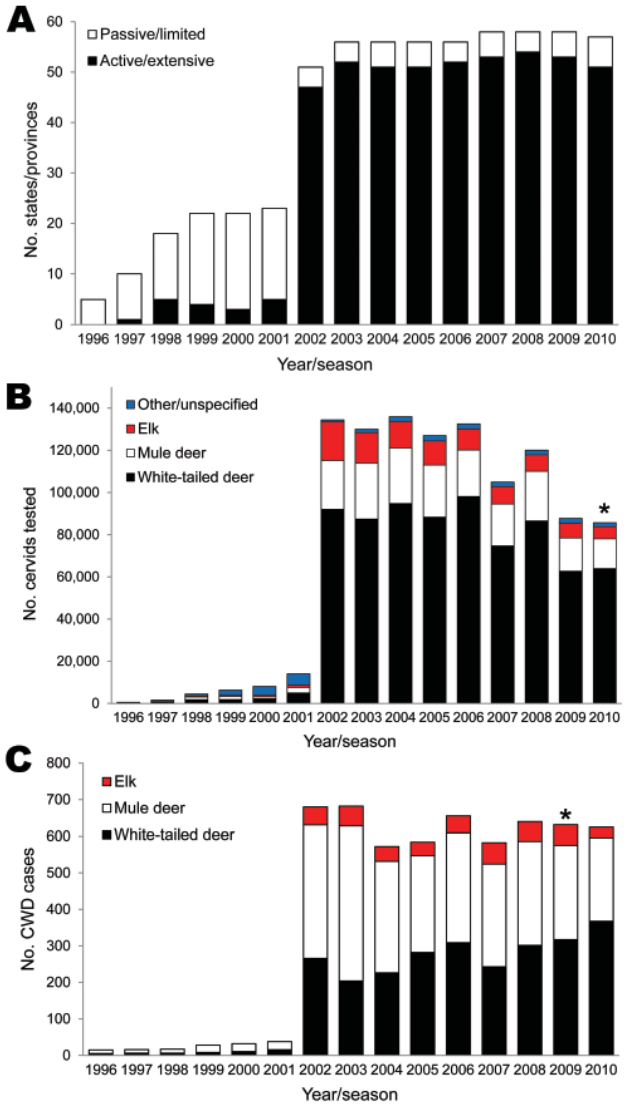

Figure 2. Annual surveillance of free-ranging cervids for chronic wasting disease (CWD). A) Number of US states and Canadian provinces conducting limited or extensive CWD surveillance of free-ranging cervids. B) Number of cervids tested by species each year/season. Other/unspecified includes black-tailed deer, moose, caribou, and data that could not be separated by species. C) Number of CWD-positive cervid samples (CWD cases) by species each year/season. Less than 5 moose were positive. Data were obtained from state and provincial wildlife agencies. Asterisks indicate preliminary or approximated 2010 data.

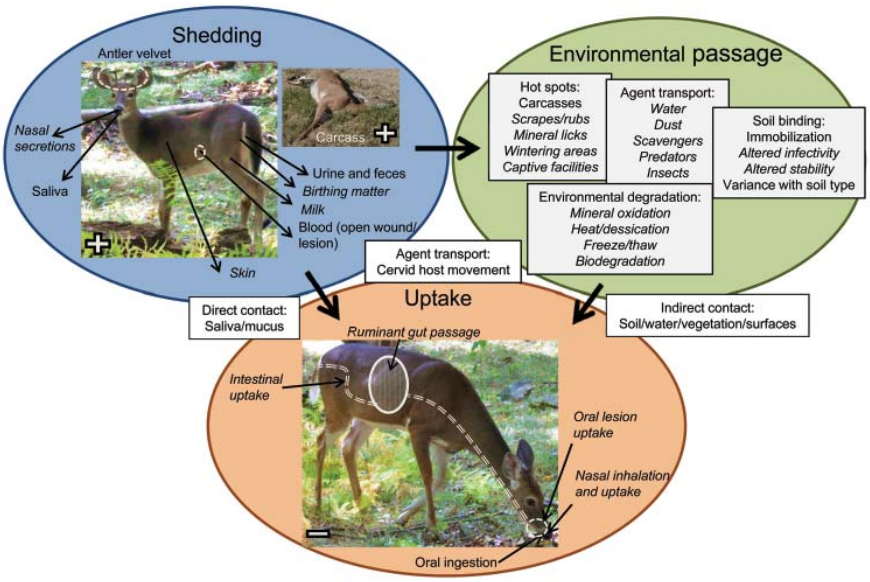

Figure 3. Conceptual model of horizontal transmission of chronic wasting disease (CWD). Items in *italics* are poorly studied or unknown in cervid CWD.

Transmission dynamics may also vary over time as CWD prevalence and ecosystem residence times continue to increase (*8*).

If the environment serves as a reservoir of CWD infectivity, hot spots of concentrated prion infectivity could be formed at areas of communal activity where shedding occurs (Figure 3) (*12*). Animal mortality sites, where highly infectious CNS matter would enter the environment, could also be hot spots (*21*). In a study of deer carcass decomposition in Wisconsin, carcasses persisted for 18–101 days depending on the season, and were visited by deer (*22*). In addition, cervid carcasses are visited by numerous scavenger species, such as raccoons, opossums, coyotes, vultures, and crows, which could consume and transport CWD-infected tissue and increase CWD spread (*21*,*22*). Thus, there is the potential for CWD to spread from sites of animal deaths. Predators may also contribute to spread of the CWD agent and transmission (*5*), as could transport by surface water (*23*) or insect vectors. Natural migration and dispersion of cervids is also a likely mechanism of geographic spread of CWD (*24*).

Given that cervids habitually ingest considerable amounts of soil, soil has been hypothesized to play a key role in CWD transmission (Figure 3) (*11*,*20*; S.E. Saunders et al., unpub. data). Inhalation of dust-bound CWD prions may also represent a route of transmission. It is known that CWD prions can bind to a range of soils and soil minerals (*25*,*26*) and retain the ability to replicate (*27*). In addition, rodent prions retain or gain infectivity when bound to soil and soil minerals (*20*,*27*; S.E. Saunders et al., unpub. data). Prion fate and transmission in soil has been recently reviewed (*20*). Although the potential for CWD transmission by soil and soil reservoirs is considerable, this transmission remains to be directly evaluated with cervids.

CWD Zoonotic Potential, Species Barriers, and Strains

Current Understanding of the CWD Species Barrier

Strong evidence of zoonotic transmission of BSE to humans has led to concerns about zoonotic transmission of CWD (*2*,*3*). As noted above, CWD prions are present nearly ubiquitously throughout diseased hosts, including in muscle, fat, various glands and organs, antler velvet, and peripheral and CNS tissue (*2*,*14*,*15*). Thus, the potential for human exposure to CWD by handling and consumption of infectious cervid material is substantial and increases with increased disease prevalence.

Interspecies transmission of prion diseases often yields a species-barrier effect, in which transmission is less efficient compared with intraspecies transmission, as shown by lower attack rates and extended incubation periods (*3*,*28*). The species barrier effect is associated with minor differences in PrP^{c} sequence and structure between the host and target species (*3*). Prion strain (discussed below) and route of inoculation also affect the species barrier (*3*,*28*). For instance, interspecies transmission by intracerebral inoculation is often possible but oral challenge is completely ineffective (*29*).

Most epidemiologic studies and experimental work have suggested that the potential for CWD transmission to humans is low, and such transmission has not been documented through ongoing surveillance (*2*,*3*). In vitro prion replication assays report a relatively low efficiency of CWD PrP^{Sc}-directed conversion of human PrP^{c} to PrP^{Sc} (*30*), and transgenic mice overexpressing human PrP^{c} are resistant to CWD infection (*31*); these findings indicate low zoonotic potential. However, squirrel monkeys are susceptible to CWD by intracerebral and oral inoculation

(*32*). Cynomolgus macaques, which are evolutionarily closer to humans than squirrel monkeys, are resistant to CWD infection (*32*). Regardless, the finding that a primate is orally susceptible to CWD is of concern.

Interspecies transmission of CWD to noncervids has not been observed under natural conditions. CWD infection of carcass scavengers such as raccoons, opossums, and coyotes was not observed in a recent study in Wisconsin (*22*). In addition, natural transmission of CWD to cattle has not been observed in experimentally controlled natural exposure studies or targeted surveillance (*2*). However, CWD has been experimentally transmitted to cattle, sheep, goats, mink, ferrets, voles, and mice by intracerebral inoculation (*2*,*29*,*33*).

CWD is likely transmitted among mule, white-tailed deer, and elk without a major species barrier (*1*), and other members of the cervid family, including reindeer, caribou, and other species of deer worldwide, may be vulnerable to CWD infection. Black-tailed deer (a subspecies of mule deer) and European red deer (*Cervus elaphus*) are susceptible to CWD by natural routes of infection (*1*,*34*). Fallow deer (*Dama dama*) are susceptible to CWD by intracerebral inoculation (*35*). Continued study of CWD susceptibility in other cervids is of considerable interest.

Reasons for Caution

There are several reasons for caution with respect to zoonotic and interspecies CWD transmission. First, there is strong evidence that distinct CWD strains exist (*36*). Prion strains are distinguished by varied incubation periods, clinical symptoms, PrP^{Sc} conformations, and CNS PrP^{Sc} depositions (*3*,*32*). Strains have been identified in other natural prion diseases, including scrapie, BSE, and CJD (*3*). Intraspecies and interspecies transmission of prions from CWD-positive deer and elk isolates resulted in identification of ≥2 strains of CWD in rodent models (*36*), indicating that CWD strains likely exist in cervids. However, nothing is currently known about natural distribution and prevalence of CWD strains. Currently, host range and pathogenicity vary with prion strain (*28*,*37*). Therefore, zoonotic potential of CWD may also vary with CWD strain. In addition, diversity in host (cervid) and target (e.g., human) genotypes further complicates definitive findings of zoonotic and interspecies transmission potentials of CWD.

Intraspecies and interspecies passage of the CWD agent may also increase the risk for zoonotic CWD transmission. The CWD prion agent is undergoing serial passage naturally as the disease continues to emerge. In vitro and in vivo intraspecies transmission of the CWD agent yields PrP^{Sc} with an increased capacity to convert human PrP^{c} to PrP^{Sc} (*30*). Interspecies prion transmission can alter CWD host range (*38*) and yield multiple novel prion strains (*3*,*28*). The potential for interspecies CWD transmission (by cohabitating mammals) will only increase as the disease spreads and CWD prions continue to be shed into the environment. This environmental passage itself may alter CWD prions or exert selective pressures on CWD strain mixtures by interactions with soil, which are known to vary with prion strain (*25*), or exposure to environmental or gut degradation.

Given that prion disease in humans can be difficult to diagnose and the asymptomatic incubation period can last decades, continued research, epidemiologic surveillance, and caution in handling risky material remain prudent as CWD continues to spread and the opportunity for interspecies transmission increases. Otherwise, similar to what occurred in the United Kingdom after detection of variant CJD and its subsequent link to BSE, years of prevention could be lost if zoonotic transmission of CWD is subsequently identified,

Management Policies

CWD will likely continue to emerge in North America. Given the current extent of CWD and the lack of an effective therapeutic, complete eradication is currently not feasible. As more is learned about disease transmission, it may be possible to manage the prevalence in CWD-endemic areas through hunting policies (*9*). However, long exposures of the environment to CWD prions may create strong environmental reservoirs of CWD capable of efficient transmission, which could sustain or heighten disease incidence (Figure 3) (*8*; S.E. Saunders et al., unpub. data).

Ostensible elimination of CWD in free-ranging cervids has been achieved in only 1 state (New York). After an intensive depopulation and surveillance effort, only 2 free-ranging deer tested were positive for CWD in New York. A similar depopulation and surveillance effort was recently conducted in Minnesota, where only 1 free-ranging deer tested was positive for CWD. Success of the effort in Minnesota and the experience in New York offer hope that new isolated CWD outbreaks can be contained and eliminated by immediate depopulation efforts. However, environmental reservoirs or unknown disease foci may hinder such efforts, and attempts to eliminate CWD in other states in addition to New York have failed. Most notably, an extensive culling effort in Wisconsin that was initiated after CWD detection in 3 free-ranging deer was most likely unsuccessful because the disease was long established in the deer population and environment (*8*,*9*).

Controlling the spread of CWD, especially by human action, is a more attainable goal than eradication. Human movement of cervids has likely led to spread of CWD in facilities for captive animals, which has most likely contributed to establishment of new disease foci in free-ranging populations (Figure 1, panel A). Thus, restrictions

on human movement of cervids from disease-endemic areas or herds continue to be warranted. Anthropogenic factors that increase cervid congregation such as baiting and feeding should also be restricted to reduce CWD transmission. Appropriate disposal of carcasses of animals with suspected CWD is necessary to limit environmental contamination (*20*), and attractive onsite disposal options such as composting and burial require further investigation to determine contamination risks. The best options for lowering the risk for recurrence in facilities for captive animals with outbreaks are complete depopulation, stringent exclusion of free-ranging cervids, and disinfection of all exposed surfaces. However, even the most extensive decontamination measures may not be sufficient to eliminate the risk for disease recurrence (*20*; S.E. Saunders et al. unpub. data)

Research Needs

The influence of environmental factors, such as local climate and habitat characteristics (e.g., vegetation and soil type), on CWD incidence has not been assessed in detail (S.E. Saunders et al., unpub. data). Epidemiologic comparisons of well-established CWD-endemic populations/habitats and newly exposed populations/habitats could yield insights on transmission dynamics. Detection and quantification of environmental CWD prions would be a key step in defining the role of indirect, environmental exposure routes in CWD transmission. Although CWD PrP^{Sc} was detected in a river water sample from an area in Colorado with endemic CWD by using protein misfolding cyclic amplification, the amount detected was below the limit of transgenic mouse bioassay detection, which complicated interpretation of data (*23*).

If environmental reservoirs were implicated in CWD transmission, it may be possible to target these reservoirs for disinfection with a topical enzymatic solution (*26*) or another yet untested treatment and thereby greatly reduce disease incidence. However, cervid density, behavior, and movement may be more significant factors in CWD transmission regardless of the environment. However, such factors also require more investigation. In either case, additional research is needed to determine the natural routes of exposure and agent uptake (Figure 3). CWD prion shedding from cervid birthing matter, milk, nasal secretions, and nonantler skin also warrants investigation because such shedding has been observed with other noncervid prion-infected animals (Figure 3) (*14*).

CWD research tools have been used to make major advances in the past 5 years. Transgenic mouse models of CWD are now invaluable tools for studying CWD infectivity and strains (*2*,*12*,*13*,*15*,*16*,*23*,*30*,*36*), and protein misfolding cyclic amplification has been used effectively for CWD detection, replication, and interspecies studies (*14*,*23*,*27*,*30*). A CWD-susceptible cell culture line is now available (*39*). Continued use of captive cervids in CWD research remains critical to understanding the disease in its natural hosts. Recent advances in premortem detection techniques, including excreta testing (*14*) and rectal biopsy (*40*), may lead to more reliable and noninvasive surveillance programs and enhance experimental capabilities.

Future CWD Surveillance

The origin of CWD is unknown but may have been either a spontaneous occurrence or caused by interspecies transmission of scrapie or another prion agent. However, because scrapie cases have been reported globally in sheep-farming countries, the potential exits for CWD to occur globally. To our knowledge, CWD surveillance outside the United States and Canada has been largely or completely confined to the industrialized countries of Europe and Asia and has not approached the extensiveness of US and Canadian efforts (Figure 2). Even within North America, surveillance of some cervids, such as caribou, has been limited, and continued enthusiasm for funding and conducting current surveillance programs is uncertain. Given that surveillance efforts are still limited compared with total cervid populations, CWD could be present at low levels in many areas considered free of CWD. At a minimum, targeted surveillance of all cervids within and outside North America should be conducted to understand the true extent of the disease geographically and its host range. This surveillance might be facilitated by more convenient premortem testing methods (*14*,*40*).

Conclusions

Much remains unknown about prion diseases and CWD in particular, especially about CWD strains (which may have varied zoonotic potentials) and the long-term effects of CWD on cervid ecosystems. CWD prevalence and geographic range appear likely to continue to increase. Moreover, the disease is inevitably fatal, and no effective therapeutic measures are presently available. As such, it would seem wise to continue research and surveillance of CWD to elucidate the details of its transmission, pathogenesis, and continued emergence in cervid populations in hopes that strategies for mitigating its negative effects on humans and cervid ecosystems can be identified.

Acknowledgments

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Dr. Saunders is a student at Stanford Law School, Stanford, California. At the time of this study, he was a student and postdoctoral researcher in the Department of Civil Engineering, University of Nebraska–Lincoln. His research interests are the fate, transmission, and mitigation of prions in the environment.

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Foodborne and Waterborne Infections in Elderly Community and Long-Term Care Facility Residents, Victoria, Australia

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We calculated rates of foodborne and waterborne infections reported to the health department in Victoria, Australia, during 2000–2009 for elderly residents of long-term care facilities (LTCFs) and the community. We used negative binomial regression to estimate incidence rate ratios, adjusting for age, sex, and reporting period. We analyzed 8,277 infections in elderly persons. Rates of campylobacteriosis, legionellosis, listeriosis, toxigenic *Escherichia coli* infections, and shigellosis were higher in community residents, and rates of *Salmonella* infection were higher in LTCF residents. Each year, 61.7 *Campylobacter* infections were reported per 100,000 LTCF residents, compared with 97.6 per 100,000 community residents. LTCF residents were at higher risk for *S. enterica* serotype Typhimurium associated with outbreaks. Rates of foodborne infections (except salmonellosis) were similar to or lower for LTCF residents than for community residents. These findings may indicate that food preparation practices in LTCFs are safer than those used by elderly persons in the community.

Infectious disease incidence varies with age, and elderly persons are considered more vulnerable than younger persons to foodborne and waterborne infections (*1*,*2*). In many countries, elderly persons unable to care for themselves live in long-term care facilities (LTCFs) where they receive assistance with meals, daily living, and health care (*3*). Food preparation practices and various exposures in LTCFs may modify the risk of foodborne and waterborne infections in these residents (*4*,*5*) when compared with elderly persons living in the community, who may have less safe food preparation practices (*6*–*8*).

A variety of pathogens transmitted by food or water, including *Campylobacter* sp., *Clostridium perfringens*, *Cryptosporidium* sp., *Legionella* spp., and *Shigella* sp., and various serotypes of *Salmonella enterica* can infect humans (*9*,*10*). Foodborne and waterborne infections predominantly manifest in elderly persons as gastroenteritis but, depending on the infectious agent, can result in pneumonia, bacteremia, and meningitis (*11*,*12*). Elderly persons can become infected by ingesting contaminated water or food or, as with *Legionella* spp., inhaling contaminated aerosols (*13*). Some infections are predominantly foodborne; others can be acquired from infected persons or animals or through contact with contaminated environments (*4*).

These agents can manifest as outbreaks in facilities, leading to community concern about the safety of residents (*14*,*15*). Although most outbreaks of gastroenteritis in LTCFs are spread from person to person and are generally mild (*16*), such outbreaks do result in higher case-fatality rates (CFRs) among residents (*17*). As a result, regulatory agencies in many countries have mandated programs to manage food safety in facilities. To prevent legionellosis in residents, health agencies commonly provide advice about disinfection of hot water systems that can be reservoirs for *Legionella* spp. (*13*).

Few studies have compared the incidence of infections caused by agents that can be transmitted by contaminated food or water consumed by elderly persons living in LTCFs and in the community. One study in the United States estimated that the lower limit of the death rate for

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nursing home residents from gastroenteritis of unknown etiology was 38.91 (95% CI 38.55–39.27) per 100,000 persons per year, compared with an estimated upper limit of 8.50 (95% CI 8.47–8.53) per 100,000 persons ≥65 years of age living in the community (*18*). Little examination has been done of the incidence of sporadic foodborne or waterborne diseases in institutionalized elderly persons. To address this gap, we estimated rates of reported infection in persons ≥65 years of age living in Victoria, Australia, infected with any of 7 different pathogens according to whether they lived in a government-subsidized LTCF or in the community, and we examined the effect of age on incidence of disease. These pathogens were *Campylobacter* sp., *Cryptosporidium* sp., *Legionella* spp., *Listeria* sp., *Salmonella enterica*, Shiga toxin–producing *Escherichia coli* (STEC), and *Shigella* sp.

Methods

Infectious Disease Surveillance

Victoria is 1 of 6 states and 2 territories in Australia. Public health legislation mandates that all physicians and pathology laboratories in Victoria report cases of notifiable conditions under the Public Health and Wellbeing Regulations 2009 (www.health.vic.gov.au/ideas/notifying/whatto) to the state's Department of Health. Health department staff members enter details about reported cases into a database. Among the 64 conditions notifiable as of April 14, 2010, a total of 14 were enteric diseases can be transmitted by contaminated food or water and 1 was *Legionella* infection that could potentially be transmitted by inhalation of contaminated water. Surveillance for these diseases has remained essentially unchanged in Victoria since the early 1990s, except for cryptosporidiosis, for which reporting was voluntary until 2001, when notification became mandatory by law.

We analyzed data on all cases of campylobacteriosis, cryptosporidiosis, legionellosis, listeriosis, shigellosis, salmonellosis, and STEC infection that were reported to Victoria's Department of Health during January 1, 2000–December 31, 2009. Surveillance officers recorded whether cases were part of an outbreak or occurred in persons who had traveled overseas during their incubation period, which varied for different diseases. In addition, to identify information that was not recorded by surveillance officers, we reviewed surveillance data for all LTCF residents. More specifically, we identified where >2 cases of the same pathogen occurred within the same facility within 2 weeks, and we recoded these cases as outbreak associated. Surveillance officers also recorded whether case-patients had died of the disease or of other concurrent conditions within the weeks after infection during public health follow-up.

Data Analysis

We categorized reported cases by residential status of the patient. An LTCF resident was a person who had a residential address of a government-subsidized LTCF, and a community resident was a person living in a private residence in Victoria (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-0311-Techapp.pdf). We excluded from analysis cases in persons without a valid address because we assumed the address was missing completely at random. We also excluded case-patients residing in privately funded facilities (supported residential services) catering to elderly or disabled persons or persons with psychiatric illness or dementia because they were not included in the denominator of LTCF residents. We counted the annual number of foodborne and waterborne infections, including those from epidemiologically important serotypes and species, in LTCF and community residents during the 10-year period. We obtained age-specific annual denominator data for residents of government-subsidized LTCFs from annual reports prepared by the Australian Institute of Health and Welfare (*19*). Denominator data for community residents were calculated by subtracting annual age-specific estimates of LTCF residents from estimated resident populations prepared by the Australian Bureau of Statistics (www.abs.gov.au/). We calculated annual rates of notification for different diseases by residential status and compared them with the total rate of notifications for the state. We calculated CFRs by dividing the number of deaths from the disease in the different groups by the total number of cases of disease, including cases for which death status was unknown, for the 10-year period. To account for age differences in LTCF and community residents, we calculated age-adjusted relative risks (RRs) for death from infection with different pathogens by using Mantel-Haenszel methods.

To estimate incidence rate ratios (IRRs), we used a negative binomial regression model of the annual count of foodborne and waterborne infections by the period of notification (2000–2004 and 2005–2009), sex, 3 categories of age (65–74, 75–84, and ≥85 years), and residence (LTCF and community). The number of persons living in LTCFs or the community in each age group for each year was entered into the model as an offset. We used robust variance estimation suited to longitudinal or clustered data to account for possible clustering from outbreaks. We assessed model fit by examining the distribution of standardized Pearson residuals. To assess the effect on incidence, we repeated regression models excluding travel-associated cases and including only the first case for each known outbreak in LTCFs and the community. We analyzed data by using Stata version 11.2 (Stata Corp., College Station, TX, USA).

The Australian National University human research ethics committee approved this study. Victoria's Department of Health approved release of the data.

Results

Incidence in Persons ≥65 Years of Age

During January 1, 2000–December 31, 2009, a total of 8,534 cases of the 7 diseases were reported in persons ≥65 years of age. During data cleaning, we excluded 238 (2.8%) persons without valid residential addresses and 19 who lived in a private institution caring for elderly or disabled persons. A total of 8,277 cases were available for analysis, including 132 (1.6%) case-patients living in retirement villages.

Infections in Institutional and Community Residents

Rates of all reported infections in LTCF residents were similar to or lower than those in community residents, except infections from *S. enterica* (Table 1). No LTCF residents were reported with *L. longbeachae*, STEC, or *Shigella* sp. infections during the surveillance period. Among persons ≥65 years of age infected with *Legionella* species other than *L. longbeachae* were more likely to live in an LTCF (crude RR 1.15, 95% CI 1.1–1.2; p = 0.27). The reported rate of *Campylobacter* spp. was lower in LTCF residents than in community residents for all years of surveillance (Figure 1). In contrast, reports of *S. enterica* serotype Typhimurium peaked because of outbreaks during the study period, which resulted in higher rates for LTCF residents overall (Figure 2).

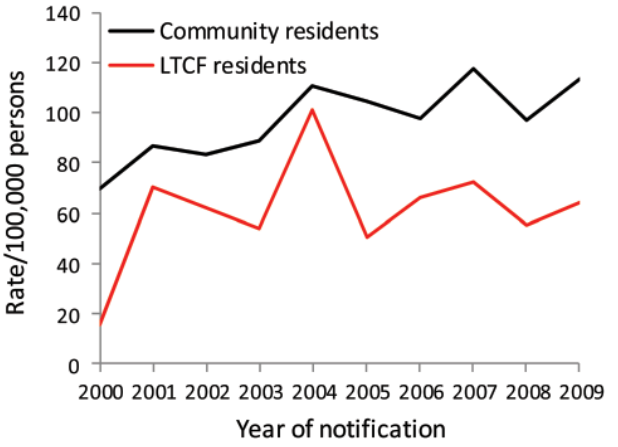

Figure 1. Notification rates for campylobacteriosis in persons ≥65 years of age, by long-term care facility (LTCF) and community residence status, Victoria, Australia, 2000–2009.

Associated Death

The CFR was highest for listeriosis and *L. longbeachae* infection, from which 17.8% and 6.7% of persons died, respectively (Table 2). The age-adjusted RR for death from *L. monocytogenes* infections was 3.5 (95% CI 1.2–10.4; p = 0.03) for LTCF residents. No *L. pneumophila*–associated deaths were recorded in LTCF residents. For salmonellosis, most deaths were considered to have resulted from other concurrent conditions. The age-adjusted RR for death in LTCF residents infected with any *S. enterica* serotype did not differ significantly from that in community residents (adjusted RR 2.8, 95% CI 0.8–9.1; p = 0.87). Death status was not routinely ascertained for persons with campylobacteriosis, although 1 death from campylobacteriosis and 11 deaths from concurrent conditions were recorded. No deaths were recorded for shigellosis or cryptosporidiosis.

Rates of Foodborne and Waterborne Infections

In multivariable analysis, the incidence of *S. enterica* serotype Typhimurium was higher in LTCF residents than

Table 1. Incidence rate for reported infections with pathogens possibly transmitted by food or water, Victoria, Australia, January 2000–December 2009*

	Persons <65 y		Persons ≥65 y					Total reports	
			LTCF residents		Community residents		Missing address/		
Pathogen	No. cases	Rate	No. cases	Rate	No. cases	Rate	excluded facility	No. cases	Rate
Campylobacter sp.	50,444	115.4	215	61.7	6,207	97.6	206	57,072	113.2
Cryptosporidium sp.	4,955	11.3	7	2.0	106	1.7	3	5,071	10.1
Legionella pneumophila/ other	457	1.0	8	2.3	293	4.6	4	762	1.5
L. longbeacheae	49	0.1	0	0.0	45	0.7	0	94	0.2
Listeria monocytogenes†	46	0.1	3	0.9	70	1.1	4	123	0.2
Salmonella enterica serotype Typhimurium	7,204	16.5	87	25.0	585	9.2	19	7,895	15.7
S. enterica, other serotypes	5,003	11.4	44	12.6	552	8.7	20	5,619	11.1
Shiga toxin–producing *Escherichia coli*	56	0.1	0	0.0	12	0.2	1	69	0.1
Shigella sp.	845	1.9	0	0.0	43	0.7	0	888	1.8

*Annual rate of reported infections per 100,000 persons. LTCF, long-term care facility.
†Total listeriosis reports exclude 16 pregnancy-associated infections.

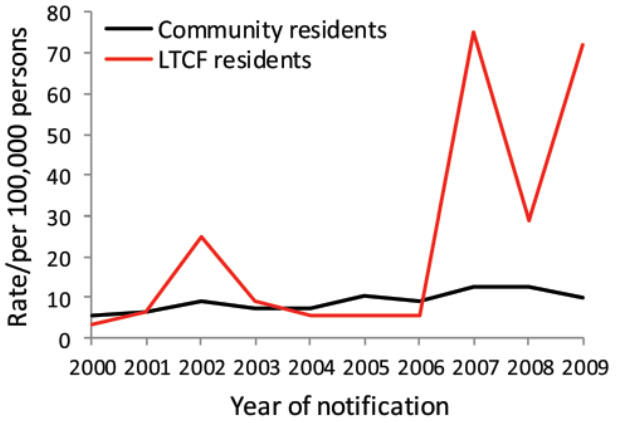

Figure 2. Notification rates for *Salmonella enterica* serotype Typhimurium infections in persons ≥65 years of age, by long-term care facility (LTCF) and community residence status, Victoria, Australia, 2000–2009.

in community residents (IRR 2.3, 95% CI 1.6–3.4; p<0.001) and non-Typhimurium serotypes of *S. enterica* (IRR 1.4, 95% CI 1.0–1.9; p = 0.04) and lower for *Campylobacter* spp. (IRR 0.63, 95% CI 0.52–0.77; p<0.001) (Table 3). IRRs could not be estimated for *L. longbeachae* and STEC infections and for shigellosis because no cases occurred in LTCF residents during the surveillance period. We observed a trend of increasing rates of reported infections for cryptosporidiosis, salmonellosis, and campylobacteriosis over time during the surveillance period.

From multivariable analysis, reported incidence rates were higher in older age groups for *L. monocytogenes*, *Campylobacter* sp., and *S. enterica* serotype Typhimurium infections than in the base age group of persons 65–74 years (Table 3). The incidence of *L. pneumophila* and non-Typhimurium serotypes of *S. enterica* infections and cryptosporidiosis did not differ significantly by age group.

Accounting for Travel and Outbreaks

During their incubation period, 105 (1.3%) of the 8,277 persons with notifiable infections traveled internationally; all were community residents. Among community residents, 16 (37.2%) of the 43 with shigellosis and 48 (8.7%) of the 552 with non-Typhimurium serotypes of *S. enterica* had traveled internationally, compared with 0 of those with *L. monocytogenes*, *L. longbeachae*, and STEC infections. Only 35 (0.6%) of the 6,207 community residents infected with *Campylobacter* sp. were recorded as traveling overseas before infection.

During the study period, 42 separate outbreaks of *S. enterica* serotype Typhimurium occurred in persons ≥65 years of age. There were also 36 outbreaks of non-Typhimurium serotypes of *S. enterica*, 14 of *L. pneumophila*/other, 10 of *Campylobacter* spp. infections, and 2 of *Shigella* sp. In total, 189 (2.4%) of 7,913 cases in community residents were recorded as outbreak associated. In LTCF residents, 111 (30.5%) of the 364 cases were outbreak associated, including 68 (78.2%) of the 87 *S. enterica* serotype Typhimurium and 33 (15.3%) of the 215 *Campylobacter* spp. infections. No cases of cryptosporidiosis, listeriosis, or STEC infection were recorded as outbreak associated in either LTCF residents or community residents.

When we repeated multivariable models, excluding travel-associated infections and including a single case for each identified outbreak, the incidence rate for *S. enterica* serotype Typhimurium was similar in LTCF residents and community residents (IRR 0.91, 95% CI 0.64–1.29; p = 0.59). For infections with non-Typhimurium serotypes of *S. enterica*, the incidence was higher in LTCF residents than in community residents (IRR 1.4, 95% CI 1.0–2.0; p = 0.05). After adjustment for travel and outbreaks, the incidence rate was lower for LTCF residents with *Campylobacter* spp. (IRR 0.57, 95% CI 0.48–0.68; p<0.001). For *L. pneumophila*, the incidence rate was lower, but not significantly, for LTCF residents (IRR 0.63, 95% CI 0.29–1.3; p = 0.23).

Discussion

Rates of foodborne and waterborne infections among LTCF residents were lower than or similar to rates among community residents, except for salmonellosis, which was higher. In particular, rates of campylobacteriosis in LTCF residents were consistently lower throughout the entire study period, which was unexpected because incidence of this infection is universally high (*20*). Despite the high

Table 2. Deaths associated with infections from foodborne and waterborne pathogens or concurrent conditions reported in residents ≥65 years of age in long-term care facilities and the community, Victoria, Australia, January 2000–December 2009

Pathogen	Died of disease	Died of concurrent condition	Death status unknown	No. cases	Case-fatality rate*
Legionella pneumophila/other	11	1	169	301	4.0
L. longbeacheae	2	1	0	45	6.7
Listeria monocytogenes	4	9	14	73	17.8
Salmonella enterica serotype Typhimurium	3	8	183	672	1.6
S. enterica, other serotypes	0	7	177	596	1.2
Shiga toxin–producing *Escherichia coli*	1	0	1	12	8.3
Shigella sp.	0	0	10	43	NA

*Per 100 cases. Case-fatality rate for the 10-year study period was calculated by dividing the total number of deaths among case-patients by all case-patients, including those with unknown death status. NA, not applicable.

Table 3. Adjusted incidence rate ratios from multivariable model for foodborne and waterborne infections reported in residents ≥65 years of age in long-term care facilities and the community, Victoria, Australia, January 2000–December 2009

Variable	Incidence rate ratio (95% CI)					
	Cryptosporidium,* n = 113	*Listeria monocytogenes*, n = 73	*Salmonella enterica* serovar Typhimurium, n = 662	*S. enterica*, other serotypes, n = 586	*L. pneumophila*/ other, n = 301	*Campylobacter* spp., n = 6,387
Sex						
F	1.0	1.0	1.0	1.0	1.0	1.0
M	0.86 (0.51–1.4)	1.5 (0.90–2.6)	0.97 (0.80–1.2)	0.97 (0.80–1.2)	2.6 (1.7–4.1)	1.2 (1.1–1.3)
Year						
2000–2004	1.0	1.0	1.0	1.0	1.0	1.0
2005–2009	3.7 (2.1–6.6)	1.2 (0.68–1.9)	1.8 (1.4–2.2)	1.7 (1.4–2.1)	0.35 (0.23–0.53)	1.2 (1.1–1.3)
Age group, y						
65–74	1.0	1.0	1.0	1.0	1.0	1.0
75–84	0.92 (0.53–1.6)	2.0 (1.1–3.5)	1.1 (0.95–1.4)	1.1 (0.86–1.3)	1.5 (0.90–2.5)	1.1 (1.0–1.2)
≥85	0.58 (0.31–1.1)	2.8 (1.2–6.4)	1.3 (0.99–1.8)	1.1 (0.80–1.4)	1.2 (0.63–2.2)	1.1 (1.0–1.2)
Long-term care facility resident						
No	1.0	1.0	1.0	1.0	1.0	1.0
Yes	1.4 (0.74–2.8)	0.56 (0.10–3.0)	2.3 (1.6–3.4)	1.4 (1.0–1.9)	0.57 (0.27–1.2)	0.63 (0.52–0.77)

*In Victoria, reporting of cryptosporidiosis was voluntary until 2001, when notification became mandatory by law.

incidence of campylobacteriosis, outbreaks are rare in Australia, possibly because of the high dose required to cause infection and because foods causing infection are thought to become contaminated through cross-contamination (*16*,*20*). In Australia, *Campylobacter* infections are the most common cause of bacterial foodborne disease, with contaminated chicken meat causing ≈30% of all infections each year (*21*–*23*).

The lower incidence of campylobacteriosis might result from the highly regulated food hygiene system for LTCFs. In 1998, Victoria was the first Australian state to implement mandatory food safety programs for food service settings, and those serving vulnerable populations require independent auditing (*24*). These programs might have resulted in better understanding and practices by LTCF staff about food storage, cooking, and cross-contamination than by elderly persons in their own homes. *Campylobacter* infections in elderly community residents have been associated with risk possibly from cross-contamination during food preparation (*25*). Even though *Campylobacter* infections are associated with travel, a case–control study in Australia found that only 23 (2.8%) of 833 persons >5 years of age with campylobacteriosis had traveled overseas during the week before illness (*21*). When we accounted for known travel history and outbreak-associated cases, the IRR for *Campylobacter* spp. infections in LTCF residents was lowered.

S. enterica is a common cause of foodborne and waterborne outbreaks in LTCFs (*17*,*26*,*27*), a finding that our study confirmed. We found that outbreaks of *S. enterica* serotype Typhimurium infections accounted for the higher incidence of these infections, but not for non-Typhimurium serotype infections, in LTCF residents. Sources of outbreaks in LTCFs often are not identified, although eggs are commonly suspected as the cause in *S. enterica* serotype Typhimurium–associated outbreaks (*17*). Although residents are at higher risk for outbreak-associated disease, ascertainment of cases is biased in the institutional setting because of the common living environment, centralized access to health care, and collection of specimens by public health staff. During outbreaks, public health investigators often collect fecal specimens from LTCF residents with diarrhea, which would not occur for elderly persons in the community. Because surveillance is well established in Victoria, LTCFs are more likely than persons in the community to report outbreaks (*16*).

We did not find any evidence to suggest that living in an LTCF increased a person's risk for legionellosis, despite the occasional occurrence of outbreaks and sporadic cases in this setting (*13*,*28*). LTCF residents reported with legionellosis were more likely to be infected with *Legionella* species other than with *L. longbeachae*. *L. longbeachae* is associated with gardening and potting mix, so we expected the incidence of this infection to be low in LTCF residents (*29*).The incidence of listeriosis was similar in LTCF and community residents. Given the food safety program requirements in facilities in Victoria, LTCF residents plausibly could be exposed to lower concentrations of *L. monocytogenes* in food, compared with community residents who may keep food longer, have poorer food preparation practices, and eat foods considered higher risk for transmitting foodborne pathogens (*8*).

Different clinical investigative approaches for LTCF and community residents with potential foodborne and waterborne disease might account for some of our findings. Although clinicians might elect not to collect specimens when LTCF residents have diarrheal illness, we think it more likely that reporting is more complete in LTCFs. Most of the diseases in our study are serious illnesses, and infected persons would have severe gastrointestinal and extraintestinal symptoms lasting for several days or weeks (*3*,*4*). In a case–control study of campylobacteriosis

in Australia, 41% of case-patients had bloody diarrhea, and 75% had fever; both of these symptoms are strong predictors for physicians ordering laboratory tests (*30*,*31*).

We were unable to control for potential confounding factors, such as concurrent conditions and factors that might predispose for infection. Many elderly persons with concurrent conditions live in the community, but the health status of LTCF residents is likely to be lower, and they are likely to be more frail. We would have partly controlled for frailty through inclusion of age in our multivariable model because elderly persons in institutions are the oldest and the most frail in society (*32*). In addition, our study was underpowered to detect an effect for diseases where notification rates were very low. The potential bias in the final estimates from lack of control of confounding would be more likely to result in increased incidence rates in LTCF residents. However, except for salmonellosis, infection rates were higher in community residents.

Our findings should not be overinterpreted because our study was a retrospective record–based study in which we manually coded surveillance data and were unable to validate case-patients' addresses. It is possible that we were unable to correctly identify residential status of case patients from addresses. In some instances, residents were recorded as living at addresses where an LTCF and retirement village were on the same grounds, making determining whether a person lived in the facility difficult. Similarly, some persons might have been infected after moving into an LTCF, but the address on a pathology report still recorded their residential address in the community where they had previously lived. However, in Victoria, physicians and laboratories were required to report these infections, making it unlikely that both sources of notification would incorrectly report the residential address. For *Campylobacter* infections, however, physicians report only 50% of notifications, with the remainder coming from laboratories (*33*).

Elderly community residents might receive meals from organizations that provide community support. In addition, elderly residents of LTCF might eat food that has been prepared outside the facility during excursions or brought in by visitors, which could result in exposure to foodborne pathogens. For both groups, these alternative routes of exposure would modify the risk for infection so that it did not truly reflect the risk in their place of residence.

The strength of our approach was that we consistently coded addresses without regard to disease-causing agent, yet we observed distinct differences in reported incidence from disease to disease. Our findings were consistent with what we know about these diseases, such as increasing incidence in older persons for diseases such as listeriosis. The CFRs were consistent with reports in the literature for elderly persons, although we assessed deaths only short term (i.e., in the weeks after infection) (*34*,*35*). In general, elderly persons have more severe outcomes from foodborne infections than do younger persons (*4*,*18*). Large-scale studies that used population-based registers have demonstrated that enteric diseases contribute to more deaths than recognized from short-term follow-up, even when controlling for concurrent conditions (*36*,*37*).

In our study, rates of surveillance reports for most infections in persons ≥65 years of age were similar to or lower than for persons <65 years of age, a finding that contradicts the common statement that elderly persons are at higher risk for foodborne disease. However, we did find that the CFRs were high for some infections and that LTCF residents were affected more severely. We believe that our findings can be generalized to other Australian states and territories with similar rates of infection and methods of surveillance (*16*,*22*). Other investigators could repeat this study by using record-linkage to compare their findings with our findings.

We observed a lower incidence of reported *Campylobacter* spp. infection in LTCF residents, which provides some reassurance for food safety regulators and the aged care industry. Our study highlights that most foodborne and waterborne infections are rare in elderly residents of LTCF and the community, but that these infections do cause occasional deaths. Primary research is needed into the specific causes of foodborne and waterborne infections in elderly persons in the community and in institutional settings that particularly accounts for the effect of concurrent conditions. In our study, elderly LTCF residents had an incidence of foodborne and waterborne infections that was similar to or lower than that that for elderly persons living in the community, except for *S. enterica* infections.

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Nonpasteurized Dairy Products, Disease Outbreaks, and State Laws—United States, 1993–2006

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Learning Objectives

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

- Evaluate the epidemiology of foodborne illness related to the consumption of dairy products
- Analyze the clinical presentation and outcomes of foodborne disease related to the consumption of dairy products
- Distinguish the organism most commonly associated with foodborne illness after consumption of unpasteurized dairy products
- Assess sources of contamination of pasteurized dairy products

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Although pasteurization eliminates pathogens and consumption of nonpasteurized dairy products is uncommon, dairy-associated disease outbreaks continue to occur. To determine the association of outbreaks caused by nonpasteurized dairy products with state laws regarding sale of these products, we reviewed dairy-associated outbreaks during 1993–2006. We found 121 outbreaks for which the product's pasteurization status was known; among these, 73 (60%) involved nonpasteurized products and resulted in 1,571 cases, 202 hospitalizations, and 2 deaths. A total of 55 (75%) outbreaks occurred in 21 states that permitted sale of nonpasteurized products; incidence of nonpasteurized product–associated outbreaks was higher in these states. Nonpasteurized products caused a disproportionate number (≈150× greater/unit of product consumed) of outbreaks and outbreak-associated illnesses and also disproportionately affected persons <20 years of age. States that restricted sale of nonpasteurized products had fewer outbreaks and illnesses; stronger restrictions and enforcement should be considered.

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In the United States, milk and other dairy products are dietary staples; the 2010 Dietary Guidelines for Americans recommend that most Americans include dairy products in their diet (*1*). However, numerous pathogens can contaminate dairy products and cause illness and death. Milkborne infections were relatively common before the advent of pasteurization in the late 19th century (*2*), and in the United States today, illness related to consumption of nonpasteurized dairy products remains a public health problem.

In 1948, Michigan enacted the first statewide requirement that dairy products be pasteurized, and many other states soon did the same (*2*). In 1987, the United States Food and Drug Administration prohibited distribution of nonpasteurized dairy products in interstate commerce for sale to consumers (*3*). However, sale of nonpasteurized dairy products within the state where they are produced is regulated by each state, and some states permit sale of these products. Despite the federal ban on the sale of nonpasteurized products in interstate commerce, the broad use of pasteurization by the dairy industry, and the infrequency with which nonpasteurized dairy products are consumed, illnesses and outbreaks associated with consumption of these products continue to occur (*4*–*23*).

State and local health departments report foodborne disease outbreaks to the Centers for Disease Control and Prevention (CDC) through the Foodborne Disease Outbreak Surveillance System. As a result of efforts to enhance outbreak surveillance starting in 1998, the total number of outbreak reports increased substantially (*24*). A recent comprehensive analysis of foodborne disease outbreaks associated with dairy products (dairy-associated outbreaks) reported to CDC reviewed outbreaks that occurred during 1973–1992 (*4*). We reviewed subsequent dairy-associated outbreaks, reported in the United States during 1993–2006. We characterized the outbreaks and examined their association with state laws regarding sale of nonpasteurized dairy products.

Methods

To compare the incidence of foodborne outbreaks involving nonpasteurized dairy products among states with differing laws with regard to the sale of these products (i.e., states that permitted their sale vs. states that prohibited their sale), we reviewed reports of foodborne disease outbreaks involving dairy products reported to CDC during 1993–2006. These reports, completed by state and local health departments, typically included the number of cases associated with the outbreak; the age and sex distribution of outbreak-associated case-patients; the number of hospitalizations and deaths; the etiologic agent associated with the outbreak; the type of dairy product implicated (e.g., fluid milk, cheese); and whether the implicated dairy product was marketed, labeled, or otherwise presented to the consumer as pasteurized or nonpasteurized. Hereafter, we refer to these products as pasteurized or nonpasteurized. Thus, any outbreak involving a dairy product that was contaminated after pasteurization or that was intended to be pasteurized but underwent inadequate pasteurization was classified as involving pasteurized product. When possible, we corrected missing or incomplete data by asking the health department that conducted the investigation for more information.

To determine whether the sale of nonpasteurized dairy products was legal at the time of each outbreak, we contacted the 50 state departments of health and agriculture and requested data on whether the state permitted the sale of nonpasteurized dairy products produced in that state for each year from 1993 through 2006. We defined an illegal state-year as a year in which a state prohibited the sale of all nonpasteurized products, and we defined a legal state-year as a year in which a state permitted the sale of nonpasteurized dairy products produced in that state. Data on the estimated population, by state, for each year were obtained from the US Census Bureau. To compare the incidence of outbreak and outbreak-associated cases during illegal state-years to that during legal state-years, we stratified the outbreaks by legal status of the state in which the outbreak occurred at the time of the outbreak and calculated incidence density ratios for reported outbreaks (Poisson model) and for outbreak-associated cases (zero-inflated negative binomial model).

Results

During 1993–2006, a total of 30 states reported 122 foodborne disease outbreaks caused by contaminated dairy products. Dairy-associated outbreaks occurred in all years except 1996, and outbreaks involving nonpasteurized dairy products occurred in all years except 1994 and 1996. The number of reported dairy-associated outbreaks increased in 1998 after surveillance for foodborne disease outbreaks was enhanced (Figure 1).

Whether the product was pasteurized or nonpasteurized was known for 121 of the 122 outbreaks, and most outbreaks (73 [60%]) involved nonpasteurized dairy products. Of the 121 outbreaks for which product pasteurization status was known, 65 (54%) involved cheese and 56 (46%) involved fluid milk. Of the 65 outbreaks involving cheese, 27 (42%) involved cheese made from nonpasteurized milk. Of the 56 outbreaks involving fluid milk, an even higher percentage (82%) involved nonpasteurized milk.

The 121 outbreaks involving dairy products for which pasteurization status was known resulted in 4,413 reported illnesses. Among these illnesses, 1,571 (36%) resulted from nonpasteurized dairy products. The median number of persons reported ill during outbreaks involving

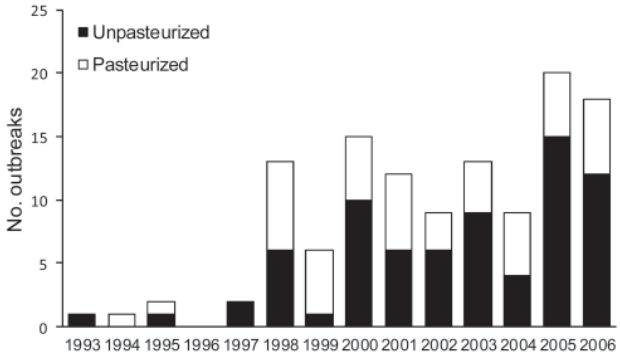

Figure 1. Number of dairy product–associated outbreaks, by year and pasteurization status of product, United States, 1993–2006.

nonpasteurized dairy products was 11 (range 2–202). Outbreaks involving nonpasteurized dairy products resulted in 202 hospitalizations (hospitalization rate 13%). In contrast, outbreaks involving pasteurized dairy products resulted in 37 hospitalizations (hospitalization rate 1%). Two deaths were associated with an outbreak caused by consuming nonpasteurized dairy products, and 1 death was associated with an outbreak caused by a pasteurized product (Table).

Ill persons in outbreaks involving nonpasteurized dairy products were generally younger than those in outbreaks involving pasteurized dairy products. For the 60 outbreaks involving nonpasteurized dairy products for which age of patients was known, 60% of patients were <20 years of age; for the 37 outbreaks involving pasteurized dairy products for which age of patients was known, 23% of patients were <20 years of age ($p<0.001$).

The causative agent was identified for all 73 outbreaks involving nonpasteurized dairy products; all were caused by bacteria. One outbreak was caused by *Campylobacter* spp. and Shiga toxin–producing *Escherichia coli*. Among the remaining 72 outbreaks, 39 (54%) were caused by *Campylobacter* spp., 16 (22%) by *Salmonella* spp., 9 (13%) by Shiga toxin–producing *E. coli*, 3 (4%) by *Brucella* spp., 3 (4%) by *Listeria* spp., and 2 (3%) by *Shigella* spp. Among the 30 outbreaks involving pasteurized dairy products for which the causative agent was reported, 13 (44%) were caused by norovirus, 6 (20%) by *Salmonella* spp., 4 (13%) by *Campylobacter* spp., 3 (10%) by *Staphylococcus aureus*, and 1 (3%) each by *Clostridium perfringens*, *Bacillus cereus*, *Listeria* spp., and *Shigella* spp.

A total of 48 reported outbreaks involved pasteurized dairy products. The source of contamination was reported for 7 (14%) of these outbreaks, of which at least 4 (57%) probably resulted from post-pasteurization contamination by an infected food handler. Failure of the consumer to store the dairy product at an appropriate temperature probably contributed to 3 other outbreaks. Such temperature abuse can enable pathogens (present because they either survived pasteurization in low numbers or were introduced after pasteurization) to multiply to concentrations capable of causing illness.

During the study period, 43 (86%) states did not change their legal status regarding the sale of nonpasteurized dairy products produced in that state. Among these 43 states, selling nonpasteurized dairy products produced in that state was legal in 21 (49%). Of the 7 states that changed their legal status, 3 changed from legal to illegal (Mississippi in 2005, Ohio in 2003, and Wisconsin in 2005), 3 changed from illegal to legal (Arkansas in 2005, Illinois in 2005, and Nevada in 2005), and 1 (Oregon) changed from legal to illegal in 1999 and then back to legal in 2005 (Figure 2).

Among the 700 state-years (14 years × 50 states) included in our analysis of the association of legal sales status and nonpasteurized dairy–associated outbreaks, sale of nonpasteurized dairy products produced in the state was legal for 342 state-years and illegal for 358 state-years. We excluded from analysis 2 outbreaks caused by nonpasteurized dairy products because each occurred in multiple states with differing laws. Of the 71 remaining outbreaks involving nonpasteurized dairy products, 55 (77%) occurred in states where sale of nonpasteurized dairy products produced in that state was legal. Among these 71 outbreaks involving nonpasteurized dairy products, 1,526 persons became ill and 1,112 (73%) of these illnesses occurred in states where it was legal to sell nonpasteurized dairy products. Also among these 71 outbreaks involving nonpasteurized dairy products, 15 occurred in states where sale of nonpasteurized dairy

Table. Characteristics of disease outbreaks after consumption of dairy products, United States, 1993–2006

Product	Outbreak characteristic, no.			
	Total	Associated illnesses	Associated hospitalizations	Associated deaths
Nonpasteurized				
Fluid milk	46	930	71	0
Cheese	27	641	131	2
Total	73	1,571	202	2
Pasteurized				
Fluid milk	10	2,098	20	0
Cheese	38	744	17	1
Total	48	2,842	37	1
All dairy	121	4,413	239	3

products was illegal. The source of the nonpasteurized dairy products was reported for 9 of these outbreaks: 7 (78%) were associated with nonpasteurized dairy products obtained directly from the producing dairy farm, 1 was associated with nonpasteurized dairy products obtained under a communal program to purchase shares in dairy cows (i.e., cow shares, a scheme used to circumvent state restrictions on commercial sales of nonpasteurized dairy products) (*11*), and 1 was limited to members of a large extended family who consumed nonpasteurized milk from their own cow.

Figure 2. Legal status of nonpasteurized dairy product sale or distribution, by state, United States, for A) 1993, B) 1999, and C) 2006. Gray shading indicates states where nonpasteurized dairy product sale or distribution was permitted. States outlined in black changed legal status during the study period.

Incidence density ratios (IDRs) for nonpasteurized product–associated outbreaks and outbreak-associated cases during legal and illegal state-years varied by the type of dairy product (milk or cheese) and are reported separately. In states where it was legal to sell nonpasteurized dairy products, the rate of outbreaks caused by nonpasteurized fluid milk was >2× as high as in states where it was illegal to sell nonpasteurized dairy products (IDR 2.20, 95% CI 1.14–4.25). The rate of outbreak-associated illnesses caused by nonpasteurized fluid milk was 15% higher in states where it was legal to sell nonpasteurized dairy products, but this result was not statistically significant (IDR 1.15, 95% CI 0.24–5.54). States where it was legal to sell nonpasteurized dairy products had nearly 6× the rate of outbreaks caused by cheese made from nonpasteurized milk (IDR 5.70, 95% CI 1.71–19.05) and nearly 6× the rate of outbreak-associated illnesses (IDR 5.77, 95% CI 0.59–56.31), although the IDR for outbreak-associated illnesses was not statistically significant.

Discussion

Incidence of outbreaks caused by nonpasteurized dairy products was higher in states that permitted the sale of nonpasteurized dairy products than in states that prohibited such sale. This association was evident for nonpasteurized fluid milk and cheese made from nonpasteurized milk. Although this association did not extend to the rates of outbreak-associated cases, factors other than whether it was legal to sell nonpasteurized dairy products probably affect the number of cases that occur in an outbreak. These factors include the volume and area of distribution of the contaminated product, the pathogen involved, the underlying health status of the exposed persons, and the ability of the responding public health agency to swiftly intervene to terminate the outbreak.

Because consumption of nonpasteurized dairy products is uncommon in the United States, the high incidence of outbreaks and outbreak-associated illness involving nonpasteurized dairy products is remarkable and greatly disproportionate to the incidence involving dairy products that were marketed, labeled, or otherwise presented as pasteurized. In a population-based survey conducted in 1996–1997, only 1.5% of respondents reported having consumed nonpasteurized dairy products in the 7 days before being interviewed; and in the 2003–2004 and 2005–2006 National Health and Nutrition Examination Surveys, only <1% of respondents who drank milk reported that they usually drank nonpasteurized milk (*21*,*25*,*26*). Because many of these respondents also reported consuming pasteurized dairy products, the proportion of dairy products consumed nonpasteurized by volume or weight is probably <1%. To illustrate this point, it is useful if we provide a hypothetical weighting of the findings in this study by the

amount of nonpasteurized and pasteurized dairy products consumed. Total milk production in the United States in 2010 was estimated at 193 billion pounds, suggesting that ≈2.7 trillion pounds of milk were consumed during the 14 years from 1993 through 2006 (*27*). If 1% of dairy products were consumed nonpasteurized, then during these 14 years, 73 outbreaks were caused by the 27 billion pounds of nonpasteurized dairy products that were consumed and 48 by the 2,673 billion pounds of pasteurized products that were consumed. Therefore, the incidence of reported outbreaks involving nonpasteurized dairy products was ≈150× greater, per unit of dairy product consumed, than the incidence involving pasteurized products. If, as is probably more likely, <1% of dairy products are consumed nonpasteurized, then the relative risk per unit of nonpasteurized dairy product consumed would be even higher.

After 1998, when surveillance for foodborne outbreaks was enhanced, the number of reported foodborne disease outbreaks caused by dairy products increased, as did the total number of reported foodborne outbreaks. Outbreaks involving nonpasteurized dairy products were all associated with bacterial enteric pathogens, most of which have known animal reservoirs. In contrast, among outbreaks in which a pasteurized dairy product was implicated, the most commonly reported causative agent was norovirus (44% of outbreaks), a pathogen with a human reservoir. These results suggest that outbreaks caused by nonpasteurized dairy products are probably caused by pathogens in the dairy environment, which would be eliminated by proper pasteurization, and that outbreaks caused by pasteurized dairy products are probably caused by contamination of the products at some point after pasteurization.

The objective of pasteurization is to eliminate from fluid milk those pathogens that originate in the dairy environment; however, pasteurization does not protect against contamination that might occur later, such as during food handling. In addition, if pasteurization is not performed properly (for appropriate times and at appropriate temperatures), pathogens might not be eliminated from the milk. Appropriate post-pasteurization food-handling practices can minimize the risk for reintroduction of pathogens into dairy products after pasteurization. In addition, other precautions, such as maintaining the dairy product at an appropriate temperature and disposing of expired products, reduce the risk to the consumer should the product become contaminated after pasteurization. When outbreaks do occur because of contamination of dairy products that are marketed as pasteurized, the source of contamination is typically traced to improper pasteurization, improper storage, or improper handling of the products after marketing (*28*–*30*). In our study, all outbreaks associated with pasteurized products for which information on the source of contamination was available were attributed to post-pasteurization mishandling.

Among outbreak-associated cases involving nonpasteurized dairy products, 60% involved persons <20 years of age. Public health and regulatory authorities are obligated to protect persons who cannot make fully informed decisions (e.g., children) from potential health hazards. Dietary decisions for younger children, in particular, are often made by caregivers. The American Academy of Pediatrics advises against giving nonpasteurized dairy products to children and recommends that pediatricians counsel caregivers against use of these products (*31*).

Proportionately more persons were hospitalized during outbreaks caused by nonpasteurized (13%) than by pasteurized dairy products (1%). This observation suggests that infections associated with nonpasteurized dairy products might be more severe, and it is consistent with the more frequent identification of bacterial, rather than viral or toxic, causative agents and with the larger proportion of illnesses affecting children.

Limitations of this analysis are primarily associated with the nature of the CDC Foodborne Disease Outbreak Surveillance System. Outbreak reporting by state and local health departments is voluntary, and outbreak reports are not always complete. For this analysis, we obtained missing data whenever possible by contacting the reporting state health department. In addition, the CDC outbreak surveillance database is dynamic; reporting agencies can submit new reports and can change or delete previous reports at any time as new information becomes available. Therefore, the results of this analysis represent data available at 1 point in time and might differ from those published earlier or subsequently.

In summary, foodborne outbreaks involving dairy products continue to be a public health problem in the United States, and this problem is disproportionately attributable to nonpasteurized dairy products. Since the US Food and Drug Administration prohibited distribution of nonpasteurized dairy products in interstate commerce for sale to consumers in 1987, all legal sale and distribution has occurred within states that permit the sale of nonpasteurized dairy products that originated in that state. How much illegal distribution in interstate commerce continues is unknown. The increased risk for outbreaks associated with legal intrastate sale of nonpasteurized dairy products demonstrated in this analysis can be weighed against the purported nutritional or other health benefits attributed to these products. Scientifically credible evidence for the health benefits of nonpasteurized dairy products beyond the benefits of those of otherwise equivalent pasteurized products is lacking (*32*). The risk for outbreaks resulting from cheese made from nonpasteurized milk in states where nonpasteurized

milk sale is legal may be higher for particular groups within those states. For example, in recent years, foodborne outbreaks involving nonpasteurized dairy products have been reported in association with traditional nonpasteurized products marketed to the growing Hispanic community in the United States (*5*,*33*).

Our analysis shows that legal intrastate sale of nonpasteurized dairy products is associated with a higher risk for dairy-related outbreaks and implies that restricting sale of nonpasteurized dairy products reduces the risk for dairy-related outbreaks within that state. Pasteurization is the most reliable and feasible way to render dairy products safe for consumption. Although warning labels and signs or government-issued permits are prudent where the sale of nonpasteurized dairy products is legal, they have not been shown to be effective and, given the results of this analysis, do not seem to reduce the incidence of outbreaks involving nonpasteurized dairy products to the degree that pasteurization does (*18*). Whether certain types of warnings or more explicit health advisories might be more effective than others is unknown. Public health officials at all levels should continue to develop innovative methods to educate consumers and caregivers about the dangers associated with nonpasteurized dairy products. State officials should consider further restricting or prohibiting the sale or distribution of nonpasteurized dairy products within their states. Federal and state regulators should continue to enforce existing regulations to prevent distribution of nonpasteurized dairy products to consumers. Consumption of nonpasteurized dairy products cannot be considered safe under any circumstances.

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Dr Langer was a CDC Preventive Medicine Fellow assigned to the Division of Foodborne, Bacterial, and Mycotic Diseases at the time of this study. He is now an epidemiologist with the CDC Division of Tuberculosis Elimination. His research interests include the investigation of infectious disease outbreaks and animal-to-human transmission of infectious agents.

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Community-associated *Clostridium difficile* Infections, Monroe County, New York, USA

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Learning Objectives

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

- Describe the relative burden of and potential risk factors for community-associated (CA) disease, based on a 6-month surveillance program for laboratory-diagnosed *Clostridium difficile* infection (CDI) cases in Monroe County, New York, in 2008
- Compare clinical characteristics of patients with CA disease with those having acquired CDI in other settings, based on the surveillance program
- Describe clinical implications of this study, including prevention strategies for CA-CDI and comparison of *C. difficile* recovery rates between refrigerated stool swabs and frozen stools

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We conducted active sentinel surveillance in Monroe County, New York, USA, to compare incidence of community-associated *Clostridium difficile* infections (CA-CDIs) with that of health care–associated infections (HA-CDIs) and identify exposure and strain type differences between CA and HA cases. Patients positive for *C. difficile* toxin and with no documented health care exposure in the previous 12 weeks were defined as possible CA case-patients. Patients with onset in a health care setting or recent health care exposure were defined as HA case-patients. Eighteen percent of CDIs were CA; 76% were in persons who reported antimicrobial drug use in the 12 weeks before CDI diagnosis. Strain type distribution was similar between CA and HA cases; North American pulsed-field 1 was the primary strain (31% CA, 42% HA; p = 0.34). CA-CDI is an

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emergent disease affecting patients recently exposed to antimicrobial drugs. Community strains are similar to those found in health care settings.

Clostridium difficile is an anaerobic, spore-forming, gram-positive bacillus that produces 2 major toxins (TcdA and TcdB). Illness caused by toxigenic *C. difficile* varies from mild diarrhea to fulminant disease and death. Infection occurs commonly in the health care setting because of concomitant exposure to the organism and antimicrobial drugs in patients with severe illnesses and concurrent conditions. Over the past several years, the incidence (*1*), severity and mortality rate (especially in elderly persons) (*2*), and treatment failure rate of *C. difficile* infection (CDI) (*3*) have increased. In addition, CDI has been more commonly observed in healthy persons often without known CDI risk factors (*4*).

The changing pattern of disease is in part being caused by the emergence of a new epidemic hypervirulent *C. difficile* strain identified as North American pulsed-field 1 (NAP1) by pulsed-field gel electrophoresis (PFGE), BI by restriction endonuclease analysis, and 027 by PCR ribotyping (*5*). NAP1 strains often demonstrate resistance to quinolones, and increased use of these drugs may provide a positive selection pressure for NAP1 relative to other strains (*6*). The incidence, risk factors, and mode of transmission of CDI in hospital-associated (HA) disease are well described. However, few studies have examined the role of the hypervirulent NAP1 strain, antimicrobial drugs, proton pump inhibitors (PPI), and foodborne transmission on the emergence of CDI (*7*–*19*).

To define the magnitude of CDI across the continuum of care (hospital, long-term care, and the community) and assess the relative incidence and possible risk factors for community-associated disease, a 6-month surveillance program for laboratory-diagnosed CDI cases was initiated in Monroe County, New York, USA, in 2008. This program was undertaken in 2 sentinel laboratories in preparation for population-based surveillance of CDI in several US states through the Emerging Infections Program of the Centers for Disease Control and Prevention (CDC). A secondary goal of this study was to compare *C. difficile* recovery rates between refrigerated fecal swab and frozen fecal specimens.

Methods

Setting

During March 1–August 31, 2008, surveillance officers reviewed all medical records associated with *C. difficile* toxin–positive fecal samples from 2 of 3 hospital laboratories in Monroe County, New York. These laboratories service long-term care facilities, doctors' offices, and the inpatient population. Electronic inpatient and outpatient medical records for all patients with fecal samples positive for *C. difficile* toxin by enzyme immunoassay were reviewed.

Case Definitions

Cases were subcategorized according to published surveillance definition guidelines (*20*). The date of CDI onset was defined as the date of the positive fecal test result, not the date of diarrhea onset. The date of diarrhea onset was not readily available in the electronic medical records. An incident case was defined as disease in a patient with a *C. difficile* toxin-positive fecal sample and no positive assay result in the preceding 8 weeks. Cases were considered recurrent if there was a positive assay result within 2–8 weeks of the most recent toxin-positive specimen. Positive assay results obtained within 2 weeks of the prior positive assay result were considered duplicates and excluded. Possible case-patients were also excluded if they had no documented diarrhea, an initial fecal sample representing recurrence of an episode before the start date of surveillance, were <12 months of age at the time of testing, or were not residents of Monroe County.

Cases were classified into 3 categories. The first category was health care facility onset (HCFO) cases. These cases were in CDI case-patients who had *C. difficile* toxin–positive fecal specimens obtained >48 hours of hospital admission or during residence in a long-term care facility.

The second category was community-onset health care–associated (CO-HCA) cases. These cases were in CDI case-patients who had *C. difficile* toxin–positive fecal specimens obtained ≤48 hours of hospital admission or as an outpatient and who had documented exposure to health care in the previous 12 weeks. Health care exposure was defined as a >24-hour stay in a hospital or 48 hours in an emergency department, residence in a long-term care facility, receipt of chronic hemodialysis, or regular hospital visits for intravenous infusion. No differentiation was made between case-patients with disease onset within 4 weeks of discharge and those with disease onset 4–12 weeks after discharge from the health care facility (i.e., indeterminate case) (*20*). Cases in the HCFO and CO-HCA categories were referred to as health care–associated (HA) CDI.

The third category was potential community-associated (potential CA) cases. These cases were in CDI case-patients who had *C. difficile* toxin–positive fecal specimens obtained within 48 hours of hospital admission or in an outpatient setting and who had no documented health care exposure in the 12 weeks before the positive test result. Potential CA case-patients were interviewed to confirm the lack of health care exposures, and to assess contact with health care personnel, medication use, travel, food consumption, and exposure to animals in the previous 12 weeks. Potential

CA case-patients who were interviewed and reported exposure to health care in the previous 12 weeks during the interview were reclassified as having CO-HCA; those who denied such exposures were confirmed as having CA-CDI. Patients who died, could not be reached, did not speak English, refused consent, or whose doctor refused consent were classified as having probable CA-CDI.

Information was obtained on demographics; previous positive assay results; dates of admission to acute-care facilities, emergency departments, and long-term care facilities; inpatient mortality rates; and CDI complications, such as toxic megacolon, renal failure, or intensive care unit admission. The study was approved by the institutional review boards at CDC, the University of Rochester Medical Center, Rochester General Hospital, and the New York State Department of Health.

Laboratory Methods

Fecal samples (unformed) were tested at clinical laboratories for *C. difficile* toxin by using the Premier Toxins A & B enzyme immunoassay (Meridian Bioscience, Inc., Cincinnati, OH, USA). Aliquots of fecal specimens were stored at –20°C until cases were reviewed. All available fecal samples of patients classified as potential CA and a random sample of the CO-HCA and HCFO (1 of each classification each week) were submitted to the New York State Public Health Laboratory (Wadsworth Center Laboratory, Albany, NY, USA) for culture. For 31 randomly chosen stool specimens, an additional specimen was obtained by using a culture swab (Copan 159C or similar). To avoid germination of *C. difficile*, no enrichment or anaerobic transport media were used. Fecal swab specimens were stored at 4°C to compare the recovery rate from a swab stored at 4°C with that for a frozen fecal aliquot. Both specimen types were stored for several weeks before shipment and were shipped on dry ice.

Culture and Molecular Characterization Methods

Fecal specimens were placed on cycloserine-cefoxitin fructose agar plates and incubated at 35°C under anaerobic conditions for 96 hours. Culture-negative fecal samples and swab specimens were treated by using alcohol shock (*21*) and recultured on cycloserine-cefoxitin fructose agar containing 0.1% sodium taurocholate (*22*). Plates were examined daily for characteristic colonies. Isolates were shipped to CDC for molecular characterization, which included PCR for binary toxin and major *tcdC* gene deletions, toxinotyping (*22*,*23*), and *Sma*I PFGE. PFGE banding patterns were analyzed by using BioNumerics version 5.10 (Applied Maths, Austin, TX, USA) and compared with the CDC *C. difficile* database (*24*). NAP types were assigned to patterns with ≈80% similarity to established NAP clusters. MICs for clindamycin, metronidazole, moxifloxacin, levofloxacin, and vancomycin were determined by using the agar dilution method, and results were interpreted by using the Clinical and Laboratory Standard Institute M11-A7 breakpoint criteria (*25*). For surveillance purposes only, levofloxacin MICs were interpreted by using criteria for moxifloxacin, and vancomycin MICs were interpreted by using criteria for *Staphylococcus aureus*.

Statistical Analysis

All statistical analyses were performed with SAS version 9.1 (SAS Institute, Cary, NC, USA) assuming a 2-tailed α of 0.05. Univariate analysis was conducted to summarize the demographic and clinical characteristics of case-patients. Bivariate analyses were used to compare these characteristics across preliminary and final classifications by using Wilcoxon rank sum, χ^2, and Fisher exact tests as appropriate.

Results

Surveillance

During the study, 366 incident CDI cases were identified after excluding 558 *C. difficile* toxin–positive stool assays for patients who did not meet eligibility criteria. The distribution of cases is shown in Figure 1. Of these cases, 196 (54%) were categorized as HCFO and 170 (46%) as CO cases. Eighty-three cases (22% of all cases) were potentially community-associated, 58 (72%) of patients with these cases were interviewed, and 16 (20%) cases were reclassified as CO-HCA. Overall, 67 cases (18% of all cases) were classified as CA; 42 of these cases were confirmed by interview and 25 were considered probable CA. Review of available electronic inpatient and outpatient records for the probable case-patients showed no previous exposure to health care. Therefore, we believe that most cases were truly CA. The probable and definite CA case-patients had similar ages, race distributions, and outcomes.

Clinical Characteristics

Clinical characteristics of CDI cases by epidemiologic classification are shown in Table 1. Compared with HCFO and CO-HCA case-patients, CA case-patients (confirmed and probable) were younger (median age 53 vs. 78 and 69 years, respectively; $p<0.001$). Illness among CA case-patients was milder; only 13 (19%) of patients required hospitalization compared with 39 (38%) CO-HCA case-patients ($p = 0.02$) (Table 2). Duration of hospitalization was 7.0 days vs. 3.5 days ($p = 0.06$) for CO-HCA and CA case-patients, respectively. None of the hospitalized CA case-patients died or had any complications. Laboratory confirmation of recurrence was documented in 22% of the HCFO and CO-HCA case-patients and 12% of the CA case-patients (Table 2).

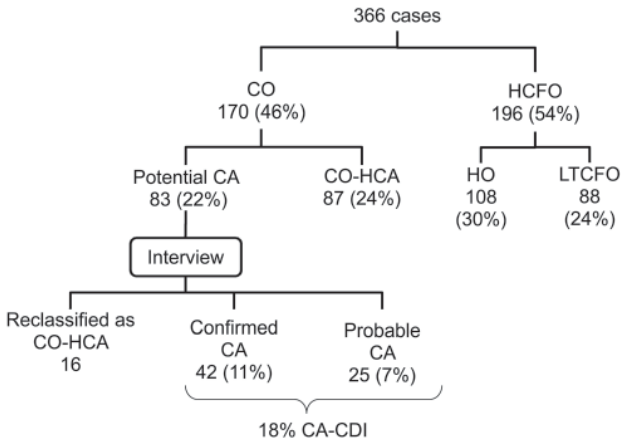

Figure 1. Classification of cases of *Clostridium difficile* infections, Monroe County, New York, USA, March 1–August 31, 2008. CO-HCA, community onset–health care associated; HO, hospital onset; LTCFO, long-term care facility onset.

Interview Results

Forty-two case-patients were confirmed as CA-CDI case-patients by interview. Median (SD) age of CA-CDI case-patients was 53 (22.6) years. CA case-patients appeared healthy, and only 30% of case-patients had an underlying illness that required regular physician visits. Severity of reported symptoms varied; the median number of bowel movements per day was 10 (range 0–50 movements), and 1 patient had syncope before diarrhea onset. Other than diarrhea, 86% of patients reported abdominal pain, 48% nausea, 40% fever and 17% bloody feces. Average time from diarrhea onset to diagnosis was 14 days (range 0–92 days). Clinical recurrence requiring a repeat course of treatment was reported by 21%; some recurrences were treated without laboratory confirmation.

Medication and health care exposures of CA case-patients in the 12 weeks before CDI are shown in Table 3. Thirty-two (76%) of the interviewed case-patients reported using antimicrobial drugs, and 20% took ≥1 type of drug. The most commonly taken drugs were penicillins, followed by clindamycin and cepholosporins (Table 3). Indications for antimicrobial drug use included upper respiratory or ear infection (26%), bronchitis/pneumonia (17%), and dental abscess treatment or dental prophylaxis (17%). Twenty-six percent of CA case-patients reported using PPIs, and only 2% reported using H_2 blockers.

Of the 42 interviewed CA case-patients, 35 (83%) reported receiving care at an outpatient office in the 12 weeks before the *C. difficile* toxin–positive test result; 13 (31%) received care only at a dental office. Nine (21%) case-patients reported visiting health care facilities without receiving care in the 12 weeks before diagnosis. Five (12%) case-patients reported no exposure to any outpatient or inpatient health care facility. Eight (19%) case-patients did not report exposure to a health care facility or to antimicrobial drugs.

Isolates

Of 145 fecal specimens cultured, 127 (87%) grew *C. difficile*. The recovery rates for HA and CA samples were 93% and 85%, respectively. The recovery of *C. difficile* was the same (80%) from fecal swab specimens and aliquots (100% agreement). One hundred nineteen *C. difficile* isolates underwent molecular and antimicrobial drug susceptibility testing. Six toxinotypes were identified; ≈50% of the isolates in each of the 3 epidemiologic classifications were wild-type toxinotype 0 (Table 4). Of the other variants, toxinotype III was the most commonly identified in all 3 epidemiologic classifications. Toxinotype V, a strain reported in food animals (*7*,*26*) was identified in 2.5%–5% of the samples and varied depending on the epidemiologic classification.

The PFGE types of 68% of the isolates were previously named NAP types; the remaining types were classified into 20 different and unnamed PFGE types (Table 5). The primary PFGE strain type among the 3 epidemiologic classes was NAP1, characteristically toxinotype III and carrying binary toxin and an 18-bp deletion in the *tcdC* gene. Three isolates identified as NAP1 by PFGE differed from the epidemic strain by having toxinotype IX/XIII and lacking the 18-bp deletion. Two of the strains from the CA isolates had characteristics that were typical of NAP1 but

Table 1. Characteristics of case-patients with CDI, Monroe County, New York, USA, March 1–August 31, 2008*

Characteristic	HCFO	CO-HCA	CA†	p value
Total	196	103	67	ND
Demographic				ND
Female sex	116 (59)	65 (63)	41 (61)	0.75
Median age, y (SD)	78 (17)	69 (17.4)	53 (20.8)	<0.001
Race				0.75
White	142 (83)	87 (87)	45 (82)	ND
Black	25 (14)	12 (12)	8 (14)	ND
Other‡	5 (3)	1 (1)	2 (4)	ND

*Values are no. (%) except as indicated. CDI, *Clostridium difficile* infection; HCFO, health care facility onset; CO-HCA, community onset–health care associated; CA, community associated; ND, not determined.
†Definite and probable CA cases.
‡Asian, American Indian/Alaska Native, and Native Hawaiian/Pacific Islander.

Table 2. Outcomes for case-patients with CDI, Monroe County, New York, USA, March 1–August 31, 2008*

Characteristic	HCFO	CO-HCA	CA†	p value
Complications				
Hospitalized for CDI	NC	39 (38)	13 (19)	0.02
Median length of stay, d (SD)	NC	7 (17)	4 (11)	0.06
Outcome‡				
Survived	158 (80)	89 (86)	44 (66)	ND
Died	23 (12)	9 (9)	0 (0)	ND
Unknown	15 (8)	5 (5)	23 (34)	ND
Deaths caused by CDI				
Yes	7/23 (30)	3/9 (33)	NC	ND
No	3/23(13)	3/9 (33)	NC	ND
Unknown	13/23 (57)	3/9 (33)	NC	ND
Laboratory-confirmed recurrence (≥1)	43 (22)	23 (22)	8 (12)	0.17

*Values are no. (%) positive or no. positive/no. tested (%) except as indicated. CDI, *Clostridium difficile* infection; HCFO, health care facility onset; CO-HCA, community onset–health care associated; CA, community associated; NC, not compared, ND, not determined.
†Definite and probable CA cases.
‡For hospitalized patients, death occurred in the hospital. For nonhospitalized patients, death occurred 8 weeks after a positive *C. difficile* assay result.

were <80% related to NAP1 by PFGE; these strains were classified as NAP1-related. NAP8, a strain associated with toxinotype V, was seen exclusively in CA isolates and at low numbers. NAP7 and NAP8 toxinotype V strains have been isolated from food animals in the United States (*7*,*26*).

A comparison of antimicrobial drug susceptibilities of the epidemic strain NAP1/toxinotype III and other strains is shown in Table 6. The NAP1 epidemic strain is more resistant to quinolones and has a slightly higher MIC_{50} to metronidazole (Figure 2).

Discussion

CA-CDI was first described in the 1980s in patients receiving outpatient antimicrobial drug treatments (*27*–*29*). In 2005, the emergence of the NAP1 epidemic strain was associated with an increase in the incidence of HA-CDI and an increase in reports of CDI in low-risk populations, such as persons living in the community, children, and peripartum women (*4*). Our laboratory surveillance showed that 18% of the CDI cases were CA, a finding that is similar to other surveillance studies reporting percentages of 20%–30% (*9*,*30*–*32*). CA-CDI case-patients were younger and healthier than those with HA exposure (median age 53 vs. 72 years). Although 20% of CA case-patients had illness severe enough to require hospitalization, no CDI-related complications or deaths were reported.

Table 3. Possible exposures to medications and health care during 12 weeks before diagnosis of CA-CDI in 42 patients, Monroe County, New York, USA, March 1–August 31, 2008*

Exposure	No. (%)
Medication†	
Antimicrobial drugs	32 (76)
Penicillins	12 (31)
Clindamycin	7 (18)
Cephalosporins	5 (13)
Quinolones	5 (13)
Macrolides	4 (10)
Sulfa	3 (8)
Metronidazole	2 (5)
H_2 blockers	1 (2)
PPI	11 (26)
Health care†	
None	5 (12)
Outpatient visit	35 (83)
Physician office	29 (69)
Dentist	13 (31)
Emergency department visit	6 (14)
Visited a hospital or LTCF	9 (21)
Health care–related job	2 (5)

*CA-CDI, community-associated *Clostridium difficile* infection; PPI, proton pump inhibitor; LTCF, long-term care facility.
†Multiple exposures could be reported in the 12 weeks before CDI.

In a population-based surveillance study in Durham North Carolina, USA, in 2005 (*9*), 59% of the CA-CDI case-patients required hospitalization, and 15% reported an emergency department visit. Similar to our findings, none of those case-patients required admission to intensive care units or surgical interventions, such as colectomy. Surveillance findings for CA-CDI in Connecticut, USA, in 2006 (*13*) showed that 111 (46%) of 241 CA-CDI case-patients required hospitalization, 29 (12%) required admission to intensive care units, 5 (2%) had toxic megacolon or colectomy, and 5 (2%) died of CDI complications. However, surveillance in Connecticut was conducted on the basis of preferential reporting by physicians and infection prevention specialists, which may have resulted in identification of the most severe disease and hospitalized case-patients. In addition, interviews were not performed to confirm lack of health care exposure.

We observed that 76% of CA-CDI case-patients were exposed to antimicrobial drugs in the 12 weeks before diagnosis. This percentage is higher than previously reported estimates of 40%–61% (*9*,*10*,*13*,*14*,*31*,*33*,*34*) and may reflect more complete information obtained during detailed case interviews. For example, several patients received antimicrobial drugs from their dentist, and such information is likely unavailable in outpatient medical records. These drugs were prescribed for common outpatient indications, and several patients received clindamycin for dental prophylaxis or infection. The role of PPI in CA-CDI remains controversial. Some studies have reported

Table 4. Toxinotypes of strains from case-patients with CDI, Monroe County, New York, USA, March 1–August 31, 2008*

Characteristic	HCFO, no. (%)	CO-HCA, no. (%)	CA, no. (%)	Total, no. (%)
Total	41	40	38	119
Toxinotype				
0	20 (48.8)	22 (55.0)	17 (44.7)	59 (49.6)
III	19 (46.3)	12 (30.0)	18 (47.4)	49 (41.2)
V	1 (2.4)	1 (2.5)	2 (5.3)	4 (3.4)
IX/XXIII	0	3 (7.5)	0	3 (2.5)
XII	1 (2.4)	1 (2.5)	0	2 (1.7)
XIV/XV	0	0	1 (2.6)	1 (0.8)
Nontoxigenic	0	1 (2.5)	1 (0.0)	1 (0.8)

*CDI, *Clostridium difficile* infection; HCFO, health care facility onset; CO-HCA, community onset–health care associated; CA, community associated.

increased risk for disease associated with their use (*12*,*35*). Twenty-six percent of CA-CDI case-patients interviewed reported PPI use. However, it is difficult to assess if this is a major risk without a control group comparison.

Molecular testing showed a similar distribution of strains between HA and CA cases, and the percentage of cases with the NAP1 epidemic strain ranged from 46% in HA cases to 32% in CA-CDI cases. The percentage of CA cases with the NAP1 strain was similar to that in other reports (18%–37%) (*8*,*36*–*38*). Similar strain distribution in health care facilities and the community suggests that in contrast to the emergence of CA-methicillin-resistant *S. aureus* strains, there was no preferential transmission of particular strains within or outside the health care setting. Health care facilities might act as a reservoir for CA disease or that the community might act as a reservoir for HA-associated disease.

Our study examined potential exposure routes for *C. difficile* acquisition in the community. There are 4 postulated sources for exposure to *C. difficile* spores (*39*): consumption of contaminated food and water, animal-to-person contact, person-to-person contact, and environment-to-person contact. Foodborne acquisition has been hypothesized as a source of CA infections on the basis of recovery of *C. difficile* spores from food products and similarities between strains recovered from animals and those known to cause disease in humans (*15*–*19*). However, there is currently insufficient evidence to support foodborne acquisition as a common source of CA-CDI (*7*).

We assessed food and animal exposure during interviews with CA-CDI case-patients and did not find any specific association. However, we were unable to compare our observations with food and animal exposure patterns among persons in the community without CDI. Other possible sources of exposures include environments contaminated by *C. difficile* spores, such as hospitals and long-term care facilities; 21% of CA-CDI case-patients reported visiting or accompanying a family member to a health care facility in the 12 weeks before diagnosis. Contact with an ill or *C. difficile*–colonized family member or a household member who worked in a health care setting (i.e., someone who might have carried *C. difficile* spores on their hands or clothes) is another possible exposure. Two case-patients reported that a family member had diarrhea or was given a diagnosis of CDI, and several had a household member who worked in a health care setting. We also observed an excellent *C. difficile* recovery rate from refrigerated stool swabs, indicating that this method could be used in epidemiologic studies in which storage and processing of *C. difficile* specimens are required.

Our findings need to be interpreted in light of several limitations. We were unable to calculate the incidence of CA-CDI because surveillance did not include all laboratories servicing the Monroe County population. This study is descriptive, and the lack of a control group prevents us from estimating the risk for various exposures in development of CDI. At the time of this study, diagnosis of *C. difficile* relied on testing with the toxins A and B enzyme

Table 5. PFGE typing of *Clostridium difficile* from case-patients with CDI, Monroe County, New York, USA, March 1–August 31, 2008*

PFGE type	HCFO, no. (%)	CO-HCA, no. (%)	CA, no. (%)	Total, no. (%)
NAP1	19 (46.4)	14 (35.0)	12 (31.6)	45 (37.8)
NAP1-related	0	0	2 (5.3)	2 (1.7)
NAP2	2 (4.9)	4 (10.0)	1 (2.6)	7 (5.9)
NAP4	1 (2.4)	0	1 (2.6)	2 (1.7)
NAP5	2 (4.9)	6 (15.0)	0 (0.0)	8 (6.7)
NAP6	3 (7.3)	1 (2.5)	3 (7.9)	7 (5.9)
NAP7	1 (2.4)	1 (2.5)	1 (2.6)	3 (2.5)
NAP8	0	0	1 (2.6)	1 (0.8)
NAP10	0	1 (2.5)	1 (2.6)	2 (1.7)
NAP11	2 (4.9)	0	1 (5.3)	4 (3.4)
Unnamed	11 (26.8)	13 (32.5)	14 (36.8)	38 (31.9)
Total	41	40	38	119

*PFGE, pulsed-field gel electrophoresis; CDI, *Clostridium difficile* infection; HCFO, health care facility onset; CO-HCA, community onset–health care associated; CA, community associated; NAP, North American pulsed-field.

Table 6. Antimicrobial drug MICs for NAP 1/toxinotype III strains and other strains from case-patients with CDI, Monroe County, New York, USA, March 1–August 31, 2008*

	NAP1/toxinotype III, μg/mL			Other strains, μg/mL		
Drug	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
Clindamycin	≤2	>32	≤2 to>32	≤2	>32	≤2 to >32
Levofloxacin	>32	>32	≤2 to >32	4	>32	≤2 to >32
Moxifloxacin	16	>32	≤2 to >32	≤2	32	≤2 to >32
Metronizadole	2	4	≤0.5 to 4	≤0.5	2	≤0.5 to 2
Vancomycin	1	2	<0.5 to 2	1	2	<0.5 to 2

*NAP, North American pulsed-field; CDI, *Clostridium difficile* infection. Clinical and Laboratory Standards Institute interpretive criteria (sensitive/intermediate/resistant): clindamycin: ≤2/4/≥8 μg/mL; levofloxacin: not available; moxifloxacin: ≤2/4/≥8 μg/mL; metronidazole: ≤8/16/≥32 μg/mL; vancomycin: not available.

immunoassay, which has a sensitivity of 60%–90% and specificity of 90%–95%. In low- prevalence populations, such as outpatients, the positive predictive value is low and the likelihood of false-positive results is higher, which might have biased some results by including patients who did not have CDI (*40*). However, this bias was minimized by laboratory refusal of formed (i.e., nondiarrheal) fecal specimens and exclusion of cases without diarrheal symptoms. We did not review medical records from physician and dental offices. Therefore, patient-reported antimicrobial drug and PPI use was not confirmed. We attempted to interview all persons with potential CA-CDI but were unable to do so in 29% of the cases. These cases were defined as probable CA and included in our clinical summary. The small number of CA-CDI cases and isolates also limited our capacity to assess difference between NAP1 and other strains in severity and outcome of CDI.

In conclusion, CA-CDI represented 18% of CDI cases in Monroe County. CA-CDI case-patients were younger and healthier than HA-CDI case-patients. Use of antimicrobial drugs in outpatient settings remains a serious exposure, and even limited exposure to the health care environment or to persons in contact with health care facilities might play a crucial role in acquisition of CDI in the community. Prevention of CA-CDI will require further studies to understand risk factors leading to CDI in patients not exposed to antimicrobial drugs and the role of various potential exposures to *C. difficile*, such as food, animals, and household environment. Our results suggest that educating outpatient clinicians, including dentists, about the risk for community-associated CDI following use of oral antimicrobial drugs and that promoting judicious use of these drugs are potentially important interventions for the prevention of CDI in the outpatient setting.

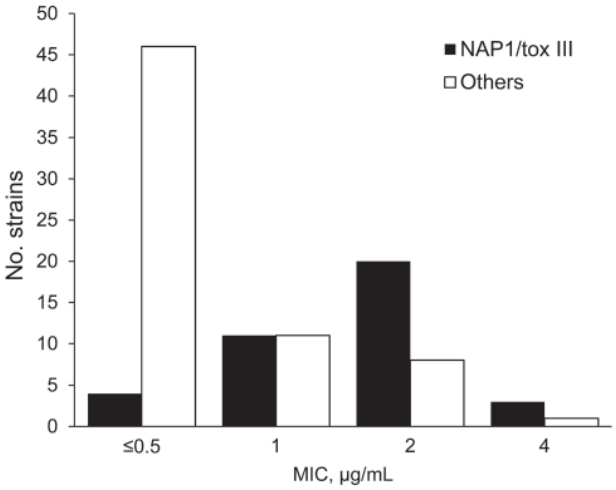

Figure 2. Metronidazole MICs (μg/mL) for North American pulsed-field 1 (NAP1) strains of *Clostridium difficile* compared with MICs for other strains, Monroe County, New York, USA, March 1–August 2008. tox, toxinotype.

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Ocozocoautla de Espinosa Virus and Hemorrhagic Fever, Mexico

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Arenavirus RNA was isolated from Mexican deer mice (*Peromyscus mexicanus*) captured near the site of a 1967 epidemic of hemorrhagic fever in southern Mexico. Analyses of nucleotide and amino acid sequence data indicated that the deer mice were infected with a novel Tacaribe serocomplex virus (proposed name Ocozocoautla de Espinosa virus), which is phylogenetically closely related to Tacaribe serocomplex viruses that cause hemorrhagic fever in humans in South America.

Tacaribe serocomplex viruses (family *Arenaviridae*, genus *Arenavirus*) comprise *Bear Canyon virus*, *Tamiami virus*, and *Whitewater Arroyo virus* in the United States; *Tacaribe virus* (TCRV) on Trinidad; *Chaparé virus* (CHPV) and *Machupo virus* (MACV) in Bolivia; *Guanarito virus* (GTOV) in Venezuela; *Junín virus* (JUNV) in Argentina; *Sabiá virus* (SABV) in Brazil; and 9 other species (*1*). Provisional species in the Tacaribe serocomplex include Big Brushy Tank virus, Catarina virus, Skinner Tank virus, and Tonto Creek virus in the United States (*2*–*4*), and Real de Catorce virus in Mexico (*5*).

Five members of the Tacaribe serocomplex (CHPV, GTOV, JUNV, MACV, and SABV) cause hemorrhagic fever in humans (*6*,*7*). Diseases caused by these viruses are zoonoses. Specific members of the rodent family Cricetidae (*8*) are the principal hosts of the Tacaribe serocomplex viruses for which natural host relationships have been well characterized. For example, the short-tailed cane mouse (*Zygodontomys brevicauda*) in western Venezuela is the principal host of GTOV (*9*,*10*), and the drylands vesper mouse (*Calomys musculinus*) in central Argentina is the principal host of JUNV (*11*).

The history of human disease in North America includes large epidemics of highly lethal hemorrhagic fever during 1545–1815 in Mexico (*12*). These epidemics primarily affected native inhabitants of the highlands. Medical historians theorized that the hemorrhagic fever was caused by Tacaribe serocomplex virus(es) or other viruses associated with rodents native to Mexico (*13*).

A recently published study reported antibody against a Tacaribe serocomplex virus in 3 (25.0%) of 12 Mexican deer mice (*Peromyscus mexicanus*) and 0 of 29 other cricetid rodents captured in the municipality of Ocozocoautla de Espinosa, State of Chiapas, Mexico (*14*). Analyses of serologic data suggested that the 3 antibody-positive deer mice were infected with an arenavirus that is antigenically more closely related to the South American hemorrhagic fever arenaviruses than to other North American Tacaribe serocomplex viruses. The objective of this study was to determine the identity of the Tacaribe serocomplex virus associated with *P. mexicanus* deer mice in western Chiapas.

Materials and Methods

Kidney samples from the 3 antibody-positive Mexican deer mice, Mexican deer mouse TK93314 (*15*) and 8 other antibody-negative Mexican deer mice, 18 southern pygmy mice (*Baiomys musculus*), and 11 Jaliscan cotton rats (*Sigmodon mascotensis*) were tested for arenavirus by cultivation in monolayers of Vero E6 cells (*16*). The 3 antibody-positive deer mice (TK93319, TK93321, TK93325) and 38 antibody-negative rodents were captured on July 16, 2000, at a locality (Universal Transverse Mercator coordinates 15–451772E, 1864243N; elevation 1,021 m) in the municipality of Ocozocoautla de Espinosa (Figure 1).

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Figure 1. Chiapas (CHP) and surrounding states in southern Mexico. The star indicates where the rodents in this study were captured; the solid circle indicates the location of the hospital that provided care for the persons affected by hemorrhagic fever in the 1967 epidemic (*17*). Inset shows the location of CHP in Mexico. CAM, Campeche; OAX, Oaxaca; TAB, Tabasco; VER, Veracruz.

Kidney samples from the 3 antibody-positive animals also were tested for arenavirus nucleocapsid (N) protein gene RNA. First-strand cDNA was synthesized by using SuperScript III Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA, USA) and oligont 19C-cons (*18*). The first-round PCR used Master*Taq* Kit (5 PRIME, Inc., Gaithersburg, MD, USA) and 19C-cons and either AVNP42 (5′-GCCGCGGACTGGGAGGGCA-3′) or AVNP122 (5′-GCCGCGGACTGGGGAGGCACTG-3′). The second-round (heminested) PCR used Master *Taq* Kit and either AVNP42 and oligont 1010C (*19*), AVNP122 and 1010C, or AVNP115 (5′-CCAATATA AGGCCAACCATCG-3′) and AVNP149 (5′-CGCACA GTGGATCCTAGGCATAGTGTC-3′). The nucleotide sequence of a 3,380-nt fragment of the small genomic segment of arenavirus AV B1030026 (GenBank accession no. JN897398) was then determined from the first-strand cDNA from antibody-positive deer mouse TK93325 by using a series of 3 heminested PCRs. The 3,380-nt fragment extended from within the 5′ noncoding region, through the glycoprotein precursor (GPC) gene, intergenic region, and N protein gene, and into the 3′ noncoding region.

Analyses of GPC sequences, N protein sequences, and nucleotide sequences included AV B1030026, 8 other viruses from North America, and 15 viruses from South America (Figure 2). Sequences in each amino acid sequence dataset were aligned by using ClustalW version 2.0.12 (*21*). The sequences in each nucleotide sequence dataset were aligned manually, and alignment was guided by the corresponding computer-generated amino acid sequence alignment. Sequence nonidentities were equivalent to uncorrected (*p*) distances.

Phylogenetic analyses of nucleotide sequences were conducted by using MRBAYES version 3.1.2 (*22*) and programs in PAUP* (*23*). Bayesian analyses used the general time reversible + proportion invariant + Γ model with a site-specific gamma distribution and the following options in MRBAYES version 3.1.2: two simultaneous runs of 4 Markov chains, 10 million generations, and sample frequency every 1,000th generation. The first 1,000 trees were discarded after review of the likelihood scores, convergence statistics, and potential scale reduction factors. A consensus tree (50% majority rule) was constructed from the remaining trees. Probability values in support of the clades were calculated a posteriori, and clades with probability values ≥0.95 were considered supported by the data (*20*).

Results

Arenavirus was not isolated from any kidney samples. However, N protein gene RNA of arenavirus AV B1030022 and N protein gene RNA of arenavirus AV B1030026 were detected in samples of kidney from Mexican deer mice TK93321 and TK93325, respectively. The sequence of a 746-nt fragment of the N protein gene of AV B1030022 (GenBank accession no. JN897399) was 96.4% identical to the nucleotide sequence of the homologous region of the N protein gene of AV B1030026.

Bayesian analyses of complete GPC gene sequences (Figure 2, panel A) and complete N protein gene sequences (Figure 2, panel B) separated the Tacaribe serocomplex viruses into 4 groups (A, B, C, D). Arenavirus AV B1030026 was included in group B with Amaparí virus (AMAV), Cupixi virus (CPXV), TCRV, and the 5 viruses from South America known to cause hemorrhagic fever in humans. Group D is exclusively North American, groups A and C are exclusively South American, and probability values calculated a posteriori indicate strong support for monophyly of viruses in each group and strong support for the sister relationship between AV B1030026 and TCRV.

Nonidentities between amino acid sequences of the GPC and N protein of AV B1030026 and amino acid sequences of homologous sequences of the 8 other viruses from North America ranged from 47.8% to 52.1% and from 44.3% to 45.4%, respectively. Similarly, nonidentities between the amino acid sequences of the GPC and N protein of AV B1030026 and homologous sequences of the 8 other

members of group B ranged from 24.7% to 46.8% and from 16.1% to 32.3%, respectively (Table). Nonidentity between the GPC amino acid sequences of AV B1030026 and TCRV was greater than the nonidentity between the GPC amino acid sequences of CHPV and SABV (Table), among the GPC amino acid sequences of the 5 viruses in group A (range 15.8%–23.7%), and between the GPC amino acid sequences of Latino virus (LATV) and Oliveros virus (20.6%). Last, nonidentity between the N protein amino acid sequences of AV B1030026 and TCRV was greater than the nonidentities between N protein amino acid sequences of 7 viruses from South America in 5 pairwise comparisons: AMAV and CPXV, AMAV and GTOV, CPXV and GTOV, CHPV and SABV, JUNV and MACV (Table).

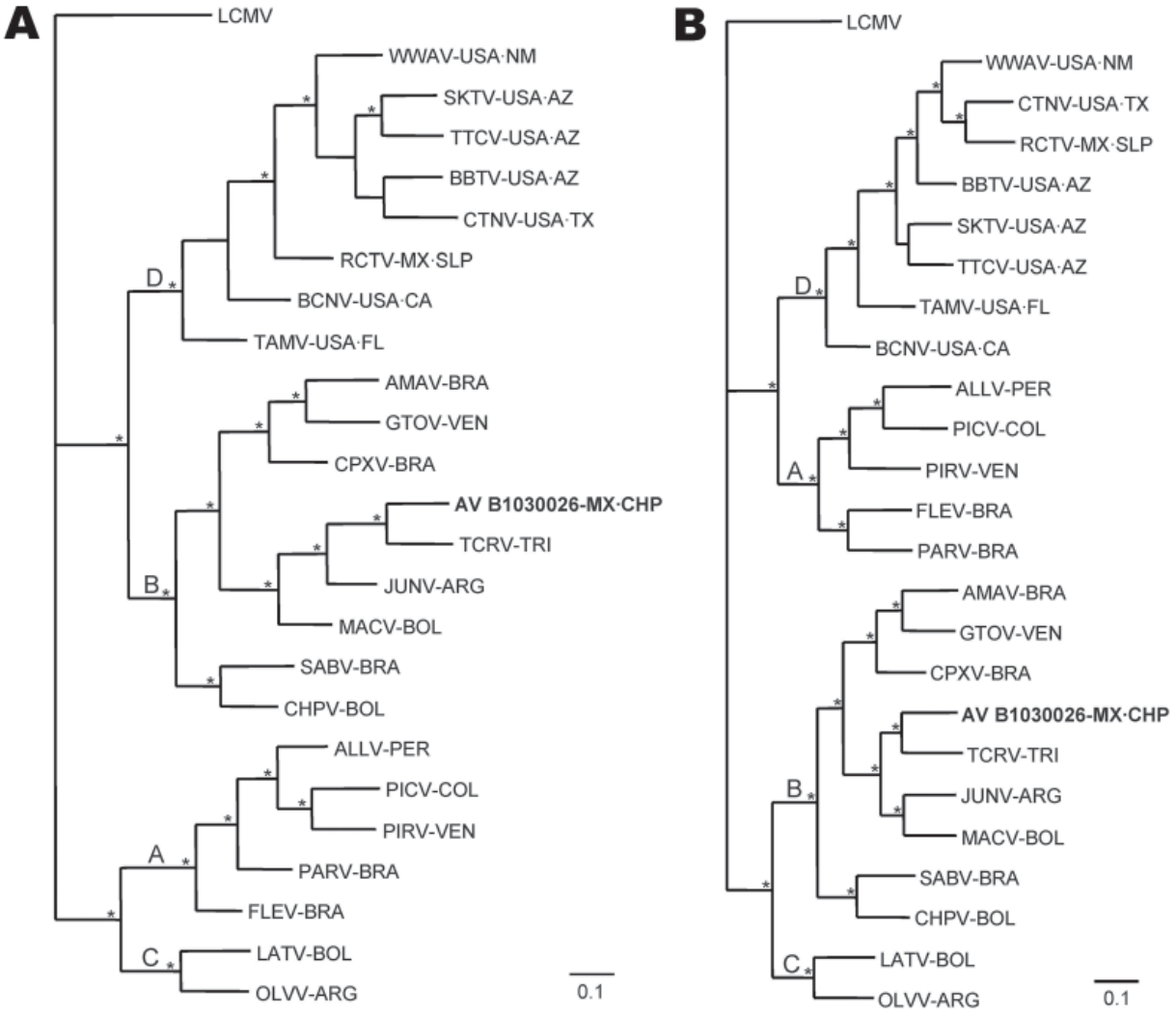

Figure 2. Phylogenetic relationships among Tacaribe serocomplex viruses from the United States, Mexico, and South America, as determined on the basis of Bayesian analyses of A) full-length glycoprotein precursor gene sequences and B) full-length nucleocapsid protein gene sequences. Arenavirus AV B1030026 is shown in **boldface**. Scale bars indicate nucleotide substitutions per site. Probability values in support of the clades were calculated a posteriori, clades with probability values ≥0.95 were considered supported by the data (*20*), and asterisks at nodes indicate that clades were supported by the data. Viruses: LCMV, lymphocytic choriomeningitis virus; WWAV, Whitewater Arroyo virus; SKTV, Skinner Tank virus; TTCV, Tonto Creek virus; BBTV, Big Brushy Tank virus; CTNV, Catarina virus; RCTV, Real de Catorce virus; BCNV, Bear Canyon virus; TAMV, Tamiami virus; AMAV, Amaparí virus; GTOV, Guanarito virus; CPXV, Cupixi virus; TCRV, Tacaribe virus; JUNV, Junín virus; MACV, Machupo virus; SABV, Sabiá virus; CHPV, Chaparé virus; ALLV, Allpahuayo virus; PICV, Pichindé virus; PIRV, Pirital virus; PARV, Paraná virus; FLEV, Flexal virus; LATV, Latino virus; OLVV, Oliveros virus. Locations: NM, New Mexico; AZ, Arizona; TX, Texas; MX·SLP, San Luis Potosí, Mexico; CA, California; FL, Florida; BRA, Brazil; VEN, Venezuela; MX·CHP, state of Chiapas Mexico; TRI, Trinidad; ARG, Argentina; BOL, Bolivia; PER, Peru; COL, Colombia. LCMV, the prototypic member of the lymphocytic choriomeningitis–Lassa (Old World) serocomplex, was the designated outgroup in the analyses. Virus strain designations and GenBank accession numbers are provided online (wwwnc.cdc.gov/EID/article/18/3/11-1602-F2.htm).

Table. Nonidentities among amino acid sequences of glycoprotein precursors and amino acid sequences of nucleocapsid proteins of Ocozocoautla de Espinosa virus strain AV B1030026, Mexico, and 8 arenaviruses from South America*

	% Amino acid sequence nonidentity								
Virus	OCEV	AMAV	CHPV	CPXV	GTOV	JUNV	MACV	SABV	TCRV
OCEV	–	43.7	45.1	44.8	46.5	31.6	33.5	46.8	24.7
AMAV	27.7	–	44.8	30.4	29.9	42.0	42.3	46.0	45.3
CHPV	31.6	27.3	–	43.3	43.9	42.7	42.8	22.3	45.1
CXPV	27.7	17.1	28.4	–	31.2	45.2	44.8	43.5	45.7
GTOV	27.0	14.6	27.9	17.1	–	43.0	44.2	44.8	45.9
JUNV	16.1	26.3	30.3	27.1	25.2	–	29.3	44.6	32.6
MACV	17.9	25.7	29.1	25.9	23.7	12.2	–	43.6	34.9
SABV	32.3	27.7	16.2	29.3	29.5	29.9	28.3	–	47.4
TCRV	18.8	29.8	31.2	30.5	28.7	21.3	20.4	33.0	–

*Nonidentities (*p* model distances) among sequences of glycoprotein precursors are listed above the diagonal and those among sequences of nucleocapsid proteins below the diagonal. OCEV, Ocozocoautla de Espinosa virus; AMAV, Amaparí virus strain BeAn 70563; CHPV, Chaparé virus, 200001071; CPXV, Cupixi virus, BeAn 119303; GTOV, Guanarito virus, INH-95551; JUNV, Junín virus, XJ13; MACV, Machupo virus, Carvallo; SABV, Sabiá virus, SPH 114202; TCRV, Tacaribe virus, TRVL 11573.

Discussion

Arenaviruses AV B1030022 and AV B1030026 are direct evidence that arenaviruses phylogenetically closely related to the South American hemorrhagic fever arenaviruses are enzootic in North America. Results of Bayesian analyses of GPC gene sequence data, Bayesian analyses of the N protein gene sequence data, and pairwise comparisons of amino acid sequences collectively indicate that AV B1030026 is a strain of a novel species (proposed name Ocozocoautla de Espinosa virus) in the family *Arenaviridae*, genus *Arenavirus* (*1*).

The hallmark of the arenaviruses is their ability to establish chronic infections in their respective principal hosts. The failure to isolate arenavirus from Mexican deer mice in this study could be caused by small sample size or poor specimen quality. Alternatively, a cricetid rodent other than the Mexican deer mouse is the principal host of Ocozocoautla de Espinosa virus (OCEV).

Members of the rodent family Cricetidae, subfamily Sigmodontinae (*8*) are the principal hosts of GTOV, JUNV, MACV, and other Tacaribe serocomplex viruses in South America for which natural host relationships have been well characterized (*24*). The available fossil record suggests that sigmodontine rodents originally invaded South America from North America after formation of the Panamanian Isthmus 2.5–3.5 million years ago.

The presence of OCEV in Mexico suggests that the last common ancestor of the 9 viruses in group B (Figure 2) emerged in North America. As such, arenaviruses phylogenetically closely related to OCEV, in association with cricetid rodents, may be widely distributed in North America. We note that antibody against AMAV has been found in northern pygmy mice (*Baiomys taylori*) captured in Texas and northern Mexico (*14*).

The history of the State of Chiapas includes an outbreak of a highly lethal hemorrhagic fever in 1967 (*17*). Antibody against Paraná virus (PARV) or LATV was found in convalescent-phase serum samples from persons who survived the fever, by an assay in which antibodies against PARV and LATV reacted with GTOV, JUNV, TCRV, and other Tacaribe serocomplex viruses (*25*). Of note, hemorrhagic fever in Chiapas clinically resembled hemorrhagic fevers caused by arenaviruses from South America, the outbreak was preceded by large-scale destruction of forested areas in the epidemic area, the *P. mexicanus* deer mouse is a relatively common species in forests of southern Mexico (*26*), and abundance of rodents in and around houses in the epidemic area had increased to disturbing proportions in the 3-year period before 1967 (*17*). Hypothetically, OCEV or an arenavirus phylogenetically closely related to OCEV was the etiologic agent in the hemorrhagic fever epidemic in Chiapas in 1967 and presently is the cause of a human disease that is clinically indistinct from dengue hemorrhagic fever and other severe febrile illnesses that are endemic to Chiapas.

It is generally accepted that humans usually become infected with arenaviruses by inhalation of virus in aerosolized droplets of secretions or excretions from infected rodents. Another source of infection may be ingestion of infected rodents (*27*).

Human consumption of wild rodents is common in rural areas in some regions of Mexico. For example, Mexican deer mice and other cricetid rodents are consumed by the Tzeltal Indians in the highlands of Chiapas (*28*). Future studies on arenaviruses native to North America should include work to assess whether humans who consume wild rodents or live or work in close association with cricetid rodents in the highlands of Mexico acquire illness from OCEV or other North American Tacaribe serocomplex viruses.

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Causes of Pneumonia Epizootics among Bighorn Sheep, Western United States, 2008–2010

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Epizootic pneumonia of bighorn sheep is a devastating disease of uncertain etiology. To help clarify the etiology, we used culture and culture-independent methods to compare the prevalence of the bacterial respiratory pathogens *Mannheimia haemolytica, Bibersteinia trehalosi, Pasteurella multocida,* and *Mycoplasma ovipneumoniae* in lung tissue from 44 bighorn sheep from herds affected by 8 outbreaks in the western United States. *M. ovipneumoniae*, the only agent detected at significantly higher prevalence in animals from outbreaks (95%) than in animals from unaffected healthy populations (0%), was the most consistently detected agent and the only agent that exhibited single strain types within each outbreak. The other respiratory pathogens were frequently but inconsistently detected, as were several obligate anaerobic bacterial species, all of which might represent secondary or opportunistic infections that could contribute to disease severity. These data provide evidence that *M. ovipneumoniae* plays a primary role in the etiology of epizootic pneumonia of bighorn sheep.

In North America, epizootic pneumonia is a devastating, population-limiting disease of bighorn sheep (*Ovis canadensis*) (*1–5*). Anecdotal and experimental evidence suggests that in at least some instances, this disease may be introduced into bighorn sheep populations by contact with domestic sheep or goats (*5,6*). When the disease is first introduced, outbreaks affect animals of all ages (*1–3*). During subsequent years or decades, sporadic cases of pneumonia in adult sheep and annual epizootics of pneumonia in lambs may continue (*7–10*).

Considering the dramatic and severe character of epizootic bighorn sheep pneumonia, the etiology is surprisingly unclear. Findings of gross and histopathologic examinations of lung tissue strongly suggest bacterial etiology: anterior–ventral distribution, suppurative inflammation, and abundant bacterial colonies. In domestic ruminants, bacterial pneumonia frequently occurs secondary to viral infections or other pulmonary insults, but extensive efforts to detect such underlying factors for bighorn sheep pneumonia have generally been nonproductive. For example, although evidence of infection or exposure to respiratory viruses, especially respiratory syncytial virus and parainfluenza virus, is frequently found in healthy and pneumonia-affected populations, no consistent association between the disease and any virus has been found (*11–13*). As a result, most research attention has been directed toward bacterial respiratory pathogens that may act as primary infectious agents, particularly leukotoxin-expressing *Mannheimia haemolytica,* which is highly lethal to bighorn sheep after experimental challenge (*5,14*). Other *Pasteurellaceae*, particularly *Bibersteinia trehalosi* and *Pasteurella multocida,* have been more frequently isolated from pneumonia-affected animals during natural outbreaks than has *M. haemolytica* (*11,12,15*). Another candidate pathogen, *Mycoplasma ovipneumoniae*, has recently been isolated from pneumonia-affected bighorn sheep during 2 epizootics (*11,16,17*); antibodies against this agent were detected in bighorn sheep from 9 populations undergoing

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pneumonia epizootics but were absent in 9 nonaffected populations (*17*). In experiments, *M. ovipneumoniae* has been shown to predispose bighorn sheep to *M. haemolytica* pneumonia (*18*). When *M. ovipneumoniae*–free domestic sheep were commingled with bighorn sheep, the bighorn sheep survived at unprecedented rates (*19*).

Development of effective methods for managing, preventing, or treating an infectious disease requires a clear understanding of its underlying etiology. However, clarifying the etiology can be difficult, particularly for primary infections (e.g., HIV) that are characteristically associated with multiple opportunistic infections that may be more lethal than the epidemic agent itself. During 2008–2010, epizootic pneumonia of bighorn sheep was detected in at least 5 western US states. These epizootics provided an opportunity to conduct a comparative study of the etiology of this disease (Table 1).

Conventional microbiological methods can fail to isolate agents because of their fastidious in vitro growth requirements or intermicrobial interactions; thus, for agent isolation, we used 2 culture-independent methods (agent-specific PCRs and 16S clone libraries) in addition to conventional bacterial cultures (*17*,*20*–*22*). We expected that primary etiologic agents could be differentiated from opportunistic agents by 1) their detection at high prevalence in affected animals, 2) the presence of single (clonal) strain types within each outbreak, and 3) their uncommon or lack of detection in animals from healthy populations (*11*,*22*–*24*). Therefore, to clarify the etiology of epizootic pneumonia, we applied these criteria to the bacterial respiratory pathogens detected in multiple bighorn sheep epizootics.

Materials and Methods

Bighorn Sheep Populations

The study sample consisted of 8 demographically independent bighorn sheep populations in 5 states (Montana, Nevada, Washington, Oregon, and South Dakota) that had been affected by epizootic pneumonia during 2008–2010 and for which lung tissue specimens from ≥4 affected animals were available (Table 1). In 6 of these populations, the disease affected bighorn sheep of all ages; in the other 2 populations, in which the disease had previously affected sheep of all ages, the disease was restricted to lambs. Convenience samples were selected among those available from each epizootic: the sample of pneumonia-affected animals consisted of the first 4–6 sheep for which pneumonia had been confirmed by gross or microscopic lesions. Sheep initially selected for analysis but later determined to have lacked gross or microscopic lesions characteristic of pneumonia were retained in the study but analyzed separately. Negative controls consisted of animals with no gross or histopathologic evidence of pneumonia that died or were culled from 2 closely observed healthy populations.

Bacteriologic Cultures

Surfaces of affected lung tissue specimens were seared, and swab samples of deeper tissues were obtained and streaked onto Columbia blood agar plates (Hardy Diagnostics, Santa Maria, CA, USA). *Pasteurellaceae* were isolated and identified by using routine methods (*25*) and then stored at −80°C in 30% buffered glycerol in brain–heart infusion agar (Hardy Diagnostics).

DNA Template Preparation

DNA was extracted from 1.0–1.5 mL of fluid collected from 1–2 g of fresh-frozen lung tissue macerated in 1 mL of phosphate buffered saline for 5 min by using a stomacher (Seward Stomacher 80 Laboratory Blender, Bohemia, NY, USA). DNA was extracted by using a QIAamp mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol.

PCR Detection of Respiratory Pathogens and *lktA*

To detect *M. haemolytica*, *P. multocida*, *B. trehalosi*, *lktA*, and *M. ovipneumoniae*, we used previously published

Table 1. Bighorn sheep populations included in study of populations affected by epizootic pneumonia, western United States, 2008–2010*

Population	Status†	Population size	% Dead or culled‡
East Fork Bitterroot, MT	Pneumonic	200–220	50
Bonner, MT	Pneumonic	160–180	68
Lower Rock Creek, MT	Pneumonic	200	43
Anaconda, MT	Pneumonic	300	50
East Humboldt/Ruby Mountains, NV	Pneumonic	160–180	80
Yakima Canyon, WA	Pneumonic	280	33
Spring Creek, SD	Pneumonic	≈40 lambs born	95 lambs
Hells Canyon, OR and WA	Pneumonic	≈170 lambs born	77 lambs
Quilomene, WA	Healthy	160	2
Asotin Creek, WA	Healthy	100	0

*MT, Montana; NV, Nevada; SD, South Dakota; WA, Washington; OR, Oregon.
†Pneumonic, populations with confirmed epizootic pneumonia restricted to lambs (Spring Creek and Hells Canyon) or not age restricted (all other pneumonic populations); healthy, populations with no evidence of epizootic pneumonia.
‡Estimated percentage of the population that died or was culled during the epizootic.

PCR protocols with minor modifications (Table 2). All reactions were conducted individually in 20-μL volumes containing 2 μL of DNA template (5–1,000 ng/μL), 10 μL of master mix (QIAGEN Hotstar mix for *P. multocida*, *M. ovipneumoniae*, and *lktA* and QIAGEN Multiplex PCR mix for *B. trehalosi* and *M. haemolytica*), and primers at 0.2 μmol (*P. multocida*, *M. haemolytica*, *and B. trehalosi*), 2 μmol (*M. ovipneumoniae*), or 0.5 μmol (*lktA*). Thermocycler conditions included an initial denaturation step at 95°C (15 min) for all agents and a final extension step at 72°C (5 min, except final extensions for *P. multocida* and *lktA* were 9 and 10 min, respectively). Cycling conditions used were as follows: for *M. ovipneumoniae*, 30 cycles at 95°C for 30 s, at 58°C for 30 s, and at 72°C for 30 s; for *B. trehalosi* and *M. haemolytica,* 35 cycles at 95°C for 30 s, at 55°C for 30 s, and at 72°C for 40 s; and for *P. multocida* and *lktA,* 30 cycles at 95°C for 60 s, at 55°C for 60 s, and at 72°C for 60 s. Amplicons were examined in UV light after electrophoresis in 1.2% agarose gel containing 0.005% ethidium bromide in 0.5× Tris/borate/EDTA buffer at 7 V/cm.

16S Analyses

To detect predominant microbial populations in the pneumonic lung tissues, we used a culture-independent method (*17*). In brief, we aseptically collected two 1-g samples of lung tissue from sites at least 10 cm apart in grossly abnormal (consolidated) tissue from 16 pneumonia-affected animals, including 2 from each outbreak. Tissues were stomached and DNA was extracted (DNeasy Blood and Tissue Kit; QIAGEN) from 100-μg aliquots of each homogenate. Segments of 16S rDNA were PCR amplified and cloned. Insert DNA was sequenced (vector primers T3 and M13, BigDye version 3.1, ABI PRISM Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) from 16 clones derived from each homogenate, resulting in 32 sequences from each animal. DNA sequences were assigned to species (≥99% identity) or genus (≥97% identity) according to BLASTN GenBank search results (*29*). Clone sequences may be accessed in GenBank under accession nos. JN857366–857894.

Pulsed-Field Gel Electrophoresis for *Pasteurellaceae*

When available, *Pasteurellaceae* isolated from the study animals were obtained from the Washington Animal Disease Diagnostic Laboratory (Pullman, WA, USA). If such isolates were unavailable, we substituted banked isolates from other bighorn sheep involved in the same outbreaks. Isolates were subjected to pulsed-field gel electrophoresis (PFGE) performed on a CHEF-DRII PFGE apparatus (Bio-Rad, Hercules, CA, USA) in 1% agarose gel (Seakem Gold Agarose; FMC Bio Products, Rockland, MD, USA) in 0.5× Tris borate EDTA buffer at 14°C for 20 h at 6 V/cm and a linear ramp of 1.0–30.0 s for *Apa*I or 0.5–40.0 s for *Sma*I. *Salmonella* serovar Braenderup H9812, digested with *Xba*I for 3 h at 37°C, was used as a size standard on each gel. Gels were stained with ethidium bromide and photographed under UV transillumination. PFGE data were analyzed by using BioNumerics version 4.6 (www.applied-maths.com/bionumerics/bionumerics.htm) with Dice coefficients and the unpaired pair group method with arithmetic means for clustering; tolerance and optimization parameters were set at 1%.

Intergenic Spacer Region Sequence typing for *M. ovipneumoniae*

In a preliminary study performed in our laboratory, ribosomal operon intergenic spacer (IGS) regions of *M. ovipneumoniae* from isolates from 6 bighorn sheep populations were amplified by using the method described by Kong et al. (*30*) and sequenced (Amplicon Express, Pullman, WA, USA). Sequences were aligned and clustered by using Lasergene software (DNASTAR, Inc., Madison WI, USA). Each isolate exhibited a different IGS sequence, demonstrating the utility of IGS sequences for identifying strain diversity (data not shown). We used Primer3 software (http://frodo.wi.mit.edu/primer3/) to develop primers flanking the variable IGS region, conserved among *M. ovipneumoniae* isolates, and divergent from IGS regions of the second most common sheep upper respiratory mycoplasma, *M. arginini* (Table 2). IGS PCR products were sequenced, and sequences were aligned and

Table 2. PCR primers used to detect etiologic agents of pneumonia in bighorn sheep, western United States, 2008–2010

Species (gene target)	Primer	Primer sequence, 5′ → 3′	Reference
Mannheimia haemolytica, Bibersteinia trehalosi, *M. haemolytica* (*gcp*)	Mhgcp	AGAGGCCAATCTGCAAACCTCG	(*21*)
	MhgcpR	GTTCGTATTGCCCAACGCCG	(*21*)
Bibersteinia trehalosi (*sodA*)	BtsodAF	GCCTGCGGACAAACGTGTTG	(*21*)
	BtsodAR	TTTCAACAGAACCAAAATCACGAATG	(*21*)
Leukotoxin (*lktA*)	F	TGTGGATGCGTTTGAAGAAGG	(*26*)
	R	ACTTGCTTTGAGGTGATCCG	(*26*)
Pasteurella multocida (*kmt1*)	KMT1T7	ATCCGCTATTTACCCAGTGG	(*27*)
	KMT1SP6	GCTGTAAACGAACTCGCCAC	(*27*)
Mycoplasma ovipneumoniae (16S)	LMF	TGAACGGAATATGTTAGCTT	(*28*)
	LMR	GACTTCATCCTGCACTCTGT	(*28*)
M. ovipneumoniae (16S–23S intergenic spacer)	MoIGSF	GGAACACCTCCTTTCTACGG	This study
	MoIGSR	CCAAGGCATCCACCAAATAC	This study

clustered by using Lasergene software. IGS sequences can be accessed in GenBank under accession nos. JN857895–857934.

Statistical Analyses

To evaluate the agreement between results of bacteriologic cultures and PCR tests for detection of *P. multocida, M. haemolytica,* and *B. trehalosi*, we used Cohen κ coefficients (*31*). To assess overall differences in prevalence of specific bacterial respiratory pathogens, we used χ^2 tests; for pairwise comparisons, we used the Marascuilo procedure (*32*) to control for multiple comparison problems, which might affect error rates. To assess associations between prevalence of different respiratory bacteria and mortality rates among different bighorn sheep populations, we used the Pearson correlation coefficient.

Results

We detected 4 previously reported bacterial respiratory pathogens of bighorn sheep. We detected *M. haemolytica, B. trehalosi,* and *P. multocida* by using aerobic culture and species-specific PCR and *M. ovipneumoniae* by using PCR alone (*20*) (Table 3; online Appendix Table, wwwnc.cdc.gov/EID/article/18/1/11-1554-TA1.htm). Agreement between detection by culture and PCR varied by agent, ranging from no agreement (*M. haemolytica*, κ –0.02), to fair agreement (*B. trehalosi*, κ 0.22), to good agreement (*P. multocida*, κ 0.76). For the purposes of the following analyses, animals for which any agent was detected by either method were considered positive for that agent. Among the targeted agents, 3 (*B. trehalosi*, *M. haemolytica*, and *M. ovipneumoniae*) were detected in ≥1 animals from all 8 outbreak-affected populations and 1 (*P. multocida*) was detected in animals from 5 outbreak-affected populations (Table 3).

Frequency of detecting *M. haemolytica*, *B. trehalosi*, *P. multocida*, and *M. ovipneumoniae* from pneumonia-affected animals differed significantly (χ^2 26.2, 3 df, p<0.0001). *M. ovipneumoniae* (n = 42, 95%) was detected significantly more often than any other agent except *B. trehalosi* (n = 35, 73%; Marascuilo procedure, p<0.05). Detection of *lktA*, a gene encoding the leukotoxin expressed by *B. trehalosi* and *M. haemolytica*, was then analyzed as a surrogate for virulent *M. haemolytica* and/or *B. trehalosi* because these species, if lacking *lktA*, would be considered to have greatly reduced or no virulence (*33*). Prevalence of *lktA* (n = 10, 22.7%) was significantly lower than that of *P. multocida* (n = 21, 47.7%, Marascuilo procedure, p<0.05).

Frequency of detecting *B. trehalosi* and *P. multocida* differed significantly among outbreaks (p = 0.002 and 0.001, respectively). Similarly, PCR-based detection of *lktA* differed among outbreaks (p = 0.003). Although such differences could potentially contribute to the significant differences in disease severity and mortality rates among the epizootics in this study ($\chi^2$184.7, 7 df, p<0.0001), the prevalence of *B. trehalosi*, *P. multocida*, or *lktA* did not correlate with estimated mortality rates in the 8 outbreaks included in this study (Tables 1, 3).

Strain typing to evaluate the genetic similarity of bacterial pathogens within and among outbreaks (*23*) detected only single IGS types of *M. ovipneumoniae* within each outbreak, whereas distinctly different IGS types were found for each epizootic with the exception of 2 populations in Montana (Figure). In contrast, the PFGE strain types of *Pasteurellaceae* isolated from within single outbreaks

Table 3. Prevalence of organisms in bighorn sheep, western United States, 2008–2010*

Population	No. tested	No. (%) detected				
		Bibersteinia trehalosi	*Mannheimia haemolytica*	*Pasteurella multocida*	*lktA*	*Mycoplasma ovipneumoniae*
Affected during epizootic						
East Fork Bitterroot, MT	5	3†	1	4	2	4
Anaconda, MT	5	5	5	5	0	5
Bonner, MT	6	2	2	0	0	6
Lower Rock Creek, MT	4	3	3	3	0	3
East Humboldt and Ruby Mountains, NV	6	6	5	0	1	6
Spring Creek, SD	5	5	3	4	3	5
Yakima Canyon, WA	8	5	4	5	0	8
Hells Canyon, OR and WA	5	5	2	0	4	5
Total	44	34 (77.3)	25 (56.8)	21 (47.7)	10 (22.7)	42 (95.5)
Healthy during epizootic						
Yakima Canyon, WA	6	5	3	0	1	2
Bonner, MT	1	0	0	0	0	1
Spring Creek, SD	1	1	1	0	0	0
Total	8	6 (75.0)	4 (50.0)	0	1 (12.5)	3 (37.5)
Healthy, no epizootic						
Quilomene, WA	3	1	3	0	0	0
Hells Canyon (Asotin Creek), OR and WA	2	2	1	1	0	0
Total	5	3 (60)	4 (80)	1 (20)	0	0

*Organisms detected by PCR and/or aerobic culture. MT, Montana; NV, Nevada; SD, South Dakota; WA, Washington; OR, Oregon.
†Number of bighorn sheep tested in which the given agent was detected by PCR and/or bacteriologic culture.

ranged from 0 to 7, including 0–7 *B. trehalosi* strains and 0–2 *P. multocida* strains (Table 4; online Appendix Table). Assessment of strain type diversity of *M. haemolytica* within outbreaks was not possible because this species was isolated only 1 time.

Among the agents and genes tested, *M. ovipneumoniae* was the only agent or gene that was detected at different frequencies for animals from epizootic-affected compared with non–epizootic-affected populations (Table 3; $p<0.001$). The frequency of *M. ovipneumoniae* and *P. multocida* detection in non–pneumonia-affected animals culled from epizootic populations was intermediate, significantly lower than that in pneumonia-affected animals ($p<0.01$).

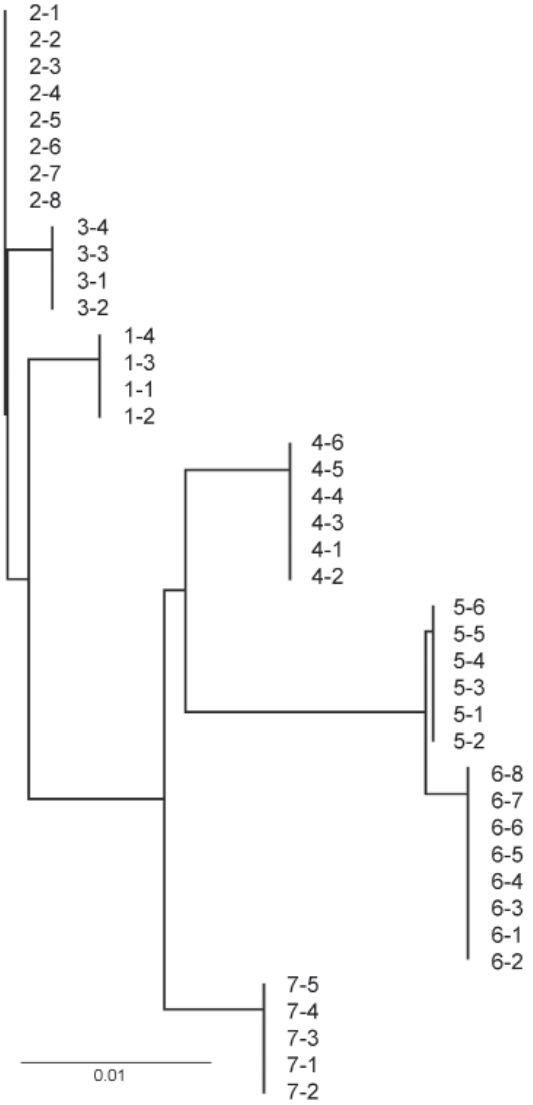

Figure. Neighbor-joining tree of ribosomal intergenic spacer region DNA sequences of *Mycoplasma ovipneumoniae* PCR-amplified from bighorn sheep lung tissues, western United States, 2008–2010. Isolate codes are those from Table 4 and the online Appendix Table (wwwnc.cdc.gov/EID/article/18/1/11-1554-TA1.htm). Scale bar indicates nucleotide substitutions per site.

Partial 16S ribosomal DNA sequences of 527 clones, including ≈30 clones from each of 2 animals from each epizootic, detected the targeted respiratory pathogens *P. multocida*, *M. ovipneumoniae*, *B. trehalosi*, and *M. haemolytica*, although the latter accounted for <1% of the identifications. Also predominant were several obligate anaerobic bacteria (*Fusobacterium necrophorum*, *Prevotella* spp., *Clostridium* spp., and *Bacteroides* spp.) (Table 5).

Discussion

The results of this study support the hypothesis that *M. ovipneumoniae* is a primary agent in the etiology of epizootic bighorn sheep pneumonia in populations across the western United States and that it acts to induce secondary infection with opportunistic pathogens. *M. ovipneumoniae* was detected in the pneumonic lungs of >95% of study animals involved in the 8 discrete pneumonia epizootics, significantly more frequently than any of the other respiratory agents sought except the bighorn sheep commensal bacterium *B. trehalosi* (*34*,*35*). We identified identical ribosomal IGS strains of *M. ovipneumoniae* within the affected animals in each outbreak, consistent with epizootic spread (*24*); *M. ovipneumoniae* was not detected in the healthy populations sampled. Of note, the 2 populations in which identical IGS strains of *M. ovipneumoniae* were detected were separated by only ≈20 miles, suggesting the possibility that this strain was transmitted among these populations by movement of ≥1 *M. ovipneumoniae*–infected bighorn sheep.

The normal host range of *M. ovipneumoniae* (members of Old World *Caprinae*, including domestic sheep and mouflon, a closely related Eurasian sheep species) is consistent with many observations that epizootic bighorn sheep pneumonia frequently follows contact with these hosts (*5*,*19*). Previous experiments in which bighorn sheep were commingled with domestic sheep or mouflon each resulted in epizootic bighorn sheep pneumonia and, cumulatively, the death of 88 (98%) of 90 bighorn sheep; similar commingling experiments with other ungulates (deer, elk, llamas, horses, cattle, goats, mountain goats) resulted at most in sporadic deaths from bighorn sheep pneumonia (4 [7%] of 56) (*19*). In a recent study in which bighorn sheep were commingled with *M. ovipneumoniae*–free domestic sheep, the lack of epizootic bighorn sheep pneumonia was unprecedented (*19*). Together, these data support the hypothesis that bighorn sheep epizootic pneumonia results from cross-species transmission of *M. ovipneumoniae* from its normal host(s) to a naive, highly susceptible host: bighorn sheep.

Each of the other specific potential respiratory pathogens targeted failed to fulfill ≥1 expectations for a primary etiologic agent. *B. trehalosi* was detected in most animals regardless of their health status; exhibited

Table 4. Strain types identified in lung tissue from bighorn sheep, western United States, 2008–2010*

Population	*Pasteurellaceae* (no. animals)†	*Mycoplasma ovipneumoniae* (no. animals)‡
East Fork Bitterroot, MT	Btre21, Mhae5, Pmul5 (1 each); Pmul24 (5)	Movi3 (5)
Bonner, MT	No isolate available	Movi4 (6)
Lower Rock Creek. MT	Btre1 (1); Pmul22 (3)	Movi6 (3)
Anaconda, MT	Pmul24 (5)	Movi6 (5)
East Humboldt and Ruby Mountains, NV	Btre7 (2); Btre8 (3)	Movi5 (4)
Yakima Canyon, WA	Btre8, Pmul 22 (1 each); Pmul20 (3); Btre9, 13 (5 each)	Movi2 (7)
Spring Creek, SD	Btre10, 11, 12, 17, 19 (1 each); Pmul20 (2)	Movi7 (5)
Hells Canyon, OR and WA	Btre 1, 2, 3, 4, 14, 15, 16 (1 each)	Movi1 (4)
Quilomene, WA	Mhae23 (1); Btre8 (2)	None detected
Asotin Creek, WA	No isolate available	None detected

*MT, Montana; NV, Nevada; SD, South Dakota; WA, Washington; OR, Oregon.
†*Pasteurellaceae* cluster assignments based on *apa*I pulsed-field gel electrophoresis profiles with Dice coefficients clustered by unpaired pair group method with arithmetic means (threshold, 90% identity).
‡*M. ovipneumoniae* strain types identified by ribosomal 16S–23S intergenic spacer region DNA sequences.

diverse strain types within epizootics; and in most instances was detected in the absence of *lktA*, consistent with the nontoxigenic strains widely distributed in healthy and pneumonic bighorn sheep (*36*). *M. haemolytica* was similarly detected in about half of the animals regardless of their health status and in the absence of *lktA*. *P. multocida* was not detected at all in animals involved in 3 of the epizootics, but in those outbreaks in which it was present, it was detected at high prevalence and somewhat more frequently in pneumonia-affected than in healthy bighorn sheep. Furthermore, multiple isolates from those epizootics in which it was detected shared a high degree of genetic similarity, consistent with epizootic transmission (*24*).

The frequencies with which *B. trehalosi*, *P. multocida*, and *lktA* were detected from animals in the different epizootics differed significantly, although this finding did not correlate with mortality rates (Table 1). This conclusion is limited, however, by the possible confounding effect of the extensive culling conducted in several areas of the epizootics examined in this study. More research into factors that affect the severity of bighorn sheep pneumonia epizootics is clearly needed.

The analysis of prevalence of bacterial respiratory pathogens in the lung tissues of healthy animals from unaffected populations was comparatively limited by the small number of control specimens available. To more clearly define the prevalence, infectivity, and virulence of *M. ovipneumoniae*, sampling of additional healthy bighorn sheep populations is needed. Although *M. ovipneumoniae* was not detected in the negative control animals examined in this study and although serologic evidence of exposure to *M. ovipneumoniae* is uncommon or rare in healthy bighorn sheep populations (*17*), several apparently healthy bighorn sheep populations with serologic and/or PCR evidence of exposure to *M. ovipneumoniae* have been identified (data not shown). This finding demonstrates that not all exposures to this agent result in epizootic bronchopneumonia or, perhaps, that unrecognized previous epizootics had occurred. To clarify these findings, more research, specifically including longitudinal observational studies and investigation of strain differences in virulence of *M. ovipneumoniae* (*37*,*38*), is needed.

Our universal eubacterial 16S rDNA approach used analysis of small clone libraries from each animal to detect those agents representing ≥10% of the 16S operons in lung tissue with high (>95%) confidence. The 3 most frequently detected aerobic bacterial agents detected by using this method were *P. multocida*, *M. ovipneumoniae*,

Table 5. Bacteria detected in lung tissue from bighorn sheep with pneumonia, western United States, 2008–2010*

Bacterial species	Clone sequences, no. (%)	No. animals†	No. populations‡
Fusobacterium spp.	112 (21.3)	8	5
Pasteurella multocida	67 (12.7)	5	4
Prevotella spp.	57 (10.8)	9	5
Mycoplasma ovipneumoniae	52 (9.9)	5	4
Bibersteinia trehalosi	46 (8.7)	4	3
Clostridium spp.	42 (8.0)	10	7
Bacteroides spp.	16 (3.0)	7	5
Acinetobacter spp.	14 (2.7)	3	3
Streptococcus spp.	13 (2.5)	1	1
Pseudomonas spp.	7 (1.3)	2	2
Eubacterium spp.	6 (1.1)	4	3
Pasteurellaceae spp.	6 (1.1)	2	2
Ruminococcus spp.	6 (1.1)	3	3

*rDNA sequence analysis was used. Only species identifications comprising >1% of sequences are reported.
†Number of animals in which the bacterial species was detected, total 16 animals (2 animals each from 8 populations).
‡Number of populations in which the bacterial species was detected, total 8 populations.

and *B. trehalosi*; detection of *M. haemolytica* was rare (0.19%) despite the high frequency of its detection by the more sensitive PCR. This finding is surprising because *M. haemolytica* has been regarded as the principal pneumonia pathogen of bighorn sheep (*5*). It has been argued that because *B. trehalosi* inhibits or kills *M. haemolytica* in coculture, the same effect in vivo may block detection of *M. haemolytica* in bighorn sheep lungs (*21*), but this argument cannot explain the dearth of *M. haemolytica* detected in this study because the proportion of lungs that were positive for *M. haemolytica* by PCR was actually lower in the absence of *B. trehalosi* (online Appendix Table). Furthermore, the lung tissues from animals affected by the 5 epizootics in Washington or Montana were obtained from bighorn sheep that were coughing and culled in an attempt to prevent further transmission of the disease; therefore, these specimens could represent animals at earlier stages of the disease when more consistent presence of causal agents would be expected.

Consistent with previous reports of bighorn sheep in Hells Canyon (*17*), the predominance of obligate anaerobes (*Fusobacterium*, *Prevotella*, *Clostridium*, and *Bacteroides* spp.) among the lung flora was consistent with decreased clearance of inhaled oral flora from the lower respiratory tract. Impaired clearance of inhaled flora is expected subsequent to infection by virulent *M. ovipneumoniae* (*38*) or by leukotoxin-expressing *Pasteurellaceae* (*39*), albeit by different mechanisms.

To our knowledge, only 1 other study of epizootic bighorn sheep pneumonia has reported comparative microbiological findings from pneumonia-affected animals involved in multiple discrete epizootics. Aune et al. (*12*) reported that *Pasteurellaceae* cultured from pneumonia-affected animals differed somewhat among 4 bighorn sheep pneumonia epizootics in Montana during 1991–1996. *P. multocida* was isolated from pneumonic lung tissues of ≥1 animals during all 4 epizootics, although prevalence exceeded 50% during only 1 epizootic. *Pasteurellaceae* biotypes corresponding to *B. trehalosi* were isolated from animals involved in 3 of the 4 outbreaks, and *Pasteurellaceae* biotypes corresponding to *M. haemolytica* were isolated from animals in only 1 outbreak. The microbiology of epizootic pneumonia in Hells Canyon also has been described (*11*,*15*,*40*); results were broadly comparable to the conventional microbiology results reported here for *Pasteurellaceae*. All these studies differed from the study reported here in that the conventional microbiological methods used failed to recognize *M. ovipneumoniae* in affected lung tissues.

In summary, of the bacterial respiratory pathogens evaluated, *M. ovipneumoniae* was the only agent for which the data consistently met the criteria for a primary etiologic agent across all outbreaks. In contrast, the data were inconsistent with regard to a primary etiologic role for any *Pasteurellaceae* species. The likelihood of *M. ovipneumoniae* having a primary role in bighorn sheep pneumonia is consistent with the association between some epizootics of this disease and contact with domestic sheep because the latter carry this agent at high prevalence. Identification of *M. ovipneumoniae* as the epizootic agent of bighorn sheep pneumonia may provide a useful focus for the development of specific preventative or therapeutic interventions.

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Chicken as Reservoir for Extraintestinal Pathogenic *Escherichia coli* in Humans, Canada

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We previously described how retail meat, particularly chicken, might be a reservoir for extraintestinal pathogenic *Escherichia coli* (ExPEC) causing urinary tract infections (UTIs) in humans. To rule out retail beef and pork as potential reservoirs, we tested 320 additional *E. coli* isolates from these meats. Isolates from beef and pork were significantly less likely than those from chicken to be genetically related to isolates from humans with UTIs. We then tested whether the reservoir for ExPEC in humans could be food animals themselves by comparing geographically and temporally matched *E. coli* isolates from 475 humans with UTIs and from cecal contents of 349 slaughtered animals. We found genetic similarities between *E. coli* from animals in abattoirs, principally chickens, and ExPEC causing UTIs in humans. ExPEC transmission from food animals could be responsible for human infections, and chickens are the most probable reservoir.

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is the leading cause of community-acquired urinary tract infections (UTIs) in humans, accounting for >85% of UTIs (*1*). Each year, 6–8 million UTIs are diagnosed in the United States, and 130–175 million are diagnosed worldwide. Estimated direct health care costs related to uncomplicated UTIs in the United States are $1–$2 billion per year (*1*,*2*). UTIs also can lead to more severe illnesses, such as pyelonephritis, bacteremia, and sepsis (*3*). During the past decade, the emergence of drug-resistant *E. coli* has dramatically increased. As a consequence, the management of UTIs, which was previously straightforward, has become more complicated; the risks for treatment failure are higher, and the cost of UTI treatment is increasing (*4*).

In the past, extraintestinal *E. coli* infections have been described as sporadic infections caused by bacteria that originate from the host's intestinal tract. However, ExPEC strains recently have been associated with possible outbreaks (*5*). Communitywide outbreaks have been described in south London (*E. coli* O15:K152:H1) (*6*); Copenhagen (*E. coli* O78:H10) (*7*); Calgary, Alberta, Canada (extended-spectrum β-lactamase–producing *E. coli*) (*8*); and California, USA (trimethoprim/sulfamethoxazole–resistant *E. coli*) (*9*). These outbreaks suggest that ExPEC can be spread to the intestinal tracts of persons in the community by a common source or vehicle.

We recently described the results of a study that characterized the genetic similarities between *E. coli* isolates recovered from retail meat, particularly chicken, and ExPEC in humans causing community-acquired UTIs (*10*). That study oversampled isolates from retail chicken because evidence suggested that chicken was likely to be the primary reservoir of ExPEC in humans (*11*–*16*). To exclude the possibility that isolates from other retail meat sources (beef and pork) might also be genetically related to

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UTI isolates from humans, we first aimed to characterize additional *E. coli* isolates recovered from retail beef and pork sources. These new isolates from retail meat were added to the preexisting collection of retail meat isolates and compared with the same UTI isolates from humans. Second, we aimed to determine whether transmission was primarily human to human through food or whether an animal source was involved. In the case of human-to-human transmission through food, *E. coli* strains from humans would be introduced during the meat preparation process by food handlers. In the case of an animal source, the *E. coli* would derive from the cecal content of the animal itself, and contamination would occur during the slaughtering process. On the basis of previous findings, we hypothesized that a food animal reservoir exits for ExPEC that cause UTIs in humans and that chicken is the primary source. To evaluate this hypothesis, we analyzed isolates from animals entering the food chain. *E. coli* isolates recovered from the cecal contents of slaughtered food animals (beef cattle, chickens, and pigs) were compared with the preexisting geographically and temporally matched collection of isolates from humans with UTIs.

Methods

Study Design

A total of 1,561 geographically and temporally matched *E. coli* isolates from animals and from humans with UTIs were used for the different comparisons (Table 1).The study target area was primarily the province of Québec, Canada; however, other regions were included as described below. The study period was 2005–2007. The McGill University Institutional Review Board approved the study protocol (A01-M04-05A).

Sampling of *E. coli* Isolates from Humans with UTI, Montreal, Québec

The 351 *E. coli* isolates recovered from humans with UTIs were collected in Montréal during June 2005–May 2007 (*17*). Women 18–45 years of age who had a suspected UTI were recruited at the McGill University Student Health Services and the Centre Local de Services Communautaires Métro Guy. UTI was defined as ≥2 symptoms or signs, including dysuria, increased urinary frequency or urgency, pyuria, hematuria, and $>10^2$ CFU of *E. coli* per mL of clean-catch urine (*18*). Specimen culture and bacterial identification have been described (*17*). One random isolate from each urine sample was selected. In case of UTI recurrence, only the isolate from the first UTI episode was included. The collection was assembled as follows. A random set of 116 fully susceptible isolates was selected. A random set of 170 isolates resistant to ≥1 antimicrobial agents was assembled; in addition, specific groups of antimicrobial-resistant *E. coli* were included. These *E. coli* strains have been closely associated with possible outbreaks of extraintestinal infections (*6*–*9*,*19*–*21*). In particular, cephalosporin-resistant *E. coli* frequently has been observed in UTI outbreaks and in poultry products (*8*,*13*,*16*,*19*–*21*). Hence, all 19 cephalothin-resistant *E. coli* isolates were included. We included 46 representative members of *E. coli* clonal groups that were known to cause clusters of UTI among unrelated women on the basis of the hypothesis that they would be more likely to be related to

Table 1. Composition of closely related *Escherichia coli* clonal groups from humans and retail meat or abattoir source isolates, Canada, 2005–2007*

	No. (%) isolates.									
	Year				Geographic area					
Source	2005	2006	2007	2008	QC	ON	BC/AB	SK/MB	Maritimes	Total
UTI in humans										
All	102 (21)	174 (37)	137 (29)	62 (13)	379 (80)	37 (8)	23 (5)	24 (5)	12 (3)	475 (100)
Manges collection†	102 (21)	174 (37)	75 (16)	0	351 (74)	0	0	0	0	351 (74)
Zhanel collection‡	0	0	62 (13)	62 (13)	28 (6)	37 (8)	23 (5)	24 (5)	12 (3)	124 (26)
Retail meat										
All	275 (37)	243 (33)	219 (30)	0	521 (71)	202 (27)	4 (1)	10 (1)	0	737 (100)
Beef	84 (11)	72 (10)	86 (12)	0	210 (28)	32 (4)	0	0	0	242 (33)
Chicken	107 (15)	101 (14)	45 (6)	0	141 (19)	99 (13)	3 (0.4)	10 (1)	0	253 (34)
Pork	84 (11)	70 (9)	88 (12)	0	170 (23)	71 (10)	1 (0.1)	0	0	242 (33)
Abattoir										
All	133 (38)	101 (29)	115 (33)	0	107 (31)	146 (42)	48 (14)	30 (9)	4 (1)	349 (100)
Beef	18 (5)	22 (6)	20 (6)	0	11 (3)	23 (7)	17 (5)	8 (2)	1 (0.3)	60 (17)
Chicken§	89 (26)	60 (17)	80 (23)	0	75 (21)	104 (30)	27 (8)	10 (3)	2 (1)	229 (66)
Pig¶	26 (7)	19 (5)	15 (4)	0	21 (6)	19 (5)	4 (1)	12 (3)	1 (0.3)	60 (17)
Total	510 (33)	518 (33)	471 (30)	62 (4)	1,007 (65)	385 (25)	75 (5)	64 (4)	16 (1)	1,561 (100)

*QC, Québec; ON, Ontario; BC, British Columbia; AB, Alberta; SK, Saskatchewan; MB, Manitoba; Maritimes, New Brunswick/Nova Scotia/Prince Edward Island; UTI, urinary tract infection.
†351 *E. coli* isolates recovered from humans with UTIs in Montréal during June 2005–May 2007.
‡124 *E. coli* isolates from humans with community- or hospital-acquired UTIs from sources throughout Canada during 2007–2008.
§The geographic area was unknown for 11 isolates from chicken.
¶The geographic area was unknown for 3 isolates from pigs.

food sources. This collection of 351 UTI-associated *E. coli* isolates is referred to as the Manges collection.

Sampling of *E. coli* Isolates from Humans with UTIs, Canada

We added 124 isolates from humans from sources across Canada to increase the diversity of the collection of *E. coli* isolates causing UTIs beyond those in Québec alone. These samples were collected from patients with community- and hospital-acquired UTIs during 2007–2008. This collection, provided by 1 of the authors (G.G.Z.), is referred to as the Zhanel collection.

Sampling of *E. coli* from Retail Meat

We systematically selected and evaluated additional isolates from retail beef and pork. The retail meat collection totaled 737 isolates from beef (242), chicken (253), and pork (242) (*10*). These isolates were collected in Montréal, areas of Québec outside Montréal, parts of Ontario, and other areas of Canada during 2005–2007. All of these isolates originated from the collection of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS). Because antimicrobial resistance has been associated with ExPEC clonal groups and outbreaks of UTIs (*6*–*9*,*17*,*22*), we oversampled antimicrobial-resistant isolates from retail meat; 60% of sampled isolates were resistant to ≥1 antimicrobial drugs, and 40% were fully susceptible. However, resistance in retail beef was fairly low; therefore the proportion of resistant *E. coli* from retail beef was only 48%.

Sampling of *E. coli* from Food Animals in Abattoirs

From the CIPARS collection, we selected 349 *E. coli* isolates from animals in abattoirs. These bacteria were isolated from the cecal contents of slaughtered food animals (*23*). Because the primary hypothesis concerned a chicken reservoir and we already had demonstrated that isolates from humans are less likely to be related to isolates from beef and pork, we included isolates in the following proportions: 20% beef cattle, 60% chickens, and 20% pigs. We chose 299 isolates from 2005–2007 as follows. Isolates from chickens were selected from abattoirs in Québec and Ontario because there are poultry abattoirs operating in Québec and Ontario and because *E. coli* from humans with UTIs was recovered primarily from women in Québec. In contrast, beef cattle and pig abattoirs are fewer and are located across Canada. Therefore, isolates were selected on the basis of the annual slaughter volume rather than on location. Sampling was conducted in proportion to the susceptibility levels for each animal species within the whole CIPARS collection. However, we included all 5 nalidixic acid–resistant isolates because this agent can be used as an indicator of resistance to fluoroquinolones (*24*).

Furthermore, because the study focused on chicken, we included a random sample of 50 additional isolates from chickens in abattoirs in other Canadian provinces. They were also selected from the CIPARS collection during the same period (2005–2007).

Antimicrobial Susceptibility Testing

We performed antimicrobial susceptibility testing on all *E. coli* isolates, except those from the Zhanel collection. Antimicrobial susceptibility screening using a panel of 15 agents (amikacin, amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulfamethoxazole) was conducted by the Public Health Agency of Canada, Laboratory for Foodborne Zoonoses. The protocol of the broth microdilution method used was fully described in the 2007 CIPARS report (*23*). Intermediate resistance for all isolates was classified as susceptible. The antimicrobial resistance patterns are provided for informational purposes only because isolates were sampled in part according to their antimicrobial resistance phenotype. Thus, the patterns observed do not reflect the prevalence of antimicrobial resistance for any of the sources.

Clonal Group Definition and Typing

Isolates were typed by multilocus variable number tandem repeat analysis (MLVA) (*25*) and enterobacterial repetitive intergenic consensus sequence 2 (ERIC2) PCR fingerprinting (*26*) in our laboratory and the McGill University and Genome Québec Innovation Center as described. A clonal group was defined as ≥2 *E. coli* isolates from human and animal sources that shared the same MLVA profile and ERIC2 PCR fingerprint. All clonal group members were further typed by phylotyping and multilocus sequence typing (MLST). Then, according to the results obtained, related isolates were selected for O:H serotyping and pulsed-field gel electrophoresis (PFGE).

Phylotyping (*27*) and MLST (*28*) (http://mlst.ucc.ie/mlst/dbs/Ecoli) were performed in our laboratory and in the McGill University and Genome Québec Innovation Center as described. Allelic profile and sequence type (ST) were assigned according to the scheme at this website. O (somatic) and H (flagellar) antigens were serotyped for clonal group isolates that shared the same phylogenetic group and MLST profile; serotyping was performed by the Public Health Agency of Canada, Laboratory for Foodborne Zoonoses, according to established protocols. Isolates that did not react with O antiserum were classified as nontypeable. PFGE of *Xba*I-digested DNA was conducted in our laboratory by using the protocol for molecular subtyping of *E. coli* O157:H7 developed by the

Centers for Disease Control and Prevention (*29*). Clonal group members that exhibited the same phylotype and MLST profile were tested. Gel fingerprints were visually compared, and strain relatedness was classified according to the Tenover criteria (*30*).

Statistical Analyses

Differences in proportions were assessed by use of the χ^2 test. 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

Isolates from Retail Meat

We identified 15 clonal groups, comprising 63 isolates. The 15 groups contained 22 isolates from humans with UTIs and 41 isolates from retail meat. Of the 41 isolates from retail meat, 6 (15%) were from retail beef, 29 (71%) from retail chicken, and 6 (15%) from retail pork. Considering the sampling proportions (66% beef and pork [484/737] and 34% chicken [253/737]), the fraction of isolates from beef and pork related to isolates from humans with UTIs was significantly lower than expected on the basis of the sampling fraction (29% observed vs. 66% expected; $p<0.001$, χ^2 test). Isolates from chicken were represented in greater numbers among clonal groups. All clonal group members had the same phylotype. According to MLST, 2 of these clonal groups (groups 1 and 3) contained isolates from newly sampled retail beef and pork (EC01DT07-0827-01 and EC01DT06-1559-01) and isolates from humans with UTIs that shared the same STs. These isolates were further typed (Table 2): *Xba*I PFGE patterns differed by ≥7 bands within the clonal groups associated with retail meat.

Isolates Collected from Abattoirs

We identified 8 clonal groups containing 46 isolates from humans and from food animals at abattoirs. These clonal groups comprised 17 isolates from humans with UTIs and 29 from abattoir animals (1 [3%] beef cattle, 23 [79%] chickens, 5 [17%] pigs). The proportion of chicken was higher than expected with 79% observed versus 60% expected, in accordance with the 60% sampling fraction ($p = 0.034$, χ^2 test). The 3 clonal groups including isolates that were further characterized, and thus more closely related, are described below (Table 2). According to the Tenover criteria (*30*), PFGE patterns of all animal strains differed from those of the human strains.

Abattoir clonal group 1 contained 11 isolates (2 from humans, 8 from chickens, 1 from a pig). All belonged to the same phylogenetic group (D) and showed the same sequence type (ST117). Two isolates from chickens (EC01AB07-0105-01 and EC01AB07-0425-01) had the same serotype (O180:H4), and the rest of the isolates had unique serotypes.

Abattoir clonal group 2 included 6 isolates (3 from humans, 1 from a chicken, 2 from pigs). They all belonged to phylogenetic group A but showed 4 different MLST profiles. Among them, ST746 was shared by 3 isolates (1 each from a human, chicken, and pig). However, they did not show the same O:H serotype.

Abattoir clonal group 3 contained 13 isolates (5 from humans, 6 from chickens, 2 from pigs), which all belonged to phylogenetic group A. The MLST profile ST10 was shared by 7 isolates (4 from humans, 3 from chickens). The 6 other isolates displayed different MLST profiles. Among the isolates exhibiting ST10, 3 from chickens (EC01AB05-0765-01, EC01AB07-0005-01, and EC01AB07-1330-01) showed the same serotype (O16:H48).

Discussion

Our first goal was to exclude retail beef and pork as a probable food source of *E. coli* causing UTIs. Our previous study (*10*), in which the sampling proportions for beef, chicken, and pork were not the same, clonal groups identified included 17% isolates from beef and pork and 83% from chicken ($p = 0.03$). In the current investigation, where the sampling proportions from retail meat were the same, 12 (29%) of isolates belonging to clonal groups were from beef and pork and 29 (71%) were isolated from chicken ($p<0.001$). Retail beef and pork isolates are much less likely than retail chicken isolates to be clonally related to isolates from humans with UTIs.

Our second goal was to determine whether the reservoir for ExPEC in humans causing community-acquired UTI was food animals, particularly chickens. The initial screening methods (MLVA and ERIC2) demonstrated that human samples and cecal samples from food animals in abattoirs can belong to the same clonal groups. Moreover, within certain abattoir clonal groups, isolates showed the same phylogenetic group and MLST sequence types, indicating that they may have originated from a recent common ancestor. The 3 major clonal groups with the highest level of similarity (groups 1, 2, and 3) included isolates from abattoir and retail meat (*10*), which suggests that food animals may serve as a reservoir for ExPEC in humans.

The 2 most common STs (ST10 and ST117), belonging to phylogenetic groups A and D, respectively, have already been reported from human and animal sources (*11*,*31*–*33*). Although phylogenetic group A is typically associated with commensal *E. coli* (*3*), most human and animal isolates from the abattoir clonal groups belonged to this phylotype. Moreno et al. (*34*) and Ewers et al. (*35*) reported data suggesting that isolates from phylogenetic group A could be responsible for extraintestinal infections. Phylogenetic group D, which has frequently been

associated with ExPEC in humans (*3*), was observed in 31% of the isolates from abattoir clonal groups. The lack of isolates from phylogenetic group B2 was unexpected because extraintestinal pathogenic strains often belong to this group (*3*). Studies from Jakobsen et al. have identified phylogroup B2 isolates from meat and animal sources, which demonstrates that B2 exists in the food animal reservoir (*36*,*37*). Our results may be explained by the fact

Table 2. Composition of closely related clonal groups containing extraintestinal pathogenic *Escherichia coli* from humans and retail meat or abattoir source isolates, Canada, 2005–2007*

Clonal group/strain	Type of sample	Isolate source	Year	Location	Phylotype	ST	Serotype	Antimicrobial drug susceptibility or resistance†
1								
MSHS 1014A	Clinical	CA-UTI	2007	QC	D	117	O114:H4	Susceptible
EC01DT05-0789-01	Retail meat	Chicken	2005	ON	D	117	O114:H4	Susceptible
EC01AB06-0065-01	Abattoir	Chicken	2006	QC	D	117	O2:H4	GEN, SIX, TET
MSHS 133	Clinical	CA-UTI	2005	QC	D	117	O24:NM	TET
EC01DT07-1090-01	Retail meat	Chicken	2007	QC	D	117	O24:H4	GEN, SIX, TET
EC01DT07-1050-01	Retail meat	Chicken	2007	ON	D	117	O45:H4	Susceptible
EC01DT07-0956-01	Retail meat	Chicken	2007	SK	D	117	O53:H4	AMP, NAL, STR, SIX, TET, TMP/SXT
EC01AB07-0840-01	Abattoir	Chicken	2007	ON	D	117	O53:NM	Susceptible
EC01AB07-0615-01	Abattoir	Chicken	2007	ON	D	117	O102:H4	AMC, AMP, FOX, TIO STR, SIX, TET
EC01AB05-1250-01	Abattoir	Chicken	2005	ON	D	117	O103:H4	AMC, AMP
EC01DT06-1887-01	Retail meat	Chicken	2006	QC	D	117	O143:H4	Susceptible
EC01AB06-1131-01	Abattoir	Pig	2006	QC	D	117	O143:H4	SIX, TET
EC01AB07-0695-01	Abattoir	Chicken	2007	ON	D	117	O149:H4	STR, SIX, TET
EC01DT05-1700-01	Retail meat	Chicken	2005	QC	D	117	O160:H4	Susceptible
EC01AB07-0105-01	Abattoir	Chicken	2007	ON	D	117	O180:H4	Susceptible
EC01AB07-0425-01	Abattoir	Chicken	2007	BC	D	117	O180:H4	KAN, STR, SIX, TET
EC01DT07-0827-01	Retail meat	Pork	2007	ON	D	117	ONT:H4	STR, SIX
EC01AB05-0695-01	Abattoir	Chicken	2005	ON	D	117	ONT:H4	GEN, SIX,
EC01DT05-0224-01	Retail meat	Chicken	2005	ON	D	117	OX182:NM	Susceptible
2								
MSHS 624	Clinical	CA-UTI	2006	QC	A	746	O20:H4	Susceptible
EC01AB07-1301-01	Abattoir	Pig	2007	ON	A	746	O20:NM	AMP, CHL, SIX, TET
EC01AB05-0990-01	Abattoir	Chicken	2005	ON	A	746	O87:NM	AMC, AMP, FOX, TIO, CHL, STR, SIX, TET
EC01DT06-0006-01	Retail meat	chicken	2006	QC	A	746	O33:NM	Susceptible
EC01AB05-0091-01	Abattoir	Pig	2005	ON	A	10		Susceptible
MSHS 254	Clinical	CA-UTI	2005	QC	A	None		CEF, TET
48-75641	Clinical	CA-UTI	2007	Maritimes	A	None		
3								
EC01AB05-0765-01	Abattoir	Chicken	2005	ON	A	10	O16:H48	Susceptible
EC01AB07-0005-01	Abattoir	Chicken	2007	ON	A	10	O16:H48	STR, SIX, TET
EC01AB07-1330-01	Abattoir	Chicken	2007	BC	A	10	O16:H48	Susceptible
MSHS 233	Clinical	CA-UTI	2005	QC	A	10	O9:H32	AMP, TET
MSHS 825A	Clinical	CA-UTI	2006	QC	A	10	O15:NM	AMP, CHL, STR, SIX, TET, TMP/SXT
EC01DT06-1559-01	Retail meat	Pork	2006	ON	A	10	O42:H37	Susceptible
MSHS 892	Clinical	CA-UTI	2006	QC	A	10	O101:NM	CIP, KAN, NAL, STR, TET
120-79443	Clinical	HA-UTI	2008	Maritimes	A	10	O101:H9	
EC01DT05-1925-01	Retail meat	Chicken	2005	QC	A	10	O106:H4	Susceptible
EC01DT05-0408-01	Retail meat	Chicken	2005	QC	A	10	O153:NM	AMC, AMP, FOX, TIO STR, SIX, TET, TMP/SXT
EC01DT07-1162-01	Retail meat	Chicken	2007	ON	A	10	OX182:NM	AMC, AMP, FOX, TIO, TET
EC01DT07-0491-01	Retail meat	Chicken	2007	QC	A	10	OX184:H4	NAL, TET
EC01AB06-0855-01	Abattoir	Chicken	2006		A	548		AMP, KAN, STR, SIX, TET
51-77552	Clinical	CA-UTI	2008	SK/MB	A	None		
EC01DT06-1546-01	Retail meat	Chicken	2006	QC	A	None		AMP, GEN, SIX, TET
EC01AB05-0320-01	Abattoir	Chicken	2005	QC	A	None		Susceptible
EC01AB06-0119-01	Abattoir	Pig	2006	SK	A	None		KAN, TET
EC01AB06-1005-01	Abattoir	Chicken	2006	ON	A	None		Susceptible
EC01AB07-0566-01	Abattoir	Pig	2007	SK	A	None		STR, SIX

*ST, sequence type; MSHS, McGill University Student Health Services; CA-UTI, community-acquired urinary tract infection; QC, Québec; ON, Ontario; AB, Alberta; GEN, gentamicin; SIX, sulfisoxazole; TET, tetracycline; NM, nonmotile; SK, Saskatchewan; AMP, ampicillin; NAL, nalidixic acid; TMP/SXT, trimethoprim/sulfamethoxazole; AMC, amoxicillin/clavulanic acid; FOX, cefoxitin; TIO ceftiofur; STR, streptomycin; CEF, cephalothin; Maritimes, New Brunswick/Nova Scotia/Prince Edward Island; BC, British Columbia; ONT, did not react with O antiserum; KAN, kanamycin; CHL, chloramphenicol; CIP, ciprofloxacin; HA-UTI, hospital-acquired urinary tract infection; MB, Manitoba; blank cells indicate isolate not tested.
†Resistance to specific antimicrobial drugs as indicated.

that the isolates collected from abattoirs are more likely to be generic or commensal *E. coli* rather than typical ExPEC because they were collected from the cecal contents of healthy animals. The lack of phylogenetic group B2 isolates also could be explained by sampling variability or our selection method (based on MLVA and ERIC2 PCR genotyping first, followed by phylotyping). Phylogenetic group A and D were predominant among the isolates collected from abattoirs, which is consistent with results obtained by Jakobsen et al. (*38*) and Cortés et al. (*11*).

Although we oversampled isolates from abattoir chickens (60%), a significantly higher proportion of the isolates collected from abattoirs (79%; p = 0.034) included in the clonal groups were from chickens than from beef cattle or pigs; this proportion was higher than expected. This study confirms our hypothesis that chickens are a likely reservoir for ExPEC in humans. However, epidemiologic data, such as diet or other exposures, were not available for the humans with UTIs. This information could have been used to search for other potential routes of transmission (e.g., travel, water sources) and to strengthen the connection between poultry consumption and UTI.

We observed more heterogeneity in the PFGE results than in results from the other typing methods. PFGE is the standard for genotyping *E. coli* in the context of outbreaks, but it is generally not useful for establishing relationships between isolates from greater distances and over longer periods (*12*). MLST results may be a more relevant as housekeeping genes evolve more slowly and are more appropriate for examining questions related to global or regional epidemiology (*39*,*40*).

This study was strengthened by use of an ecologic design in which all isolates were systematically and purposively selected over the same period of time and geographic area (*17*,*23*), rather than sampling haphazardly by using existing clinical laboratory collections. The results suggest that potential ExPEC transmission from food animal sources is likely to be implicated in human infections and that chicken is a major reservoir. The possibility that ExPEC causing UTIs and other extraintestinal infections in humans could originate from a food animal reservoir raises public health concern. New interventions may be needed to reduce the level of food contamination and risk for transmission.

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A Systematic Approach for Discovering Novel, Clinically Relevant Bacteria

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Sequencing of the 16S rRNA gene (16S) is a reference method for bacterial identification. Its expanded use has led to increased recognition of novel bacterial species. In most clinical laboratories, novel species are infrequently encountered, and their pathogenic potential is often difficult to assess. We reviewed partial 16S sequences from >26,000 clinical isolates, analyzed during February 2006–June 2010, and identified 673 that have <99% sequence identity with valid reference sequences and are thus possibly novel species. Of these 673 isolates, 111 may represent novel genera (<95% identity). Isolates from 95 novel taxa were recovered from multiple patients, indicating possible clinical relevance. Most repeatedly encountered novel taxa belonged to the genera *Nocardia* (14 novel taxa, 42 isolates) and *Actinomyces* (12 novel taxa, 52 isolates). This systematic approach for recognition of novel species with potential diagnostic or therapeutic relevance provides a basis for epidemiologic surveys and improvement of sequence databases and may lead to identification of new clinical entities.

Broad-range PCR amplification and sequencing of the 16S rRNA gene (16S sequencing) is not only widely used as a taxonomic tool but is recognized as an effective reference method for bacterial identification. It has been used to identify novel and emerging pathogens (*1*–*4*) and to define complex microbial communities (*5*,*6*). The method has also revolutionized our understanding of microbial diversity (*7*–*9*). In clinical microbiology laboratories, 16S sequencing is useful for classifying microorganisms from pure culture (*10*,*11*). Molecular identification is especially valuable for bacteria that are slow growing, biochemically inert or variable, and fastidious, and it has also enhanced our understanding of previously unrecognized, often opportunistic pathogens (*1*,*10*,*12*).

Sequence-based identification relies on limited, yet phylogenetically informative, 16S sequence variation between related bacterial taxa. The entire 16S rRNA gene is ≈1,500 nt long (*11*); however, sequencing the 5′ third (partial 16S) generally provides sufficient taxonomic information while limiting costs (*10*). Partial 16S sequences are compared with reference libraries to determine the species with maximum similarity (*10*,*11*). The largest library is the nucleotide database hosted by the National Center for Biotechnology Information (NCBI) (*13*). Depending on their similarity to reference sequences, unknown isolates can be identified to different taxonomic levels by using interpretive guidelines published by the Clinical and Laboratory Standards Institute (CLSI) (*14*). For most taxa, sequence identity ≥99% with a valid reference sequence is required for species-level identification. Although this cutoff is widely used to identify isolates of the same species, a uniform cutoff for defining isolates as belonging to separate species is more controversial (*1*,*10*,*15*–*17*). Values of 99.5% to 97.0% have been proposed in the past (*12*,*15*,*17*–*22*), with more recent evidence and recommendations supporting values between 98.7% and 99.0% (*10*,*17*,*23*).

In our laboratory, as in many others, 16S sequencing is performed when morphologic and phenotypic identification is inconclusive or difficult or when it is

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specifically requested. By using CLSI guidelines and an NCBI nucleotide-based reference library (*24*), >90% of these isolates can be identified to the species level. However, clinical isolates belonging to as-yet-undescribed taxa are regularly encountered. Whether they represent emerging pathogens (*1*) or environmental contaminants is often difficult to determine in individual cases. Therefore, we conducted a systematic analysis of large numbers of unidentified strains to screen for novel taxa of potential clinical relevance. We reviewed partial 16S sequences from >26,000 clinical isolates to identify and characterize novel species with possible clinical significance. We identified 673 isolates that belong to as-yet-undescribed species, including 348 isolates of 95 novel taxa that were isolated from multiple patients. Repeated isolation of these undescribed organisms may indicate their clinical relevance and warrant their formal description as species.

Methods

Clinical Isolates

From results reported for ≈26,000 clinical isolates identified by 16S rRNA gene sequencing during February 2006–June 2010, we searched for those isolates that could not be identified to the species level by using SmartGene software (*24*) and CLSI guidelines (*14*). Phenotypic characteristics were routinely compared with those expected for closely related taxa. Species-level identification might have been unsuccessful for several reasons, including lack of separation between closely related species (which resulted in a report of >1 species), poor sequence quality on multiple attempts, insertions or deletions in multiple nonidentical copies of the 16S rRNA gene (which compromised sequence quality, length, or both), unpublished or unsubstantiated references, or a lack of similar sequences in reference databases. After multiple isolates recovered from the same patients were eliminated, 1,678 (≈6%) isolates were found that had not been identified to the species level. A cutoff of <99% identity with a known species was used to define isolates that may represent novel taxa (*17*,*23*). On the basis of provided information, anatomical sites were classified as follows: blood, bones (including bone marrow), central nervous system (brain, cerebrospinal fluid), eye, gastrointestinal tract (abdomen, gallbladder, stool), genitourinary tract (genitals, placenta, urine), oral cavity/paranasal sinus (including throat), respiratory tract (invasive: bronchoalveolar lavage, bronchial brush/wash, lung; other: sputum, endotracheal aspirate, respiratory specimen), tissue, wound/abscess (including bite wounds, lesion, scraping), other (aspirate, biopsy, body and dialysis fluids, ear, heart valve, medical devices), or unknown.

Sequence Assembly

Partial 16S rRNA gene sequencing had been performed as reported (*25*). Original chromatogram files were reanalyzed with MicroSeq 500 software (version 2.0; Applied Biosystems, Foster City, CA, USA). Consensus sequences of <400 bp in length were eliminated from further analyses. Remaining sequences with average phred quality scores ≥35 were included without manual review. Sequences with quality scores <35 were reviewed manually and included only if quality was sufficient, as determined by visual inspection. Sequences were converted to FASTA format (http://blast.ncbi.nlm.nih.gov/blastcgihelp.shtml) for comparison with reference sequences and submitted to GenBank under accession nos. JQ259197–JQ259857X and JN986812–JN986825. Sequences were annotated with taxonomic information from the best match with species-level identification by using CLSI guidelines (*14*). In brief, isolates with 97% to <99% identity were annotated at the genus level, isolates with 95% to <97% identity were annotated at the family level, and isolates with <95% identity were annotated at the order level. Aerobic actinomycetes (*26*), members of the family *Enterobacteriaceae*, and mycobacteria with identities of 95%–99% were annotated at the family level (*14*).

Comparison to Reference Sequences

NCBI stand-alone-BLASTn version 2.2.23+ with default parameters and internally developed software applications were used to compare sequences to a local copy of the NCBI nucleotide database (*13*) (downloaded July 2010). Information from 3 matches per isolate was parsed from XML-formatted BLASTn output files into a database by using custom python code and biopython libraries (*27*): 1) top match with valid species-level annotation (e.g., *Streptococcus sanguinis*); 2) top match with valid genus-level annotation (e.g., *Streptococcus* sp. oral strain T4-E3); and 3) top BLASTn match irrespective of annotation (e.g., uncultured bacterium). Valid nomenclature was determined by comparing annotations in the GenBank organism field to a list of approved bacterial taxa (*28*). Values in the following GenBank database fields or BLAST XML results were retrieved from each of the 3 matches: organism, taxonomy, associated publication, publication date, alignment length, number of identities, and position in the hit list. Reference sequences with species-level annotation were used, whether they were linked to a publication or not. For each of the 3 matches, the number of ambiguous bases (International Union of Pure and Applied Chemistry codes) and the percent aligned (alignment length as percentage of query length) were calculated. Percent identity was calculated by considering International Union of Pure and Applied Chemistry ambiguity codes as

matching any corresponding bases (e.g., Y matched C or T). N symbols were always recorded as mismatches.

Only sequences that had <99% identity with a valid species-level reference were included in subsequent analyses. Since BLASTn uses a local alignment algorithm, resulting alignments may be based on truncated query or match sequences if similarities are low at either end of the sequences. This practice may cause inflated pairwise sequence identity values. To control for this effect, we also retrieved the 3 matches described above using a minimum alignment length cutoff of 98%, on the basis of the query sequence length. Manual reviews were performed when this filter resulted in different best matches. For sequences with percent identity values close to the 99% cutoff and BLASTn alignment length of <100%, pairwise alignments with the best species-level match were analyzed by using MEGA4.1 (*29*). Percent identity was calculated manually for these isolates on the basis of a full-length alignment of query and match sequences.

Phylogenetic Analysis to Determine Repeatedly Encountered Taxa

Isolates that likely belonged to the same undescribed species were recognized by constructing phylogenetic trees with related isolates in MEGA. Groups of isolates with high sequence identity were specified from phylogenetic trees, and percent identity was calculated from multiple sequence alignments by using MEGA. Isolates that shared ≥99.0% sequence identity with each other were considered part of the same cluster. For all clusters containing ≥5 isolates, BLASTn matches were manually reviewed. Phylogenetic trees were constructed by using sequences from clinical isolates in the same cluster and related type strains as identified by the The All-Species Living Tree Project (release 102) (*30*) and/or List of Prokaryotic Names with Standing in Nomenclature (*31*).

Results

Clinical Study Isolates

During a 4-year period, 1,678 clinical isolates (≈6%) were not identified to the species level by routine 16S sequence analysis. Reanalysis of these sequences showed that 315 isolates (19%) were unidentified because of inadequate sequence quality; they were excluded from this study. The remaining 1,363 sequences were re-screened by using a current NCBI nucleotide database, and 690 (50.6%) were found to share >99% identity with ≥1 species-level annotated GenBank reference. The remaining 673 isolates were marked as probable novel taxa and included in this study. Of these 673 isolates, 52 (7.7%) were obtained at the University of Utah Medical Center, and the remaining isolates were referred from hospitals in 41 different US states. Nearly half of the isolates (47.3%) originated from blood cultures. Anatomical sources of the isolates are shown in Figure 1.

Sequence Length and Quality

Most sequences (84%) for the 673 isolates had lengths of 460 to 500 bp, as expected on the basis of the PCR and sequencing primers used (Figure 2, panel A). The median sequence phred quality score for the isolates suspected of representing novel taxa was 45, indicating high-quality sequences (Figure 2, panel B). One to 18 ambiguous nucleotide positions were observed in 38% of isolates (Figure 2, panel C), indicating multiple nonidentical copies of the 16S rRNA gene.

Similarity of Clinical Isolates to Reference Sequences

BLASTn identities were 80.9%–98.9% for references with valid species annotation (Figure 3, panel A), 84.5%–100% for references with valid genus annotation (Figure 3, panel B), and 86.7%–100% for any reference (Figure 3, panel C). A total of 448 isolates (66.6%) ranged from ≥97% to <99% identity to a valid species reference (*23*), likely indicating new species. However, fully one third of the isolates (n = 225) were <97% identical to a validly described species, satisfying a more conservative threshold for novel species (Figure 3, panel A) (*15*). Identities of 111 isolates (16.5%) were <95%, indicating novel genera (*21*). Using reference sequences with at least a genus-level annotation, we found that identities were ≥99% for 279 isolates (41.5%), ≥97% to <99% for 259 (38.5%),

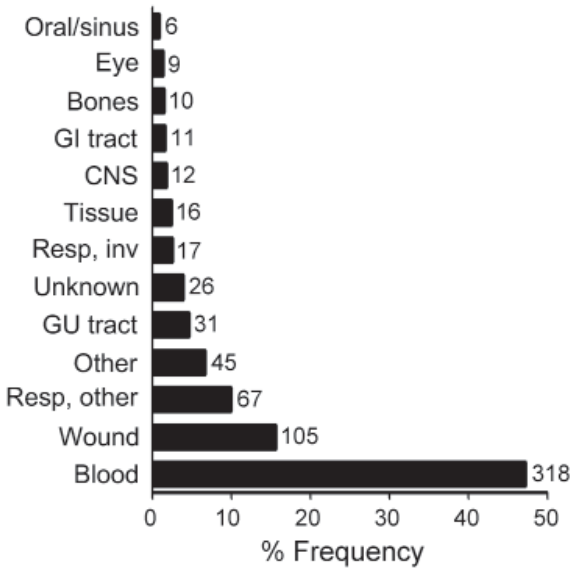

Figure 1. Anatomical sites that yielded 673 unidentified clinical bacterial isolates. The x-axis indicates relative frequency in percent. Numbers to the right of bars represent isolate counts. GI, gastrointestinal; CNS, central nervous system; Resp; respiratory; inv, invasive; GU, genitourinary.

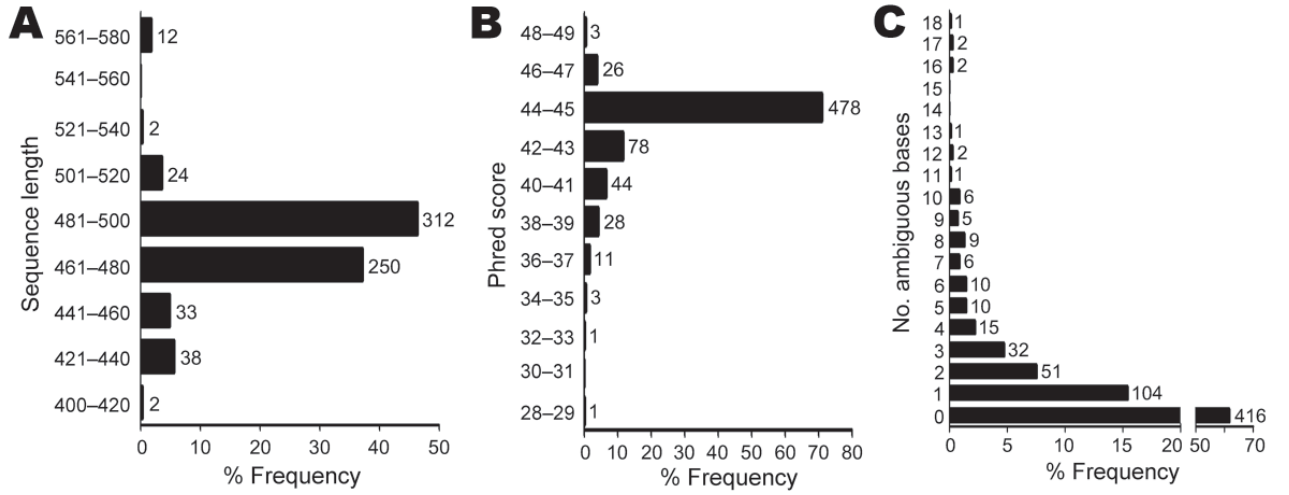

Figure 2. Sequence quality and number of ambiguous bases for 673 unidentified bacterial isolates. The median sequence length was 480 bases, with 84% of sequences in the range of 461 to 500 bases (A). The median phred sequence quality score was 45 (B). Most sequences had no ambiguous positions (n = 416, 61.8%). Up to 18 ambiguous positions were seen in isolates with multiple, nonidentical copies of the 16S rRNA gene (C). The x-axes indicate relative frequency in percent. Numbers to the right of bars represent isolate counts.

and <97% for 135 (20.1%) isolates (Figure 3, panel B). The same comparison with any reference, regardless of annotation, yielded values of 445 (66.1%), 165 (24.5%), and 61 (9.1%) isolates (Figure 3, panel C), with the latter group representing isolates highly divergent from any previously sequenced organisms.

Taxonomic Analysis of Clinical Isolates Representing Novel Taxa

Taxonomy of the 673 isolates was inferred from best database matches with species-level annotation (Table 1). The largest number of isolates (n = 294, 43.7%) belonged to the order *Actinomycetales*, followed by *Bacillales* (n = 61) and *Pseudomonadales* (n = 56). Within the order *Actinomycetales*, the most common families were *Actinomycetaceae* (n = 73), *Corynebacteriaceae* (n = 59), and *Nocardiaceae* (n = 53) (online Appendix Table 1, wwwnc.cdc.gov/EID/article/18/3/11-1481-TA1.htm). Taxonomic information by source is summarized in the online Appendix Figure (wwwnc.cdc.gov/EID/article/18/3/11-1481-FA1.htm).

Taxonomic Analysis of Novel Taxa Represented by Multiple Clinical Isolates

Overall, 348 isolates (52%) belonged to 95 novel taxa represented by >1 isolate. Cluster sizes ranged from 2 to 15,

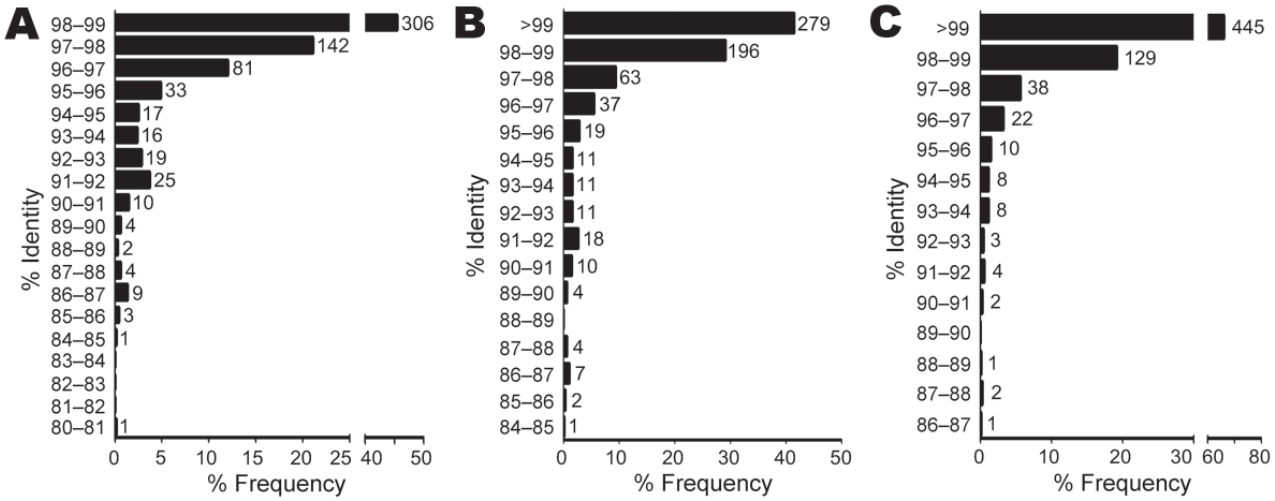

Figure 3. Identities of 673 unidentified bacterial isolates to best match in BLASTn database (*23*) with species-level (A) or genus-level annotation (B) and identity to best match in database, regardless of annotation status (C). The x-axes indicate relative frequency. Numbers to the right of bars represent isolate counts.

Table 1. Taxonomic distribution, by order of best species-level matches, for 673 isolates of possibly novel species of bacteria

Order	No. isolates
Actinomycetales	294
Bacillales	61
Pseudomonadales	56
Flavobacteriales	41
Burkholderiales	39
Lactobacillales	38
Enterobacteriales	33
Neisseriales	15
Pasteurellales	14
Rhizobiales	14
Clostridiales	10
Cardiobacteriales	9
Sphingomonadales	8
Caulobacterales	7
Rhodospirillales	7
Xanthomonadales	7
Fusobacteriales	6
Bacteroidales	5
Sphingobacteriales	4
Rhodocyclales	2
Desulfovibrionales	1
Micrococcineae	1
Rhodobacterales	1

and sequence identities to species-level references ranged from 86.5% to 98.9% (Table 2; online Appendix Table 2, wwwnc.cdc.gov/EID/article/18/3/11-1481-TA2.htm). Clusters within the order *Flavobacteriales* showed the greatest divergence from known species, with only 92.9% average identity. Not surprisingly, given the preponderance of isolates in this order, the largest number of clusters (n = 45) was identified among the *Actinomycetales* (online Appendix Table 1). Fourteen clusters with up to 9 members were seen in the family *Nocardiaceae*, 12 clusters with up to 12 members in *Actinomycetaceae*, and 9 clusters with up to 10 members in *Corynebacteriaceae*.

Nineteen novel taxa were represented by ≥5 isolates (Table 2). Upon manual review, 12 were confirmed without changes, 2 clusters contained at least 1 isolate with >1% sequence difference in pairwise comparisons, 2 clusters were split because of >1% sequence heterogeneity, and isolates of 3 clusters could be identified to validly described species: *Rothia aeria*, *Cardiobacterium hominis*, and *Streptomyces thermoviolaceus* subsp. *thermoviolaceus*. One cluster of 12 isolates belonged to a novel genus and species, *Kroppenstedtia eburnea*, which was described subsequent to our initial analysis (*32*).

Anatomical Source of Unidentified Isolates

In addition to the frequency with which isolates of novel taxa are encountered in clinical specimens, their importance may also be judged by their anatomical source. Isolates cultured from the following normally sterile sites were considered clinically relevant: cerebrospinal fluid, pericardial fluid, synovial fluid, and tissues (brain, heart valve, or biopsy tissues). A total of 32 isolates were identified from these key sites. A manual analysis showed 3 isolates that were not identified because of short reference sequences and 1 isolate that was subsequently identified as *K. eburnea*. Of the remaining 28 isolates, 17 (61%) belonged to taxa that were repeatedly encountered. Taxonomic information for all 32 isolates is summarized in Table 3.

Discussion

Broad-range molecular identification methods have facilitated the discovery of novel bacterial species and have resulted in a rapid increase in recognized bacterial taxa (*28*). The use of these methods in diagnostic laboratories may lead to the detection of bacterial strains that belong to novel species. We reviewed 16S sequencing results for >26,000 clinical isolates in a systematic approach to recognize novel species that may be pathogenic. Their formal description will provide the basis for improvements of sequence databases, antimicrobial susceptibility studies, and epidemiologic surveys to characterize their pathogenicity.

A sequence identity cutoff of <98.7%–99.0% for species discrimination has been shown to correlate with DNA-DNA hybridization results and is recommended for taxonomic purposes (*17*,*23*). In this study, 673 isolates showed <99% sequence identity and 535 isolates showed <98.7% sequence identity to any reference sequence with species-level annotation in the NCBI nucleotide database and could thus be considered novel taxa. Comparison of these sequences against the NCBI nucleotide database, the largest reference sequence repository (*10*,*11*), which contains 16S sequences for all newly described bacterial species, ensured a robust analysis of possibly novel species. Our algorithm employed 2 quality assurance criteria for reference sequences identified in BLASTn analysis: minimal alignment length of 98% and annotation as a validly described bacterial taxon (*28*). Because a more stringent manual review of reference sequences, as performed in diagnostic practice (*14*), was not feasible for this large study, the 673 isolates detected by this algorithm represent a conservative estimate of the total number of novel species encountered.

To ensure that sequence quality was not limiting, we confirmed that sequences were of expected length (Figure 2, panel A) and had phred scores showing a median accuracy of >99.99% per base (Figure 2, panel B). It has been recommended that sequences used for bacterial identification should contain <1% ambiguous positions (*19*), which was the case in 92% of the sequences in our study (Figure 2, panel C). However, ambiguous positions can be seen in bacteria with multiple, nonidentical 16S alleles. We observed up to 18 ambiguous positions in a small number of isolates (Figure 2, panel C), which is

Table 2. Tentative novel taxa represented by ≥5 clinical isolates*†

Family	Identity, %	Initial cluster size	Reviewed cluster size	Gram stain morphology	Result
Micrococcaceae	98.5	15	0	GPR	*Rothia aeria*, short reference sequence
Actinomycetaceae	98.7	12	11	GPR	1 strain with >1% dissimilarity
Thermoactinomycetaceae	91.8	12	12	GPR	Belong to *Kroppenstedtia eburnea* gen. nov., sp. nov.
Moraxellaceae	96.4	11	11	GNR	Most similar to *Acinetobacter ursingii*
Corynebacteriaceae	98.1	10	10	GPR	Most similar to *Corynebacterium. mucifaciens*
Corynebacteriaceae	98.6	10	5	GPR	Most similar to *C. jeikeium,* 5 isolates are *C. jeikeium*
Enterobacteriaceae	98.9	10	10	GNR	Most similar to *Enterobacter cloacae*
Streptomycetaceae	98.5	9	0	GPR	*Streptomyces thermoviolaceus subsp. thermoviolaceus*
Nocardiaceae	98.9	9	9	GPR	Most similar to *Nocardia vermiculata*
Cardiobacteriaceae	98.9	8	0	GNR	Belong to *Cardiobacterium hominis*, poor reference sequence
Flavobacteriaceae	86.5	7	7	GNR	Most similar to *Chryseobacterium daecheongense*
Actinomycetaceae	96.9	7	7	GPR	Most similar to *Actinomyces odontolyticus*
Actinomycetaceae	98.5	6	6	GPR	Most similar to *Actinomyces meyeri*
Thermoactinomycetaceae	90.8	5	5	GPR	Most similar to *Laceyella putida*
Actinomycetaceae	95.0	5	3+2	GPR	2 separate taxa
Streptococcaceae	96.7	5	5	GPC	Most similar to *Streptococcus oralis*
Enterobacteriaceae	97.3	5	5	GNR	Most similar to *Dickeya dieffenbachiae*
Actinomycetaceae	97.8	5	3+2	GPR	2 separate taxa
Streptococcaceae	97.9	5	5	GPC	Most similar to *Streptococcus mitis*

*GPR, gram-positive rods; GNR, gram-negative rods; GPC, gram-positive cocci.
†initial and reviewed clusters sizes indicate number of isolates in each cluster before and after manual review, outcome of manual review, and most similar valid species names are listed. Manual review was performed for all clusters with at least 5 isolates. Sequences were aligned with type strain sequences, and manual BLAST (*23*) analysis was performed to calculate pairwise sequence identities.

consistent with whole-genome sequencing data that indicate ≥19 nucleotide differences in bacteria with multiple rRNA operons (*33*,*34*). Although full-length 16S sequencing might have facilitated the identification of some isolates, partial 16S sequencing is considered robust (*10*) and is an unlikely reason for incomplete identification in most cases.

To determine taxonomic properties of all 673 isolates, we calculated 16S sequence identities to reference sequences with valid species-level (Figure 3, panel A), genus-level (Figure 3, panel B), or any annotation (Figure 3, panel C). Consistent with results of previous smaller studies, our results showed that most isolates were gram-positive rods and nonfermenting gram-negative rods (Table 1) (*22*,*35*). A total of 294 isolates belonged to the order *Actinomycetales*, with *Actinomyces* (n = 71), *Corynebacterium* (n = 59), and *Nocardia* (n = 52) being the most common genera. Molecular identification methods have resulted in a dramatic increase in the number of recognized species in these genera, and our results indicate that more species of possible clinical relevance are yet to be described (*28*). A total of 535 (79.5%) and 225 isolates (33.4%) belonged to novel species even when more conservative cutoffs of 98.7% and 97% identity, respectively, were used (*15*,*23*). Of these, 111 isolates (16.5%) represented novel genera at the conservative 95% identity cutoff (*10*,*21*).

To determine the isolates most likely to be of clinical importance, we identified novel taxa that were isolated repeatedly or were from normally sterile, clinically relevant anatomical sites. More than half of the unidentified organisms were isolated at least twice, forming clusters that represented 95 novel taxa. Most clusters belonged to the order *Actinomycetales* (45 clusters, 176 isolates), with 14 clusters (42 isolates) in the genus *Nocardia* and 12 clusters (52 isolates) in the genus *Actinomyces*. A total of 19 clusters that contained ≥5 members were initially identified (total of 156 isolates, Table 2). After manual review, isolates in 2 of these clusters were found to belong to validly described species (Table 2). These species were not identified in the automated analysis due to short reference sequences or because they had a subspecies annotation not covered in the algorithm. The validity of our approach was confirmed, however, when a novel thermoactinomycete, *Kroppenstedtia eburnea* (*32*), was formally described during preparation of this article. The 16S sequence of this organism showed ≈99.5% identity to a large cluster of 12 isolates in our study (Table 2).

While this study only included bacterial strains from clinical specimens (Figure 1), isolates from some anatomical sites (e.g., central nervous system) may be more likely to represent pathogens than others (e.g., upper respiratory tract). When highly stringent criteria are used (e.g., recovery from a normally sterile fluid or tissue), a minimum of 28 isolates may represent novel pathogens (Table 3). The presence of multiple isolates for 17 of these novel species further supports their status as potential pathogens. While proving pathogenicity is

Table 3. Anatomical sites and possible novel bacterial isolates*

Source	Identity, %	Best species-level match	Cluster	Gram stain morphology	Comment†
Tissue	98.8	*Acidovorax delafieldii*	N	GNR	
Tissue	97.2	*Actinoallomurus fulvus*	N	GPR	
CSF	98.3	*Actinomyces meyeri*	Y	GPR	
Pericardial fluid	94.4	*Anaerococcus prevotii*	N	GPC	
Tissue	94.8	*Capnocytophaga sputigena*	N	GNR	
CSF	93.8	*Chryseobacterium taiwanense*	Y	GNR	*Planobacterium taklimakanense*, short reference sequence
CSF	98.3	*Corynebacterium mucifaciens*	Y	GPR	
Tissue	98.6	*Cupriavidus gilardii*	Y	GNR	
CSF	97.1	*Erwinia chrysanthemi*	Y‡	GNR	
Tissue	97.7	*E. chrysanthemi*	Y‡	GNR	
CSF	97.2	*Globicatella sanguinis*	Y	GPC	
Tissue	96.2	*Kocuria kristinae*	Y	GPC	
CSF	92.4	*Desmospora activa*	Y	GPR	*Kroppenstedtia eburnea*
Synovial fluid	91.2	*Laceyella sacchari*	N	GVR	
Tissue	96.0	*Microbacterium thalassium*	Y‡	GPR	
Tissue	95.9	*M. thalassium*	Y‡	GVR	
Tissue	96.9	*Neisseria canis*	N	GNC	
Tissue	97.9	*Neisseria zoodegmatis*	Y	GNCB	
Biopsy specimen	98.7	*Nocardia beijingensis*	Y	GPR	
Brain	98.9	*Nocardia nova*	Y	GPR	
Tissue	98.9	*Nocardia transvalensis*	N	GPR	
CSF	96.1	*Phenylobacterium immobile*	N	GNR	
Tissue	97.5	*Prosthecomicrobium enhydrum*	N	GVR	
Tissue	95.2	*Pseudomonas pohangensis*	Y‡	GNR	
Tissue	95.2	*Pseudomonas pohangensis*	Y‡	GNR	
CSF	98.5	*Rothia dentocariosa*	Y	GPR	*Rothia aeria*, short reference sequence
Tissue	98.0	*Streptococcus mitis*	Y	GPC	
Valve	96.8	*Streptococcus oralis*	Y	GPC	
CSF	98.0	*Streptococcus sanguinis*	Y	GPC	
CSF	96.4	*Streptomyces prasinopilosus*	N	GPR	
CSF	96.8	*Terrabacter terrae*	N	GPC	
Tissue	97.8	*Williamsia serinedens*	N	GPR	*Williamsia deligens*, short reference sequence

*GNR, gram-negative rods; GPR, gram-positive rods; CSF, cerebrospinal fluid; GPC, gram-positive cocci; GVR, gram-variable rods; GNC, gram-negative cocci; GNCB, gram-negative coccobacilli; Y, isolates belonging to tentative novel taxa represented multiple times in this study.
†Results of manual review of BLASTn analysis (*23*).
‡These pairs of isolates belong to same 3 respective clusters.

beyond the scope of this study, our analysis may serve as a sentinel for novel organisms with pathogenic potential and provide a rationale for further studies to define their pathogenicity.

During 2001–2007, a total of 215 novel bacterial species and 29 novel genera isolated from clinical samples were formally described (*1*). Only 100 of these new species were represented by at least 4 isolates, of which *Mycobacterium* and *Nocardia* were the most common genera. In contrast to our study, most new species were isolated from nonsterile body sites, such as the oral cavity and gastrointestinal tract, and may thus be commensal or from the environment. Using a proposed minimum of 3 to 5 isolates to describe novel bacterial species (*10*,*36*,*37*), the present study may include up to 46 novel species (<99% identity) and up to 4 novel genera (<95% identity). Alternatively, it has been argued that even a single isolate from a human specimen should be reported to allow for more rapid identification of additional isolates in other laboratories (*1*,*12*,*22*). By this strategy, several hundred novel taxa may be represented in this study. Although our study does not prove that these isolates represent novel species, it provides a framework for screening large numbers of sequences for possible novel taxa that may be of clinical importance. Candidate isolates will require rigorous polyphasic validation, including full 16S rRNA gene sequencing, to confirm that they are new bacterial species. By providing information on morphologic characteristics, antimicrobial drug susceptibility profiles, virulence factors, and spectrum of disease, future studies will facilitate clinical decision making. Results of our phylogenetic analysis may thus help focus efforts to formally describe novel, clinically relevant species and to improve the diagnostic utility of reference databases.

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Seroprevalence of Antibodies against *Taenia solium* Cysticerci among Refugees Resettled in United States

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Neurocysticercosis (NCC) is a disease caused by central nervous system infection by the larval stage of the pork tapeworm, *Taenia solium*. In developing countries, NCC is a leading cause of adult-onset epilepsy. Case reports of NCC are increasing among refugees resettled to the United States and other nations, but the underlying prevalence among refugee groups is unknown. We tested stored serum samples from the Centers for Disease Control and Prevention Migrant Serum Bank for antibodies against *T. solium* cysts by using the enzyme-linked immunoelectrotransfer blot. Seroprevalence was high among all 4 populations tested: refugees from Burma (23.2%), Lao People's Democratic Republic (18.3%), Bhutan (22.8%), and Burundi (25.8%). Clinicians caring for refugee populations should suspect NCC in patients with seizure, chronic headache, or unexplained neurologic manifestations. Improved understanding of the prevalence of epilepsy and other associated diseases among refugees could guide recommendations for their evaluation and treatment before, during, and after resettlement.

Cysticercosis is a disease caused by infection with the larval stage of the pork tapeworm, *Taenia solium*. Humans and pigs acquire cysticercosis by ingesting *T. solium* eggs shed in the feces of humans with taeniasis (i.e., infected with an adult intestinal tapeworm). Upon ingestion, tapeworm eggs release oncospheres, which invade the intestinal wall and disseminate through the bloodstream to form cysts throughout the body. The natural lifecycle of *T. solium* tapeworms completes when a human eats pork contaminated by *T. solium* larval cysts because these can then develop into adult egg-producing intestinal tapeworms. This endemic lifecycle occurs primarily in regions where sanitation is poor and where pigs are allowed to roam and access raw human sewage.

Neurocysticercosis (NCC) occurs when cysts develop within the central nervous system (CNS); NCC is the primary cause of illness in *T. solium* infection. The clinical features of NCC cover a diverse range of neurologic manifestations, including seizures, headache, intracranial hypertension, hydrocephalus, encephalitis, stroke, cognitive impairment, and psychiatric disturbances (*1*,*2*). In areas in which *T. solium* infection is endemic, it is a major cause of epilepsy, with 30% of seizure disorder attributable to NCC (*3*–*5*).

Numerous reports document that cysticercosis in the United States occurs primarily among migrants and travelers who are presumed to have acquired their infection in another country (*6*–*9*). Refugees represent a large group of migrants in which the frequency of *T. solium* infection has not been described. Approximately 690,000 refugees resettled in the United States during 2000–2010 (*10*). Resettlement from regions with known pockets of *T. solium* tapeworm endemicity, including Southeast Asia, central Asia, and sub-Saharan Africa, is common. Cysticercosis among resettled refugees has been reported, but the underlying prevalence in refugee populations is unknown

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(*11*–*15*). Understanding the prevalence of *T. solium* infection could guide recommendations on evaluating and treating refugees before, during, and after resettlement.

During 2010, we used the classic enzyme-linked immunoelectrotransfer blot for lentil-lectin purified glycoprotein (EITB LLGP) to measure the seroprevalence of antibodies against *T. solium* cysts among several refugee populations resettled to the United States in previous years. We present the results, discuss clinical and public health implications, and suggest topics for further research.

Methods

Study Populations

Refugees who apply for resettlement to the United States are required to undergo a predeparture medical screening examination that includes collection of a peripheral blood sample from persons ≥15 years of age. The Migrant Serum Bank, established by the Division of Global Migration and Quarantine at the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) in 2002, retains a convenience sample of de-identified serum samples from these examinations. Each sample has associated demographic information, including refugee group, age, birth country, refugee camp, and site and date of specimen collection. At the time of this study, ≈31,000 serum samples were available that represented resettled refugee populations from the Middle East, Southeast Asia, and Africa. We identified refugee populations represented in the Migrant Serum Bank in which cases of human cysticercosis or NCC have been reported in the countries of origin (*16*). We then randomly selected serum samples from each of these identified populations to test by EITB LLGP for antibodies against *T. solium* cysts. Populations with limited numbers of samples were excluded because lack of statistical power could impede prevalence estimations. Our final sample comprised 2,001 serum samples from resettled refugees from Laos, Burma (renamed Myanmar in 1989), Bhutan, and Burundi (Figure 1). The institutional review boards at CDC and at Oregon Health & Science University reviewed and approved this study.

Laboratory Methods

Individual 100-μL aliquots of each sample were separated at the CDC Central Repository, stored in microtubes, and shipped on dry ice to the CNS Parasitic Diseases Research Unit, Universidad Peruana Cayetano Heredia (Lima, Peru), for processing. Serum samples were analyzed by EITB for the presence of antibodies against *T. solium* cysts (EITB LLGP) as described (*17*). The EITB LLGP uses a semipurified fraction of homogenized *T. solium* cysts containing 7 *T. solium* glycoprotein antigens named after the Kda molecular weights of the corresponding reactive bands (GP50, GP42, GP24, GP21, GP18, GP14, GP13). Reaction to any of these 7 glycoprotein antigens is considered positive. When applied in community settings, the EITB LLGP provides an estimate of population exposure to *T. solium* cyst antigens. A positive EITB LLGP result alone does not definitely establish active infection

Figure 1. Geographic location and background of refugee populations sampled for antibodies against *Taenia solium* cysticerci by using the classic enzyme-linked immunoelectrotransfer blot for lentil-lectin purified glycoprotein. Countries of origin are shaded dark grey (Burundi, Bhutan, Burma [Myanmar], Laos). Host countries are shaded light grey (Tanzania, Nepal, Thailand). Burundi: ≈14,000 Burundian refugees who lived in camps in Tanzania since 1972 were resettled during 2006–2008. Resettled refugees were primarily ethnic Hutu. Bhutan: ethnic Lhotshampa Bhutanese refugees arrived in Nepal ≈1990. Resettlement began in 2008 and is ongoing, with ≈40,000 resettled thus far. Burma: there has been intermittent influx of refugees into Thailand from Burma since 1984. Resettlement began in 2004 and is ongoing, with ≈90,000 resettled thus far. Resettled refugees in this group are primarily ethnic Karen and Karenni. Laos: refugees from Laos arrived in Thailand as early as 1975, and many resettled soon thereafter. The most recent round of resettlement from the Wat Tham Krabok camp occurred during 2004–2006 with resettlement of ≈16,000 ethnic Hmong refugees.

because antibodies can persist even after parasite clearance. The clinical significance of specific glycoprotein bands or combinations of bands in community studies has not been described. Although a highly sensitive and specific EITB is available to detect serum antibodies against adult *T. solium* intestinal infection, the unknown duration of antibody persistence after parasite clearance and the large sample size required for reasonable confidence intervals precluded our use of this assay in this study (*18*).

Data Analysis

Data were analyzed by using Stata version 10 (StataCorp LP, College Station, TX, USA). Direct standardization was used to facilitate comparison across refugee groups, with age–sex standardized seroprevalence calculated as the weighted average of stratum-specific seroprevalence. Continuous variables were assessed by using Kruskal-Wallis for differences among groups of interest. Pearson χ^2 and Fisher exact tests were used to compare distributions of proportions or to examine association between pairs of categoric measures. Logistic regression models were constructed for each refugee population to calculate odds ratios for seropositivity among strata (refugee camp or birth country) while controlling for age and sex. All tests are 2-sided, and significance was set at 0.05.

Results

A total of 2,001 samples were distributed approximately equally among refugees from Burma (499 [24.9%] refugees), Laos (502 [25.1%]), Bhutan (500 [25.0%]), and Burundi (500 [25.0%]). The median age of refugees sampled was 26 years (interquartile range 20–40 years, range 15–99 years). No significant difference existed between the proportions of samples from male (984 [49.2%]) and female (1,017 [50.8%]) refugees (p = 0.30). Of the 2,001 samples, 22.5% (95% CI 20.7%–24.4%) were EITB LLGP–positive for antibodies against *T. solium* cysts.

The aggregate seroprevalence was statistically homogenous across categories of age and sex. However, within individual refugee groups, seroprevalence differed across strata of age (Figure 2) and sex (Table 1). Male refugees from Burma were 2× more likely than female refugees from Burma to be seropositive (odds ratio [OR] 2.0, 95% CI 1.3–3.1). This association between male sex and positive serologic test results was not present in the other refugee groups. The proportion of seropositive results also varied by age category in refugees from Laos (p = 0.04) and Bhutan (p = 0.12).

Refugees from Burundi were significantly younger than those from the other countries (p<0.01), but Burma had a higher proportion of male refugees (p = 0.04) (Table 2). The crude seroprevalence (25.8%, 95% CI 22.0–29.6) and age–sex standardized seroprevalence (27.4%, 95% CI 22.8–32.0) were highest among refugees from Burundi. Samples from Burundian refugees were collected during 2006–2007 from persons in a single camp (Kibondo, Tanzania).

Of the 499 samples from refugees from Burma, 459 (92.0%) were collected during 2006–2007, a period of increased resettlement; the remaining 40 were collected during 2004–2005. The refugees came from 5 refugee camps and 2 urban populations, with most samples from

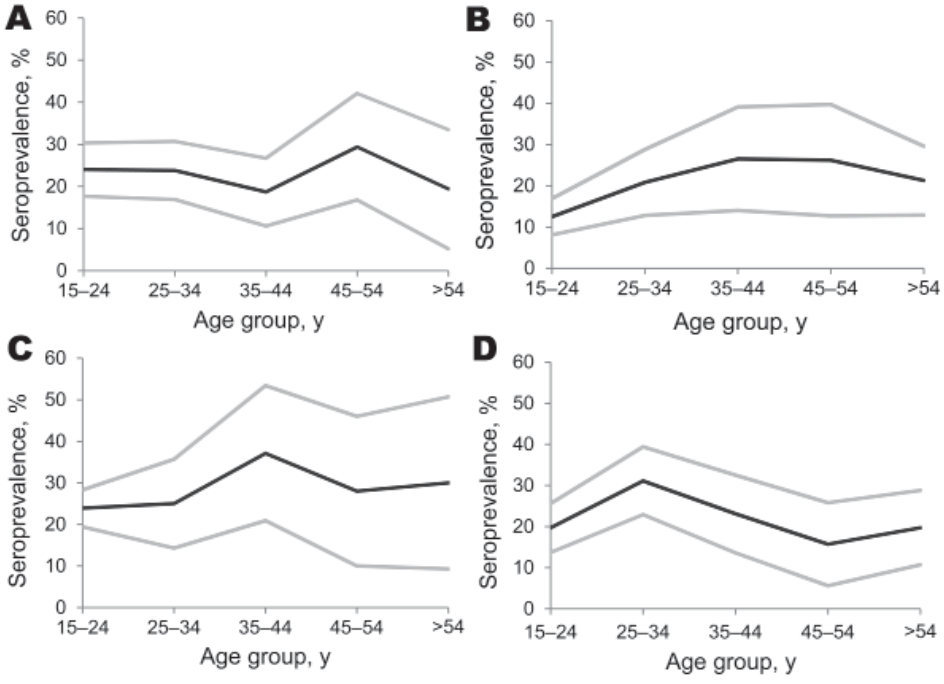

Figure 2. Distribution of positive results from the classic enzyme-linked immunoelectrotransfer blot for lentil-lectin purified glycoprotein for antibodies against *Taenia solium* cysticerci by age category among US-bound refugees from A) Burma (Myanmar), p = 0.65; B) Laos (Hmong), p = 0.04; C) Burundi, p = 0.56; and D) Bhutan, p = 0.12. Black lines represent seroprevalence estimates across age categories; gray lines represent upper and lower bounds of the corresponding 95% CI. Two-sided p values were determined by using likelihood ratio χ^2.

Table 1. Relationship between sex and seroprevalence of antibodies against *Taenia solium* cysts among refugees resettled in the United States

Country of origin	Male refugees		Female refugees		Odds ratio*	
	No. positive/total no.	% (95% CI)	No. positive/total no.	% (95% CI)	(95% CI)	p value†
Burma (Myanmar)	78/273	28.6 (23.2–34.0)	38/226	16.8 (11.9–21.7)	2.0 (1.3–3.1)	<0.01
Laos	41/240	17.1 (12.3–21.9)	51/262	19.5 (14.7–24.3)	0.9 (0.5–1.3)	0.49
Burundi	57/234	24.4 (18.8–29.9)	72/266	27.1 (21.7–32.4)	0.9 (0.6–1.3)	0.49
Bhutan	56/237	23.6 (18.2–29.1)	58/263	22.1 (17.0–27.1)	1.1 (0.7–1.7)	0.68
Total	232/984	23.6 (20.9–26.2)	219/1,017	21.5 (19.0–24.1)	1.1 (0.9–1.4)	0.27

*Odds of positive result for enzyme-linked immunoelectrotransfer blot for lentil-lectin purified glycoprotein (EITB LLGP) testing of samples from male refugees compared with samples from female refugees.
†Pearson χ^2.

Mae La Camp (326 [65.3%]) and Tham Hin Camp (130 [26.1%]). The proportion of seropositive refugees was not equal between camps (p<0.01). The crude seroprevalence was significantly higher in Mae La (28.5%, 95% CI 23.6%–33.4%) than in Tham Hin (12.3%, 95% CI 6.6%–18.0%). After controlling for age and sex, we found that refugees from Mae La were >3× more likely than refugees from Tham Hin to be seropositive (OR 3.5, 95% CI 1.7–7.5) (Figure 3, panel A). The remaining 40 samples from Burmese refugees were distributed among 5 refugee sites (Nupo, Umpiem, Ban Don Yang, urban Bangkok, and urban Kuala Lumpur), but sample sizes were insufficient to calculate reliable seroprevalence estimates.

All 500 refugees from Bhutan sampled resettled during 2007–2008. The crude seroprevalence was highest in Beldangi-1 Camp (26.6%, 95% CI 18.8%–34.4%) and lowest in Timai Camp (6.3%, 95% CI 0.0–14.5) (Figure 3, panel B). After adjusting for age and sex, we found that refugees from Beldangi-1 were >5× more likely than refugees from Timai to be seropositive (OR = 5.4, 95% CI 1.2–23.9).

The 502 samples from the Hmong refugees were collected during 2004–2005, and all were from 1 refugee camp (Wat Tham Krabok) in central Thailand. This group was the only one in which refugees who provided samples were born in different countries; 260 (51.8%) were born in Thailand and 242 (48.2%) were born in Laos. The crude seroprevalence was higher for Hmong refugees born in Laos (22.7%, 95% CI 17.4%–28.0%) than for those born in Thailand (14.2%, 95% CI 10.0%–18.5%). After adjusting for age and sex, we found that Hmong refugees born in Laos were 2× more likely than those born in Thailand to be seropositive (OR 2.0, 95% CI 1.0–4.2).

Of the 451 positive samples, 247 (54.8%) were reactive to a single glycoprotein antigen band only. In all 247 instances, the single-band–positive samples reacted to GP50. The remaining 204 (45.2%) positive samples reacted to multiple bands: 51 (11.3% of the 451 positive samples) with 2 bands, 119 (26.4%) with 3 bands, and 34 (7.5%) with ≥4 positive bands. On 24 (5.3%) of the positive samples, an atypical band pattern was noted, in which reactivity with the lower molecular weight proteins (GP13, GP14, and GP18) were present in the absence of reactivity to higher molecular weight proteins (GP50, GP42, GP24, and GP 21). This pattern was more frequent among positive samples from Burundi (16 [12.4%], p<0.01) than from Burma (6 [5.2%]), Laos (2 [2.2%]), or Bhutan (0). The odds of an atypical reaction occurring in a sample from Burundi were 6× greater than for all of the other groups combined (OR 6.2, 95% CI 2.6–14.6). The proportional distribution of atypical reactions did not differ with respect to age (p = 0.94) or sex (p = 0.54).

Discussion

We demonstrated that exposure to *T. solium* parasitic infection is common among refugees from Burma, Laos, Burundi, and Bhutan who resettled to the United States. All 4 populations had seroprevalence of antibodies against *T. solium* cysts comparable to or higher than the seroprevalence in well-characterized *T. solium*–endemic communities in Latin America where illness attributable to NCC is common (*4*,*5*,*19*,*20*). The widespread exposure among these groups has clinical and public health implications because these populations are resettling to the United States, where the infection is not endemic and where many clinical providers are not familiar with the disease manifestations, diagnosis, or treatment.

Table 2. Crude and age–sex standardized seroprevalence of antibodies against *Taenia solium* cysts among refugees resettled in the United States

Variable	Burma, n = 499	Laos, n = 502	Bhutan, n = 500	Burundi, n = 500	p value*
Age, y, median (interquartile range)	29 (22–40)	28 (20–47)	30 (22–45)	21 (18–25)	<0.01†
Male, no. (%)	273 (54.7)	240 (47.8)	237 (47.4)	234 (46.8)	0.04
Seroprevalence, % (95% CI)					
Crude	23.2 (19.5–27.0)	18.3 (14.9–21.7)	22.8 (19.1–26.5)	25.8 (22.0–29.6)	0.04
Age–sex standardized‡	23.0 (19.1–26.8)	18.3 (14.9–21.7)	22.3 (18.5–26.0)	27.4 (22.8–32.0)	<0.01

*Pearson χ^2 unless otherwise noted.
†Kruskall-Wallis χ^2.
‡Direct standardization method.

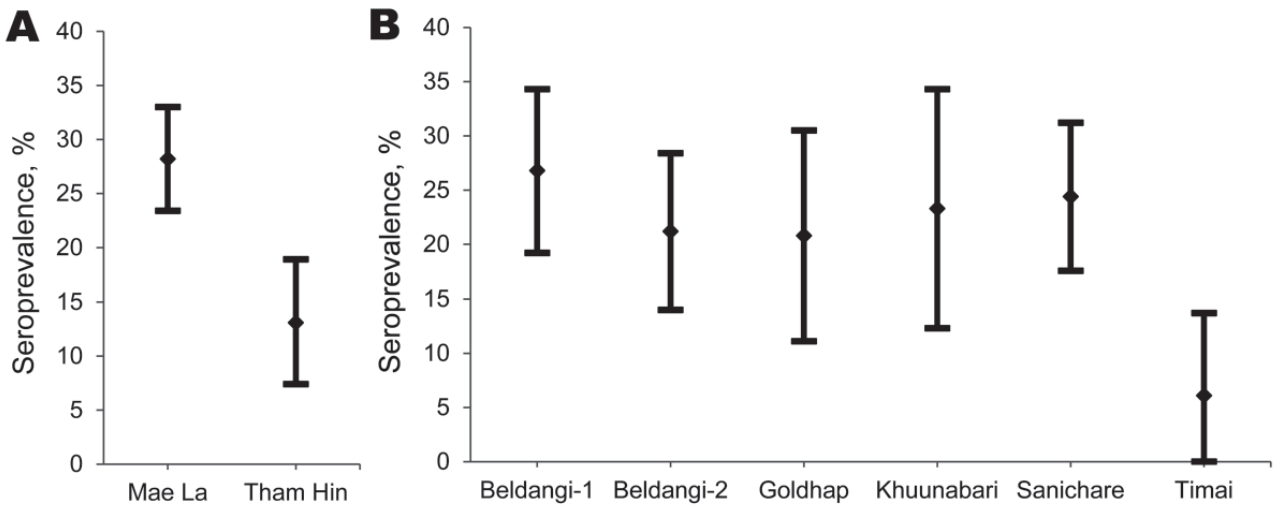

Figure 3. Age- and sex-adjusted seroprevalence of antibodies against *Taenia solium* cysticerci, by refugee camp among US-bound refugees from A) Burma (Myanmar) and B) Bhutan. Adjustment is by direct standardization within each refugee group. Point estimates and corresponding 95% CIs are shown.

Epilepsy and other neurologic diseases associated with *T. solium* infection may be prevalent among certain populations of resettled refugees. We were unable to characterize the prevalence of *T. solium*–related disease because of the retrospective nature of this study and the lack of clinical data accompanying the anonymous samples. However, case reports of symptomatic NCC among resettled refugees are now being reported in the literature (*11*–*15*). Clinicians who care for migrants from countries where *T. solium* infection is endemic should have a high index of suspicion for NCC when encountering seizure disorder, chronic headache, or other neurologic deficits of unknown cause. Up-to-date information on diagnosis and treatment of NCC is available in recent reviews (*21*–*23*).

Although human cysticercosis is considered a dead end in the *T. solium* life cycle, a person with taeniasis can transmit infection to others by shedding infective eggs in feces. An adult-stage tapeworm can live for several years within the human intestine and intermittently releasing proglottids containing tens of thousands of potentially infective eggs. The infrequent reports of *Taenia* spp. infections in fecal samples of resettled refugees may not reliably indicate the true prevalence of taeniasis (*24*). Routine screening of fecal samples is done by light microscopy, which has low sensitivity (<40%) for *Taenia* spp. (*25*,*26*). The number of imported *T. solium* taeniasis infections can be estimated among the populations we tested by extrapolation from other communities with similar seroprevalence in areas in which it is endemic. Multiple studies using the highly sensitive (99%) coproantigen ELISA in Latin America have shown the prevalence of *Taenia* spp. taeniasis in *T. solium*–endemic communities to be 2.0%–3.5% (*19*,*27*–*30*). These estimates include both *T. solium* and *T. saginata* tapeworms because the coproantigen ELISA used in those studies does not differentiate between these species (*26*). Approximately 87,000 refugees resettled to the United States from the 4 refugee populations we sampled during 2004–2009 (*10*). By using a conservative estimate of 1% prevalence, ≈870 refugees with *T. solium* taeniasis may have entered the United States from these populations alone.

Identifying and treating taeniasis among resettling refugees could prevent further transmission of cysticercosis in destination countries. The current approach for controlling intestinal helminthes among resettling refugees involves presumptive treatment before resettlement rather than routine fecal screening (www.cdc.gov/immigrantrefugeehealth/guidelines/overseas/intestinal-parasites-overseas.html). *Taenia* spp. tapeworms are not specifically targeted, although refugees from *Schistosoma* spp.–endemic areas in Africa may receive presumptive treatment with praziquantel, which is an effective treatment for *T. solium* taeniasis. Household screening for taeniasis when cysticercosis is diagnosed in an area to which it is not endemic is an alternate approach that can identify persons with *T. solium* intestinal tapeworm infection (*6*,*31*–*35*). Clinicians who diagnose cysticercosis should consider screening the index case-patient for taeniasis and household members for taeniasis and NCC. A combination of clinical history, laboratory analysis of feces and serum, and neuroimaging may be required.

The demonstration of widespread exposure to *T. solium* tapeworms among certain refugee populations is of concern because of the potential for severe adverse events related to presumptive treatment for intestinal helminthes. Refugees resettling to the United States from Africa and Asia receive presumptive treatment for intestinal roundworms. All

refugees without contraindication receive a single dose of albendazole, and refugees originating in sub-Saharan Africa receive additional treatment for schistosomiasis with praziquantel before departure. These guidelines are consistent with program strategies of presumptive treatment in parasite-endemic areas used by the World Health Organization for soil helminth infections (www.who.int/intestinal_worms/strategy/en/) and schistosomiasis (www.who.int/schistosomiasis/strategy/en/). Both medications are used in the treatment of NCC because of their ability to penetrate the CNS and to damage *T. solium* cysts. Corticosteroids are typically administered simultaneously in treatment of NCC to control resulting inflammation and to prevent neurologic complications (22). The long latency between CNS infection and development of symptoms means that some persons with NCC will have occult viable brain cysts. Inadvertent damage to these occult brain cysts during presumptive treatment for intestinal helminthes could precipitate an inflammatory CNS reaction in patients for whom presumptive treatment would otherwise have been contraindicated had their infection been known. Multiple case reports describe new-onset seizures in persons with underlying NCC who receive treatment with these agents (*12*,*35*–*39*). The Food and Drug Administration recently updated label precautions for albendazole and praziquantel to inform clinicians about this potential adverse event (www.accessdata.fda.gov/drugsatfda_docs/appletter/2009/020666s005,s006ltr.pdf and www.accessdata.fda.gov/drugsatfda_docs/appletter/2010/018714s012ltr.pdf).

The current CDC refugee predeparture health guidelines advise avoiding presumptive treatment for intestinal helminthes in patients with known history of cysticercosis or previous seizure (www.cdc.gov/immigrantrefugeehealth/guidelines/refugee-guidelines.html). However, refugees from *T. solium* tapeworm–endemic regions may harbor occult viable CNS cysts that could increase their risk for severe adverse events. Refugees are observed for 1–3 days after drug administration before departure; however, enhanced surveillance with systematic data collection would help inform risk-benefit analyses of these programs. Prospective studies that monitor adverse neurologic reactions after mass treatment with albendazole and/or praziquantel in *T. solium* tapeworm–endemic areas are needed to quantify the actual risk. Clinicians should be aware of potential adverse treatment events when evaluating refugees who develop neurologic symptoms after presumptive therapy and should report suspected cases by email to CDC (RefGuidelines@cdc.gov) or telephone (1-404-498-1600) and to the Food and Drug Administration though the Adverse Events Reporting System (www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Surveillance/AdverseDrugEffects/ucm115894.htm).

Although we cannot confirm that active transmission is occurring within the refugee camps and/or within surrounding communities on the basis of seroprevalence alone, we do suspect that active transmission is occurring for 2 reasons: 1) the populations we sampled lived in the refugee camps for years to decades before resettlement, and 2) the antibody response to *T. solium* cysts detected on EITB LLGP has been demonstrated to be transient, with ≈40% reversion from seropositive to seronegative after 1 year in serial community studies (*40*). Although we were unable to adequately explore risk factors for positive serologic findings in this study, we did detect significant differences in the odds of exposure between refugee camps. Further investigation within the camps and surrounding communities may clarify reasons for the variation observed. Areas for further study include characterizing 1) the prevalence of epilepsy and other neurologic disease associated with NCC, 2) the prevalence of and risk factors for taeniasis, 3) the prevalence of and risk factors for porcine cysticercosis, and 4) animal husbandry practices and market structure for sale of pork. Interventions, such as screening for and treatment of taeniasis, use of corrals for raising pigs, and improved sanitation infrastructure and education, may reduce transmission among refugees and ultimately prevent disease.

This study has limitations. The EITB LLGP is known to have low sensitivity for detecting single parenchymal cysts and calcified cysts alone. On the other hand, the 100% specificity to the larval stage of *T. solium* means that false-positive reactions are unlikely. Seroprevalence estimates based on the EITB LLGP are therefore likely to underestimate the overall prevalence of exposure to *T. solium* eggs in a community. Although *T. asiatica* tapeworms are co-endemic in Southeast Asia, there is no evidence for or against potential cross-reactivity of this related species on the EITB LLGP. However, *T. asiatica* cysticercosis has not been reported among humans. We preselected our sample to include refugee populations in which we expected to find evidence of endemic *T. solium* transmission. Our seroprevalence estimates should not be generalized to all resettling refugee populations, particularly those from Middle Eastern or northern African countries, to which *T. solium* tapeworms are not thought to be endemic. Our seroprevalence estimates also may not be generalizable to the broader population in the refugees' countries of origin. Refugee populations often include ethnic minority groups whose compilation of risk factors may not represent those of the majority population in their countries of origin. Nevertheless, our study does provide seroprevalence estimates for regions in which little to no data were previously available.

Exposure to *T. solium* parasitic infection is widespread among specific refugee groups resettled to the United States. Clinicians should suspect NCC in patients from these

regions who have seizure, headache, or other unexplained neurologic manifestations and should consider screening household members for additional cases. Systematic screening and treatment for taeniasis among refugees may prevent additional cases of NCC. Further investigation is needed to characterize illness and risk factors associated with *T. solium* infection in refugee populations. Additional serologic testing of stored samples from different regions of the world with the EITB LLGP can improve understanding of the global distribution of *T. solium* infection and may highlight regions that could benefit from control or elimination interventions.

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Pathogenic Potential to Humans of Bovine *Escherichia coli* O26, Scotland

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Escherichia coli O26 and O157 have similar overall prevalences in cattle in Scotland, but in humans, Shiga toxin–producing *E. coli* O26 infections are fewer and clinically less severe than *E. coli* O157 infections. To investigate this discrepancy, we genotyped *E. coli* O26 isolates from cattle and humans in Scotland and continental Europe. The genetic background of some strains from Scotland was closely related to that of strains causing severe infections in Europe. Nonmetric multidimensional scaling found an association between hemolytic uremic syndrome (HUS) and multilocus sequence type 21 strains and confirmed the role of stx_2 in severe human disease. Although the prevalences of *E. coli* O26 and O157 on cattle farms in Scotland are equivalent, prevalence of more virulent strains is low, reducing human infection risk. However, new data on *E. coli* O26–associated HUS in humans highlight the need for surveillance of non-O157 enterohemorrhagic *E. coli* and for understanding stx_2 phage acquisition.

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are gastrointestinal pathogens associated with asymptomatic carriage and human diseases ranging from mild diarrhea to hemorrhagic colitis and hemolytic uremic syndrome (HUS) (*1*). Worldwide, EHEC serogroup O157 strains are responsible for most human Shiga toxin–producing *E. coli* (STEC) infections (*2*). However, recent outbreaks in Germany (*3*) and the United States (*4*) have highlighted the increasing role of other Shiga toxin–producing serogroups (termed non-O157 strains) in causing human disease.

The pathogenesis of EHEC strains is associated with production of Shiga toxins expressed from lysogenic bacteriophages in the EHEC genome. There are 2 predominant classes of Shiga toxins (1 and 2), each encoded from 2 genes, *stxAB*, with the genotypes simplified to stx_1 or stx_2 in this study. Whereas EHEC is regarded as an emerging zoonotic pathogen, the related EPEC strains cause diarrhea, especially in infants in developing countries (*1*).

EPEC and EHEC express a type III secretion (T3S) system that translocates multiple effector proteins into host cells and manipulate host innate responses, which are needed for colonization (*5*–*7*). The T3S system is central to the formation of attaching and effacing lesions on the intestinal epithelium that requires the bacterial outer membrane protein intimin (encoded by *eae*) and the secreted bacterial protein—the translocated intimin receptor (Tir)—that is injected into the host cell. Studies of EPEC and EHEC O157 show that these pathogens trigger different actin polymerization pathways respectively involving Tir cytoskeleton coupling proteins (*tccP* or *tccP2*) (*5*). The formation of attaching and effacing lesions is needed for bacterial colonization by EHEC O157 and EHEC O26 in cattle. However, Shiga toxin (Stx) is the principal factor responsible for severe human illness, including HUS (*8*). Strains with stx_2 alone appear more strongly associated with HUS than do strains with only stx_1 (*8*–*10*). These

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observations have been supported by mouse (*11*) and primate models (*12*).

Among non-O157 EHEC, Stx-producing *E. coli* O26 also causes human disease (*13–15*) and has been isolated from livestock (*16*). However, unlike EHEC serogroup O157, it may be pathogenic for both cattle and humans (*17*). Although the origin of human *E. coli* O26 infections is rarely identified (*18*), evidence exists of person-to-person spread (*19*) and foodborne transmission (*20*). Furthermore, because EHEC O26 has been isolated from the feces of cattle and other animals (*16*,*21*,*22*), potential exists for direct and indirect zoonotic transmission to humans (*20*).

In the United Kingdom, Stx-producing human *E. coli* O26 infections are usually uncommon and not clinically severe. Most identified EHEC infections are associated with serogroup O157, and incidence rates in Scotland are among the highest, compared with rates in countries with comparable surveillance (*23*). In contrast with human infection rates, farm and animal prevalence of *E. coli* serogroups O26 and O157 are similar in Scotland (*21*).

Our first objective was to identify the cause of the disparity between the incidence of *E. coli* O157 and O26 infections among humans in Scotland by examining the natural heterogeneity among serogroup O26 strains and reexamining their prevalence in cattle. As a second objective, we used different molecular techniques to compare the relationships between *E. coli* O26 isolates recovered from humans and cattle.

Methods

Bacterial Isolates

All bacterial isolates used in this research are shown in Table 1. All isolates from cattle were collected in parallel with a prevalence study of *E. coli* O157 in cattle in Scotland, conducted during 2002–2004 (*21*). Fecal samples were obtained from cattle on 338 farms to test for non-O157 *E. coli* strains, including serogroup O26, as described by Pearce et al. (*21*). Human *E. coli* O26 isolates came from collections within the United Kingdom, Ireland, and continental Europe.

PCR for Virulence Genes

Genes encoding stx_1, stx_2, *eae*, and *hlyA* were detected by using multiplex PCR (*24*). Possession of the locus of enterocyte effacement (LEE) pathogenicity island was confirmed by PCR screening for *sepL*. Genes encoding *tccP* and *tccP2* were detected with PCR by using universal primer pair univtccP/tccP2-F and tccP-R (*25*). Primers and PCR conditions used in this study are listed in Table 2.

Pulsed-field Gel Electrophoresis

We performed pulsed-field gel electrophoresis (PFGE) on 187 isolates (33 from humans and 154 from cattle) (Table 1) by using the method of Willshaw et al. (*26*), standardized by the Scottish *E. coli* O157/VTEC Reference Laboratory (SERL; Edinburgh, UK) and the Laboratory of Gastrointestinal Pathogens (Colindale, UK). A phage λ ladder (48.5 kb) was used as a DNA size standard, and gels were run with a linearly ramped switch time of 5–50 s applied for 38 h at a voltage of 5.4 V/cm and an included angle of 120°. PFGE profiles were analyzed by using BioNumerics (version 3.0) software (Applied Maths, Kortrijk, Belgium). The degree of similarity between profiles was determined by the Jaccard coefficient, and dendrograms were generated by using the unweighted pair group method with arithmetic mean with 1.3% tolerance and 1% optimization settings.

Multilocus Sequence Typing

We performed multilocus sequence typing (MLST) on 63 isolates (30 from humans and 33 from cattle) (Table

Table 1. Source of *Escherichia coli* O26 isolates and sample sizes for the various statistical analyses, Scotland*

Source	Country of origin	Analyses	No. isolates	Clinical information
Bovine†	Scotland	Prevalence	249	NR
Bovine†	Scotland	PFGE	154	NR
Bovine†	Scotland	MLST	33	NR
Human‡	Scotland	PFGE	12	NK
Human‡	Scotland	MLST	11	D (n = 8); BD (n = 2); NK (n = 1)
Human§	England	PFGE	4	NK
Human§	Ireland	PFGE	11	NK
Human§	Belgium	PFGE	2	NK
Human§	Sweden	PFGE	3	NK
Human§	Italy	PFGE	1	NK
Human¶	Italy	MLST	5	HC (n = 1); HUS (n = 4)
Human#	Germany	MLST	14	D (n = 6); HUS (n = 8)

*NR, not relevant; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; NK, not known; D, diarrhea; BD, bloody diarrhea, HC, hemorragic colitis; HUS, hemolytic uremic syndrome. Where appropriate, the number of isolates from each clinical designation is provided.
†Isolated 2002–2004 (*21*).
‡L.J. Allison, Scottish *E. coli* O157/VTEC Reference Laboratory, Edinburgh, UK; isolated 2002–2003.
§H. Smith, Laboratory of Gastrointestinal Pathogens, Colindale, UK. The years isolated are unknown.
¶A. Caprioli, Instituto Superiore di Sanità, Rome, Italy. The years isolated are unknown.
#H. Karch, University of Műnster, Műnster, Germany; isolated 1994–2000.

Table 2. Primers and PCR conditions for *Escherichia coli* O26, Scotland

Primer name	Primer sequence, 5′ → 3′	Target gene	Annealing	Amplicon size, bp	Reference
stx1F	ATAAATCGCCATTCGTTGACTAC	*stx*$_1$	60°C,45 s	180	(*24*)
stx1R	AGAACGCCCACTGAGATCATC				
stx2F	GGCACTGTCTGAAACTGCTCC	*stx*$_2$	60°C,45 s	255	(*24*)
stx2R	TCGCCAGTTATCTGACATTCTG				
eaeAF	GACCCGGCACAAGCATAAGC	*eae*	60°C,45 s	384	(*24*)
eaeAR	CCACCTGCAGCAACAAGAGG				
hlyAF	GCATCATCAAGCGTACGTTCC	*hlyA*	60°C,45 s	534	(*24*)
hlyAR	AATGAGCCAAGCTGGTTAAGCT				
sepLF	GCTAAGCCTGGGATATCGC	*sepL*	60°C,45 s	725	This study
sepLR	ACAATCGATACCCGAGAAGG				
univ tccP/tccp2-F	GTAAAAACCAGCTCACCTTTTTC	*tccp*	64°C,60 s	Variable	(*25*)
tccP-R	TCACGAGCGCTTAGATGTATTAAT	*tccP2*			
espAF	CCTTCTCGGGTATCGATTGTCG	*espA*	58°C,60 s	1012	This study
espAR	CAGAGGGCGTCACTAATGAGTG				
LEE1promF	CGAATGGTACGGTTATGCGGG	*LEE1*	58°C,60 s	645	This study
LEE1promR	GCTCTCGCAGTCGCTTTGCTTCC				

1) by using a method similar to that of Wirth et al. (*27*). Internal fragments of 7 housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) were sequenced, and the allele numbers and sequence types (STs) were assigned in accordance with the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli). The T3S system of *E. coli* O26 is encoded on the LEE, and this region was originally sequenced from *E. coli* O26 isolate 413/89–1 (GenBank accession no. AJ277443). From this sequence, primers were designed to amplify *espA* encoding the main translocation filament protein of the T3S system and a 644-bp region (5082–5726) including the promoter controlling expression of the first LEE operon (*LEE1*) (Table 2). Sequence data for *espA* and the *LEE1* promoter region were compared by using ClustalX (www.clustal.org). Each unique sequence was given a different allele number assigned in the order in which they were discovered.

Data Management and Statistical Analyses

Prevalence of *E. coli* O26

E. coli O26 isolated from cattle are more heterogeneous than *E. coli* O157 with respect to the presence of *stx* and *eae* (Figure 1). Therefore, we determined the prevalence of *E. coli* O26 for 4 different groups with potential differences in virulence: 1) all *E. coli* O26, similar to Pearce et al. (*21*); 2) *stx*$_1$+; 3) *stx*$_1$+*stx*$_2$+; and 4) *stx*$_2$+*eae*+. Farm-level and fecal pat–level prevalences were calculated by using the method of Pearce et al. (*28*). SAS version 9.1.3 (SAS Institute, Cary, NC, USA) was used to fit generalized linear mixed models and to generate bootstrap-based estimates of key parameters. Excel 2000 (Microsoft Corporation, Redmond, WA, USA) was used to implement a Latin hypercube sampling to convert results from generalized linear mixed models into prevalence taking into account random effects (*29*).

PFGE Analysis

Differences between isolates from humans and cattle were evaluated by assigning isolates to statistically distinct groups. The methods used are described in (*30*). Group membership was determined by estimating an optimum cutoff value using dendrogram-based distances. Statistical support for each group was evaluated by using the cophenetic correlation. The statistical significance of the cophenetic correlation was evaluated by using a Mantel test. Program zt (*31*) was used with 1,000 simulations, and the probability of the observed cophenetic correlation occurring by chance was <5% for groups of isolates regarded as statistically distinct.

A subset of 41 isolates from Scotland (8 from humans and 33 from cattle) were included in the MLST and PFGE analyses. The PFGE data for 3 isolates from humans were missing and therefore could not be included in the comparison. Agreement between these 2 techniques was examined.

Nonmetric Multidimensional Scaling

To identify patterns in data associated with the clinical severity of infection in humans, we used nonmetric multidimensional scaling (NMS) to analyze all genotype characteristics measured. PC-ORD software version 6.03 (MjM Software Design, Gleneden Beach, OR, USA) was used. The main matrix was created from the following isolate data: MLST type (ST, ST complex), *tccP* and *tccP2*, Stx status, *hlyA*, *eae*, LEE, and sequence upstream region of *LEE1*. A second matrix that used isolate data and an indicator of clinical severity was also created. Isolates were separated into 2 groups: 1) HUS and those associated with HUS and 2) non-HUS, including those from diarrhea, bloody diarrhea, and hemorrhagic colitis. The grouping was chosen because HUS has clearly identifiable clinical characteristics and is a reliable indicator of severe illness.

We used NMS with a Euclidian distance measure after standardizing each variable by division by its

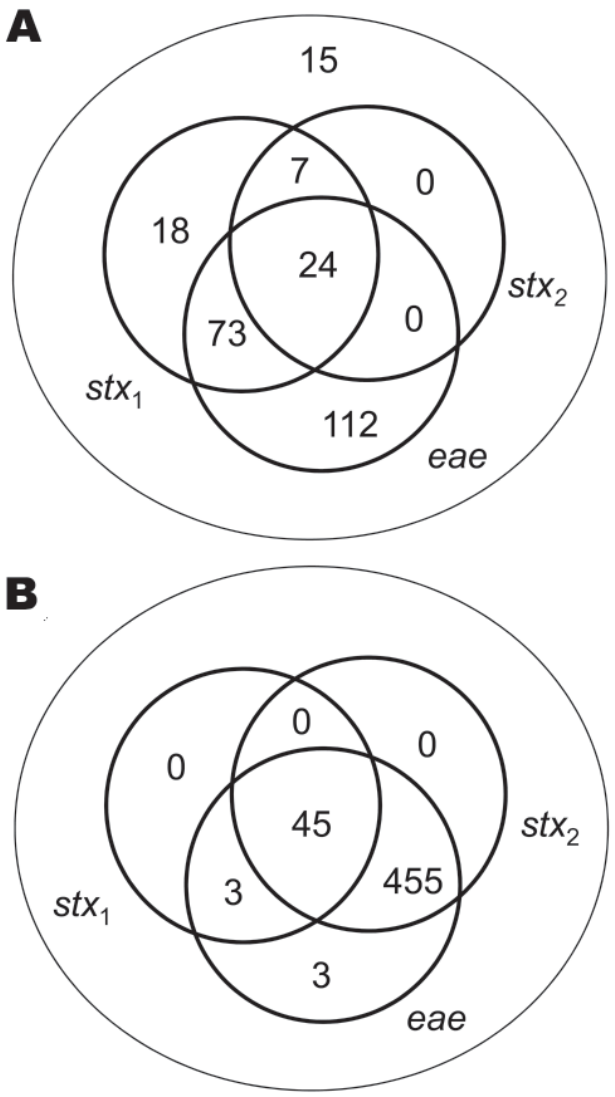

Figure 1. Isolates of *Escherichia coli* O26 (A; n = 249) and O157 (B; n = 507), collected from a 2002–2004 field survey, that illustrate the differences between the 2 serogroups from Scotland with reference to the presence or absence of Shiga toxin gene (stx_1 and stx_2) and the *eae* gene.

standard deviation. The dimensionality of the dataset was determined by plotting an inverse measure of fit ("stress") to the number of dimensions. Optimal dimensionality was based on the number of dimensions with the lowest stress. A 3- dimensional solution was shown to be optimal. We performed several NMS runs to ensure that the solution was stable and probably represented a configuration with the best possible fit. For each NMS run, we used 500 iterations with random starting coordinates. The overall significance of the difference between HUS and non-HUS groups was assessed by using multiresponse permutation procedures (*32*).

Results

Prevalence in Cattle

Of the 338 farms visited, 68 were positive for *E. coli* O26 (mean prevalence 20.1%, exact binomial 95% CI 16.0–24.8); these 68 farms were evenly distributed across Scotland (Figure 2, panel A). Farms were classified by their isolates' virulence groups: *stx*–, stx_1+, and stx_1+stx_2+. No farm had stx_2+ only strains. Farms with *stx*– isolates clustered in southern Scotland. Farms that were stx_1+ were fairly evenly distributed, but those with stx_1+stx_2+ isolates were found mainly in the northeast (Figure 2, panel B). Analysis of the farms grouped according to their Scottish animal health district (*21*) significantly supported this observation (p = 0.005). No seasonal differences were observed (p = 0.59).

The adjusted mean overall prevalence of *E. coli* O26 was 0.22 (95% CI 0.18–0.27) and 0.046 (95% CI 0.031–0.062), respectively (Table 3). However, the adjusted mean prevalences for farms and fecal pats of stx_2+*eae*+ *E. coli* O26 were lower at 0.05 (0.03–0.09) and 0.004 (0.001–0.007), respectively.

PFGE Genotyping

PFGE profiles of the 187 *E. coli* O26 isolates from 154 cattle and 33 humans were compared (online Technical Appendix Figure 1, wwnc.cdc.gov/EID/pdfs/11-1236-Techapp.pdf). This comparison identified 4 groups comprising 5, 19, 142, and 16 isolates, which all included *E. coli* O26 isolates from cattle and humans. The 4 groups are characterized by statistically significant cophenetic correlations ranging from 0.807 to 0.945. Isolates from groups 2 and 4 were mainly *stx*–, whereas groups 1 and 3 were mainly *stx*+. Five isolates were not assigned to a group. All of the *stx*+ isolates from outside Scotland were in group 2, along with the 5 *stx*+ isolates from Scotland. The remaining isolates from Scotland were in group 1 (2 isolates), 2 (3 isolates), and 4 (1 isolates).

We compared PFGE profiles of the 41 *E. coli* O26 isolates from 33 cattle and 8 humans. PFGE grouping correlated strongly with MLST ST (online Technical Appendix Figure 2). PFGE was more discriminative than MLST because each ST was represented by >1 PFGE pattern.

MLST Analysis

Irrespective of source, most isolates had a close genetic background (Table 4). Ninety percent of all isolates were either ST21 or ST29, both belonging to ST complex 29 and differing by 1 nt in the *adk* locus. Most isolates from continental Europe were ST21 (74%) and stx_2+ (53%). Considerably fewer isolates from humans in Scotland were ST21 (36%) and stx_2+ (18%). Other differences

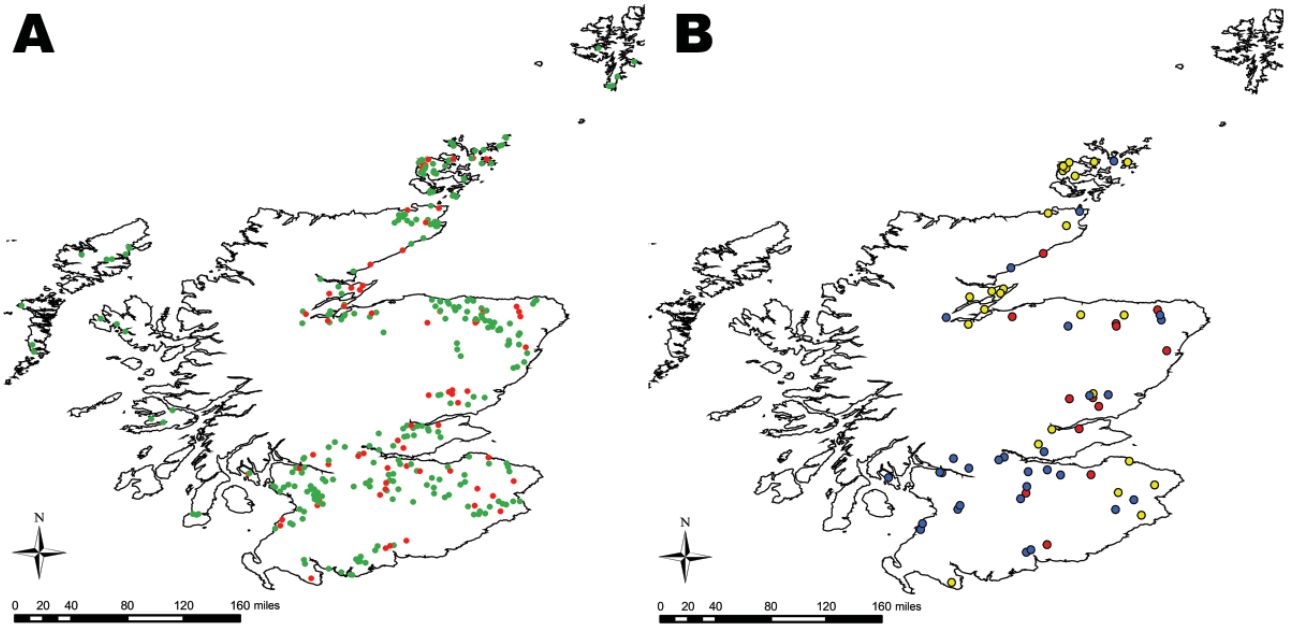

Figure 2. Location of farms sampled in 2002–2004 field survey for *Escherichia coli* O26, Scotland. A) Farms that were positive for *E. coli* O26 are shown in red; farms negative for *E. coli* O26 are shown in green. B) The positive farms were subdivided according to differences in virulent properties of *E. coli* O26 (farm status) based on the possession of stx. Farms were designated as *stx*– (blue), stx_1+ (yellow), or stx_1+stx_2+ (red).

between Scotland and continental Europe were related to the relative absence of *tccP2* in isolates from Scotland and the presence of different alleles upstream of LEE (Table 4). Isolates from cattle were genotypically more similar to those from humans from Scotland than from continental Europe (Table 4), except for the proportion of isolates that were ST21 and stx_2+. This similarity may be explained by the fact that the isolates analyzed from cattle (33 [13%] of 249) were not a random sample of all isolates from cattle but were selected on their virulence properties (i.e., *stx*+ and *eae*+) to assess their potential to cause human infection rather than to provide a direct comparison with isolates from humans in Scotland.

Disease Severity Association

Before NMS analysis, 3 isolates were excluded because of missing data. ST complex, LEE, *tccP*, and *eae* were removed because no variation existed in the data (Table 4). The final matrix comprised 5 variables and 27 isolates. The final NMS solution was 3-dimensional and explained 90.8% of the variation in clinical severity and explained more variation than expected by chance (Monte Carlo, p = 0.008). Final stress for the 3-dimensional solution was 8.53 with no real risk for drawing false associations (*32*), and the final instability was 0 with 53 iterations.

The results of the NMS models are shown in joint graphs (Figure 3, panel A) and 3-dimensional ordination graphs (Figure 3, panel B) of the distance between sample units, which approximates dissimilarity in clinical severity. Where appropriate, R package version 2.12.1 (*33*) was used to add 80% confidence ellipsoids to discriminate between groups of interest. Variables used in the NMS analysis are shown as vectors; the direction indicates positive and negative correlation (Figure 3, panel A). Although some

Table 3. Farm-level and fecal pat–level prevalence of *Escherichia coli* O26, Scotland*

	Farms, n = 338			Fecal pats, n = 6,086		
E. coli O26 status	No. positive	Observed prevalence	Adjusted prevalence (95% CI)	No. positive	Observed prevalence	Adjusted prevalence (95% CI)
E. coli O26*	68	0.20	0.22 (0.18–0.27)	249	0.041	0.046 (0.031–0.062)
stx+ *E. coli* O26	38	0.11	0.12 (0.09–0.16)	122	0.020	0.020 (0.012–0.029)
stx_1 + stx_2 + *E. coli* O26	13	0.04	0.06 (0.031–0.09)	97	0.016	0.004 (0.001–0.008)
stx_1 + stx_2 + *eae* + *E. coli* O26	12	0.04	0.05 (0.03–0.09)	24	0.004	0.004 (0.001–0.007)

*Minor differences in the farm-level prevalence estimates for *E. coli* O26 between Pearce et al. (*21*) and this study resulted from use of different statistical models. Pearce et al. (*21*) aimed to provide national prevalence estimates; thus, weighted estimates of mean prevalence were generated that accounted for the fractions of the national herd found in different Animal Health Districts (AHDs). By contrast, we reported the mean of the sample collected in a stratified fashion across the AHDs. Because there were only relatively small differences in mean prevalence in the different AHDs, the effect on the 2 means is negligible and the effect on the standard errors relatively small.

overlap exists, the difference between HUS and non-HUS groups was statistically significant (multiresponse permutation procedures, p = 0.007) (Figure 3, panel B). HUS was associated with ST21 and stx_2, whereas non-HUS cases were associated with ST29 and the absence of stx_2. The *hlyA* gene was not associated with either condition because 69% of non-HUS isolates possess *hlyA* and 67% of HUS isolates possess *hlyA* (Figure 3, panel A).

Discussion

Although Stx-producing *E. coli* O26 is a major cause of HUS in continental Europe, human infection with this pathogen is uncommon in Scotland and has resulted in less severe illness. In contrast, *E. coli* O157 is a major cause of human infections and HUS (*1*). Previous reports of a high prevalence of *E. coli* O26 in cattle (*21*) do not appear to be matched by a high level of infection in humans. Our first objective was to determine whether this discrepancy could

Table 4. Results of genotypic characterization by MLST and the presence of virulence genes for *Escherichia coli* O26 isolates, Scotland*

Genotypic characterization	No. (%) isolates from humans, n = 30		No. (%) isolates from cattle,
	Scotland	Germany/Italy	Scotland, n = 33
MLST			
ST			
21	4 (36.4)	14 (73.7)	22 (66.6)
29	4 (36.4)	5 (26.3)	9 (27.3)
Other	3 (27.2)	0	2 (6.1)
ST complex†			
29	10 (90.9)	19 (100.0)	31 (93.9)
10	1 (9.1)	0	2 (6.1)
espA‡			
Allele 1	9 (81.8)	19 (100.0)	31 (100.0)
Other	1 (9.1)	0	0
ND	1 (9.1)	0	0
Upstream of *LEE1*§			
Allele1	2 (18.2)	14 (73.7)	7 (21.2)
Allele2	5 (45.4)	5 (26.3)	20 (60.6)
allele3	0	0	1 (3.0)
allele4	1 (9.1)	0	3 (9.1)
allele5	1 (9.1)	0	0
allele6	1 (9.1)	0	0
ND	1 (9.1)	0	2 (6.1)
Presence of virulence genes			
stx			
stx–	6 (54.5)	4 (21.1)	7 (21.2)
stx_1 +	3 (27.3)	5 (26.3)	16 (48.5)
stx_2+	2 (18.2)	10 (52.6)	10 (30.3)
Eae¶			
Absent	1 (9.1)	0	2 (6.1)
Present	10 (90.9)	19 (100.0)	31 (93.9)
sepL#			
Absent	1 (9.1)	0	2 (6.1)
Present	10 (90.9)	19 (100.0)	31 (93.9)
*hlyA***			
Absent	3 (27.3)	7 (36.8)	6 (18.2)
Present	8 (72.7)	12 (63.2)	27 (81.8)
tccP††			
Absent	11 (100.0)	19 (100.0)	33 (100.0)
Present	0	0	0
tccP2††			
Absent	6 (54.5)	2 (10.5)	16 (48.5)
Present	5 (45.5)	17 (89.5)	17 (51.5)

*MLST, multilocus sequence typing; ST, sequence type; ND, not determined; stx, Shiga toxin; +, *stx* gene present; –, *stx* gene absent. *LEE1* encodes the first operon of the locus of enterocyte effacement (LEE) and the 644-bp region sequenced includes the promoter for this operon amplified using the defined LEE primer pair in Table 2.
†The ST and ST complex were assigned in accordance with the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli).
‡*espA* encodes for the surface-associated protein, espA. The allele numbers at each loci were assigned in the order in which they were discovered.
§Six different sequences were discovered for the region upstream of *LEE1* in the *E. coli* O26 isolates. Approximately 644 bp of sequence data were determined, and all sequence variation between the *E. coli* O26 alleles occurs within a 91-bp region upstream of *LEE1* in the region where regulators act in *E. coli* O157:H7. The allele numbers at each locus were assigned in the order in which they were discovered.
¶*eae*, gene that encodes intimin.
#*sepL*, gene confirming the presence of LEE pathogenicity island.
***hlyA*, enterohemolysin.
††*tccP/tccP2,* genes encoding Tir-cytoskeleton coupling protein and Tir-cytoskeleton coupling protein 2, which are used in actin polymerization and subsequent attaching and effacing lesion formation.

be explained by the natural heterogeneity in strains by examining virulence determinants associated with serious human disease in strains recovered from cattle in Scotland.

The overall percentage of farms on which *E. coli* shedding was detected and the overall proportion of fecal pats positive were comparable for both organisms, but for *E. coli* O26, the farm and pat-level prevalence becomes smaller as the carriage of the virulence determinants *eae* and *stx* are taken into account (Figure 4). In contrast, the prevalence of virulent strains of *E. coli* O157 (*stx*+*eae*+) is not smaller. Thus, because there are fewer virulent strains at farm and animal levels, the risk for human illness is expected to be much lower for *E. coli* O26 than for *E. coli* O157 in Scotland.

Our second objective was to determine the genetic relationship of strains from different sources. PFGE characterization demonstrated that PFGE profiles of isolates from humans differed from those from cattle. This result was expected in the absence of a demonstrable epidemiologic link. However, human strains are widely distributed among groupings of isolates from cattle. Although an exact interpretation of relatedness of isolates from humans to animals is not possible, the isolates cannot be regarded as distinct populations. Further characterization by MLST confirmed that the genetic backbone of bovine and human *E. coli* O26 strains in Scotland match and that isolates from cattle in Scotland are closely related to highly pathogenic isolates from humans in Germany and Italy.

Characterization of human strains from Scotland and continental Europe and of bovine strains from Scotland showed that most *E. coli* O26 strains were ST29 and were likely to be capable of expressing a T3S system because they were *eae* and *sepL* positive. Although less virulent strains generally are present in cattle in Scotland, the unexpected finding of this study was that the genetic background of many of the strains shows a close relationship to strains causing severe human infections in continental Europe.

The use of NMS enables identification of an association between HUS and ST21 strains and confirms the role of stx_2 alone in severe human disease. As previously suggested, the *hlyA* gene was not associated with HUS (*8*). Our results indicate that, in addition to stx_2, *tccP2* is linked to the severity of clinical disease. Therefore, the risk to humans in Scotland from isolates from cattle might be even lower for *E. coli* O26 if ST21 and the carriage of *tccP2* are considered.

In the absence of *stx* genes, isolates from cattle feces are less of a threat to human health than *E. coli* O157 strains, but there are *E. coli* O26 in Scotland of ST21 that are LEE positive and carry *tccP2*. These need only to acquire the stx_2-encoding bacteriophage and they could prove to be highly pathogenic because *E. coli* O26 is a highly dynamic group capable of acquiring *stx*-encoding phages (*34*,*35*).

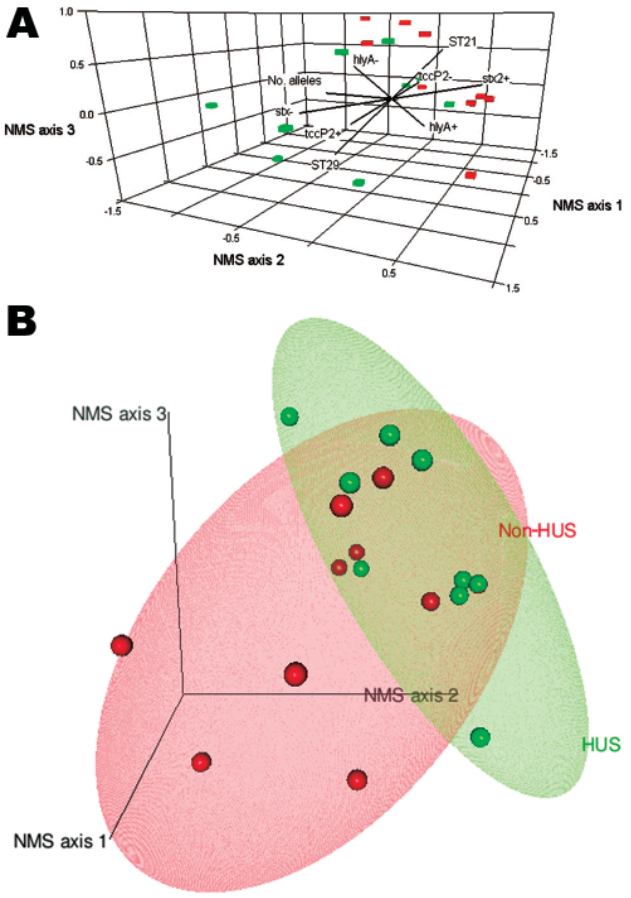

Figure 3. Patterns in data associated with the clinical severity of *Escherichia coli* O26 infection in humans, as identified by nonmetric multidimensional scaling, Scotland. A) Joint graph illustrating the association between the multilocus sequence typing and genotypic variables measured and the severity of the human infection (hemolytic uremic syndrome [HUS] in red and non-HUS [diarrhea, bloody diarrhea] in green). B) 3-dimensional scatterplot and 80% confidence ellipses (R rgl package [*33*]) around the cases in space illustrating the separation between individual classes as HUS (red) and non-HUS (green).

Zhang et al. (*14*) described a shift in *stx* genotype from stx_1 to stx_2: before 1994, only the stx_1 gene was identified in their STEC O26 isolate collection, but after 1997, stx_2 was the only genotype identified in 71% of the isolates. The trigger for this shift was not established.

At the time of our study, there was no correlation between farms in Scotland with *E. coli* O157 and *E. coli* O26 ($p = 0.28$) or those with *E. coli* O26 stx_1+ stx_2+ and *E. coli* O157 ($p = 0.10$). However, release of free *stx*-encoding bacteriophages into the intestinal environment (*36*,*37*) by STEC O157 or other EHEC may permit horizontal transfer of virulence genes to *E. coli* O26. Acquisition of stx_2-encoding bacteriophages could confer *E. coli* O26 with

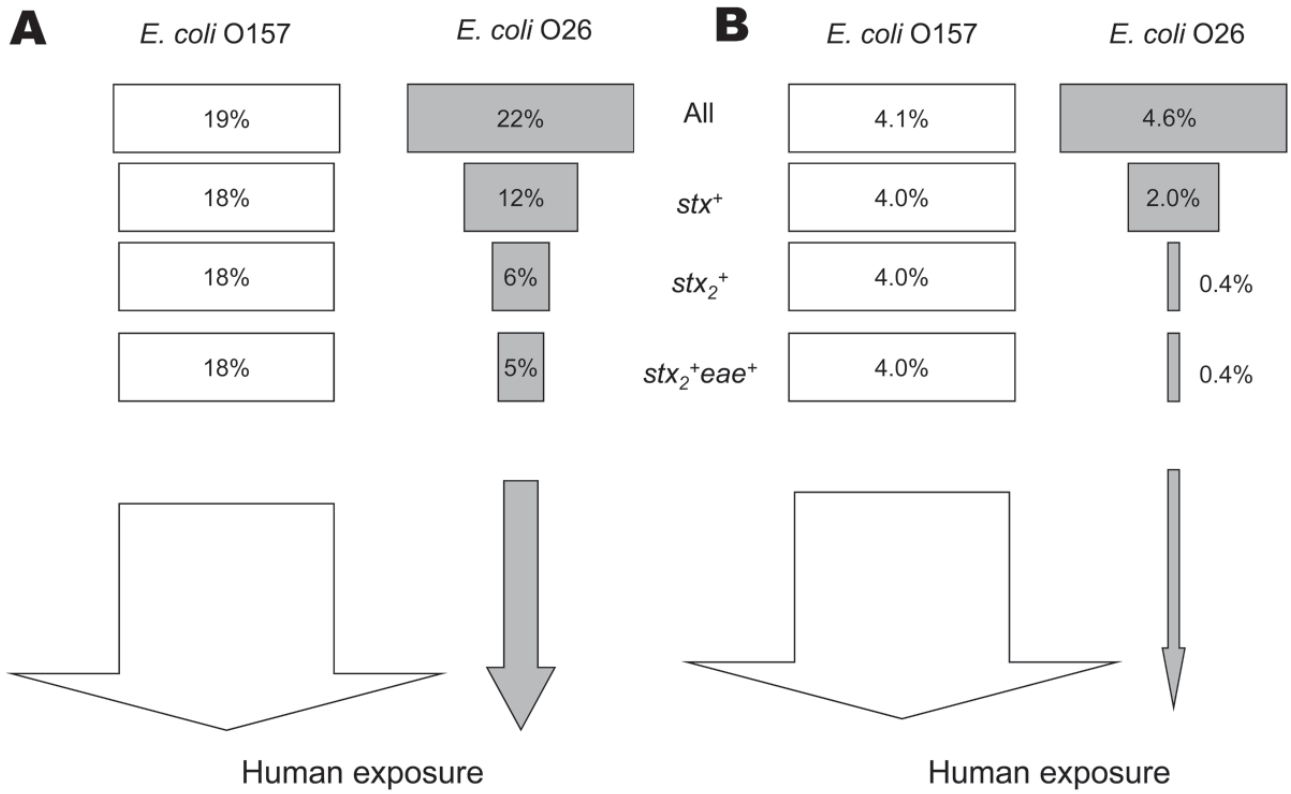

Figure 4. Schematic comparing farm-level (A) and animal-level (B) prevalences of *Escherichia coli* O157 and *E. coli* O26 in Scotland for different virulence levels. Analysis was done by using only the 338 farms sampled for both *E. coli* O157 and *E. coli* O26.

greater pathogenic potential for humans, and possession of stx_2 may alter the bovine immune response and lead to longer shedding and to larger quantities of the shed bacterium, resulting in greater exposure to humans. Changes in virulence characteristics of strains may also affect pathogen transmission in cattle (*38*) and thus pose a greater threat to humans from an increase in bacterial prevalence.

We suggest that limited human exposure in Scotland to *E. coli* O26 with virulence genes explains the recorded difference in the number of human infections attributed to *E. coli* O26 or *E. coli* O157. In addition, the more virulent forms of *E. coli* O26 (stx_2+*eae*+) in cattle in Scotland were clustered in the sparsely populated northeast region. However, exposure routes and exposure doses for *E. coli* O26 and serogroup O157 may differ, although no evidence confirms this alternative explanation for the human infection rate differences. Although cattle are hypothesized as a potential reservoir of *E. coli* O26 (*18*), little is known about the transmission routes to humans and the only evidence linking human infection with cattle is 2 HUS cases in Austria indirectly linked to consumption of unpasteurized milk (*20*).

The approach to laboratory diagnosis of non-O157 *E. coli* gastrointestinal infection in humans has changed in recent years. At the time of this study, testing of samples from humans for non-O157 *E. coli* was conducted by a small number of clinical diagnostic laboratories by using polyvalent antiserum. *E. coli* O26 isolates were forwarded to the SERL for virulence typing by PCR. SERL identified additional *E. coli* O26 isolates by testing feces from patients with suspected STEC infection but who had negative routine culture results. However, since 2006–2007, non-O157 STEC detection in submitted fecal samples has been conducted solely by SERL by using PCR followed by culture and characterization of STEC. Since 2006–2007, the reported incidence of *E. coli* O26 in Scotland has increased from 1.6% of all STEC identified at SERL to 6% in 2010–2011 (Figure 5). The virulence patterns of *E. coli* O26 strains isolated from humans in Scotland also appear to have altered. The stx_1+ *E. coli* O26 isolates previously predominated, but in 2010–2011, >50% of *E. coli* O26 isolates possessed both stx_1 and stx_2, and for the first time, 1 isolate from human feces was solely positive for stx_2.

In 2010, three particularly severe sporadic cases of HUS associated with *E. coli* O26 in children were reported in Scotland (*39*). The characteristics of 2 of the *E. coli* O26 strains included stx_1 and stx_2 (*39*). To our knowledge, this was the first time >1 *E. coli* O26–attributed HUS case

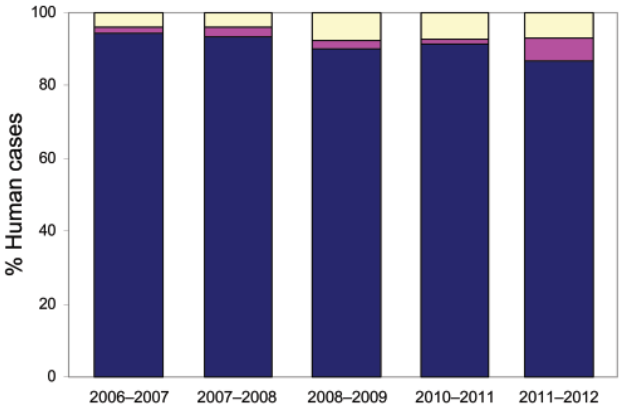

Figure 5. *Escherichia coli* O157 and Shiga toxin–producing non-O157 *E. coli* infection in humans in Scotland identified or confirmed by the Scottish *E. coli* O157/VTEC Reference Laboratory, Edinburgh, UK, by financial year (April–March). Samples include isolates, feces, and serum. Non-O157 isolates were serotyped by the Laboratory of Gastrointestinal Pathogens. Data are presented as percentage of all cases in humans that are *E. coli* O157 (blue), Shiga toxin-producing non-O157 (excluding *E. coli* O26) (yellow) and Shiga toxin–producing *E. coli* O26 (pink). The mean number of cases per financial year during 2006–2011 was 248 (221–275). This time frame was selected to ensure application of consistent method.

was reported in Scotland in a calendar year, reinforcing Bettelheim's (*40*) warning that we ignore these strains at our peril. Worldwide, reports of human outbreaks of non-O157 EHEC, including *E. coli* O26, are common (*2*). In addition, the recent outbreak *of E. coli* O104:H4 in Germany highlights the potential for Shiga toxin prophage acquisition into different *E. coli* genetic backgrounds with serious consequences for human health (*3*).

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Lineage-specific Virulence Determinants of *Haemophilus influenzae* Biogroup aegyptius

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An emergent clone of *Haemophilus influenzae* biogroup aegyptius (*Hae*) is responsible for outbreaks of Brazilian purpuric fever (BPF). First recorded in Brazil in 1984, the so-called BPF clone of *Hae* caused a fulminant disease that started with conjunctivitis but developed into septicemic shock; mortality rates were as high as 70%. To identify virulence determinants, we conducted a pan-genomic analysis. Sequencing of the genomes of the BPF clone strain F3031 and a noninvasive conjunctivitis strain, F3047, and comparison of these sequences with 5 other complete *H. influenzae* genomes showed that >77% of the F3031 genome is shared among all *H. influenzae* strains. Delineation of the *Hae* accessory genome enabled characterization of 163 predicted protein-coding genes; identified differences in established autotransporter adhesins; and revealed a suite of novel adhesins unique to *Hae*, including novel trimeric autotransporter adhesins and 4 new fimbrial operons. These novel adhesins might play a critical role in host–pathogen interactions.

For more than a century, *Haemophilus influenzae* biogroup aegyptius (*Hae*) has caused worldwide seasonal epidemics of acute, purulent conjunctivitis (*1*,*2*). In 1984, an entirely new syndrome, Brazilian purpuric fever (BPF), emerged in the town of Promissão, São Paulo State, Brazil. Caused by an emergent clone of *Hae*, the virulence of BPF in children was unprecedented and fatal. Invasive infection was preceded by purulent conjunctivitis that resolved before the onset of an acute bacteremic illness, which rapidly evolved into septic shock complicated by purpura fulminans (*3*). In the 11 years to 1995, several hundred cases of BPF were reported, of which all but 3 were in Brazil (*4*,*5*); overall mortality rate was 40%. Cases occurred sporadically and in outbreaks, mainly in small towns, although some were in the state capital, where an epidemic was feared because of crowding and deprivation. A collaborative task force by the Brazilian Health Authorities and the US Centers for Disease Control and Prevention was created to investigate this emergent infection and identified the cause as the BPF clone of *Hae* (*Hae*BPF) (*6*).

After 1995, no more cases were reported for more than a decade, although cases may have been missed, submerged in periodic surges of clinically indistinguishable hyperendemic or epidemic meningococcal disease. The potential of the disease to reappear with devastating effect is, however, underscored by the recent report of a suspected outbreak (7 cases, 5 fatal within 24 hours) in 2007 in the town of Anajás in the previously unaffected Brazilian Amazon region (*7*); thus, it cannot be assumed that this emergent infection has gone away.

The emergence of new pathogens causing human and animal diseases represents a constant threat. Distinguishing invasive strains from their noninvasive relatives is relevant for diagnosis, treatment, and prevention of the spread of emerging infectious diseases. *Hae*BPF constitutes a unique *H. influenzae* clade separate from the usual conjunctivitis-causing *Hae* strains (*8*); in experimental infections, it has caused sustained septicemia (*9*) and endothelial cytotoxicity (*10*).

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However, despite intensive research spanning 2 decades, these phenotypes remain unexplained. *Hae*BPF, a strain of nontypeable *H. influenzae* (NTHI), lacks genes encoding the polysaccharide capsule, a major virulence determinant of invasive *H. influenzae.* Although 1 animal study has indicated that a phase-variable lipopolysaccharide structure might play a part in the serum resistance of *Hae*BPF (*11*), in other respects, a novel lipopolysaccharide has not convincingly explained its virulence (*12*). With regard to adhesins, Farley et al. (*13*) identified duplication of fimbrial (*haf*) genes, with sequences differing from *H. influenzae* type b pilin (*hif*), but could find no systematic difference in binding of *Hae*BPF and conventional *Hae* strains to human epithelial cells and could not conclusively implicate this locus in virulence. Various other BPF-specific outer membrane proteins potentially involved in host–pathogen interactions have been identified, including a partially characterized hemagglutinin (*14*) and an ≈145-kDa phase-variable protein eliciting protective immunity (*15*), but none have been fully characterized, and their role in disease has not been established. *Hae*BPF (but not other *Hae* strains) has a copy of the *Haemophilus* insertion element IS*1016* (*16*), which has been implicated in acquisition of capsulation genes and other unspecified virulence factors in other *H. influenzae* strains (*17*,*18*), but its role has not been defined.

To better define the role of *Hae*BPF, we conducted a pan-genomic analysis. This comparison with 5 other complete *H. influenzae* genomes available in public databases has enabled delineation of the accessory genome for *Hae* and *Hae*BPF, characterizing all *Hae*-specific features that might contribute to the differences in the biology of this lineage of *H. influenzae*. This study goes beyond other *H. influenzae* pan-genome studies (*19*) by comparing only complete genomes and provides an absolute genomic comparison among the strains. Analysis of differences in genome content between the *Hae* strains and other *H. influenzae* revealed a plethora of novel adhesins that might play a critical role in host–pathogen interactions.

Materials and Methods

We first sequenced and annotated the genomes of the *Hae*BPF strain F3031 and a contemporaneous, non–BPF-associated conjunctivitis strain from Brazil, F3047. We compared strains F3031 and F3047 with *H. influenzae* strain Rd KW20, the type d capsule-deficient laboratory strain that was the first free-living organism to have its genome sequence determined; with *H. influenzae* strain 10810, a serotype b meningitis strain; with NTHI strains 86–028NP and R2846 (strain 12) (*20*), isolated from middle ear secretions from patients with otitis media; and with NTHI strain R2866, an unusually virulent NTHI strain isolated from a child with meningitis.

Bacterial Strains Sequenced

F3031 (GenBank accession no. FQ670178) is a BPF clone strain that is indistinguishable from other isolates by various typing systems, including multilocus sequence typing. F3047 (GenBank accession no. FQ670204) is a conjunctivitis isolate from Brazil that was established by typing to be unrelated to the BPF clone. F3031 and F3047 are described in more detail elsewhere (*21*).

Sequencing and Assembly

Bacterial genomes were sequenced at the Wellcome Trust Sanger Institute, Cambridge, UK. The first drafts of the F3031 and F3047 genomes were assembled from sequence to ≈7-fold coverage, from pOTWI2 and pMAQ1Sac_BstXI genomic shotgun libraries, by using BigDye Terminator chemistry on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). End sequences from large insert fosmid libraries in pCC1FOS (insert size 38–42 kb) were used as a scaffold for each strain. Further sequencing was performed on the Illumina Genome Analyzer (Illumina, Inc., San Diego, CA, USA). Assemblies were created and gaps and repeat regions were bridged by read pairs and end-sequenced PCR products.

Annotation and Analysis

Coding sequences were predicted by using Glimmer 3 (www.cbcb.umd.edu/software/glimmer). Automated annotation by similarity was done by searching the Glimmer 3 coding sequence set against the National Center for Biotechnology Information Clusters of Orthologous Groups database and the SwissProt dataset (www.uniprot.org). Annotation by similarity was done by importing the NTHI strain 86–028NP annotation and comparing it with the F3031 coding sequence set by using reciprocal FASTA (www.ebi.ac.uk/Tools/sss/fasta). Automated annotation was confirmed by manual curation with the Artemis genome visualization tool (*22*). Gene definitions and functional classes were added manually by using FASTA analyses of the primary automated comparisons. tRNA genes were predicted by using tRNAScan-SE version 1.2 (*23*). Identification of the rRNA operons was based on similarity to homologs in the NTHI strain 86–028NP genome.

Pan-Genome Comparison

Generation of pairwise comparisons of complete genome sequences was based on alignment of basepairs in MAUVE (*24*), which enabled alignment of whole genome sequences despite rearrangements. For each pairwise comparison of whole-genome sequences, the length of the alignment between the 2 strains was calculated and a distance matrix was created. The distance matrix, based on the lengths of the sequence alignments, was used to create a heat map showing the clustering of strains.

Phylogenetic Analysis

Evolutionary relationships between protein-coding sequences from different strains were inferred by using MEGA version 5.02 (*25*). Phylogenetic trees were constructed by using sequence alignments, and a neighbor-joining tree was built under a Poisson correction substitution model assuming uniform rates of substitutions among sites.

Results

The 7 Complete *H. influenzae* Genomes

Genome sizes ranged from 1.83 to 2.0 Mb (Table 1). The F3031 genome comprises 1,985,832 bp, is 8% larger than Rd KW20, and encodes 1,892 genes. The F3047 genome is larger (2,007,018 bp) and encodes 1,896 genes. All strains have a genome G+C content of 38%, typical of *H. influenzae*. *Hae*BPF strain F3031 contains an ≈24-MDa plasmid, previously sequenced and annotated (*26*), with average G+C content of 36.7%. This plasmid sequence has been excluded from analysis.

Whole-genome alignment of *Hae* strains F3031 and F3047 revealed substantial colinearity with 1 major rearrangement and 3 small inversions (Figure 1). Pairwise nucleotide alignments of the 7 sequences indicated a closer relatedness of the 2 *Hae* strains to each other than to the 5 other *H. influenzae* genomes (Figure 2). A core genome of 77% was shared across all 7 strains.

The *Hae* Accessory Genome

F3031 shares 10.6% of its genomic sequence with 1 other strain and 88% of this shared sequence (9.3% of total) with F3047, emphasizing the closer relatedness of these 2 strains to each other than to the other *H. influenzae* strains. A total of 163 predicted coding sequences lie within this *Hae*-specific DNA. A total of 99 (61%) coding sequences lie within regions of previously characterized *Haemophilus* bacteriophages, encoding proteins inferred by the similarity of their deduced sequences to be phage components associated with coexpressed genes transported by the phage (phage cargo). These proteins are are either homologs of conserved hypothetical proteins in other organisms or previously unidentified proteins of unknown function. Of all *Hae*-specific genes, >22% encoded homologs of

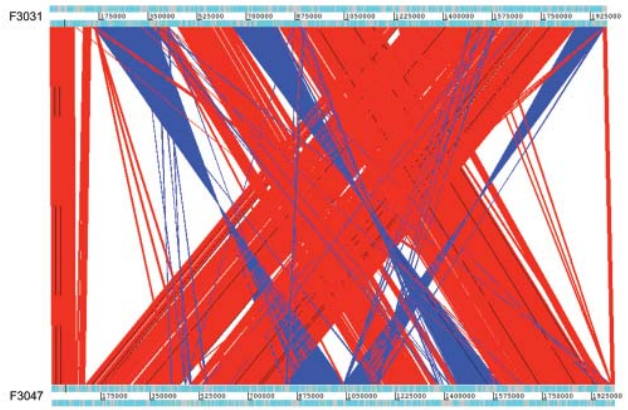

Figure 1. Comparison of the whole genome of Brazilian purpuric fever clone of *Haemophilus influenzae* biogroup aegyptius (*Hae*BPF) strain F3031 and *Hae* conjunctivitis strain F3047 with Artemis Comparison Tool (*22*). Red, syntenic regions; blue, inverted regions of the genome.

products identified elsewhere as being involved in host–pathogen interactions; prominent members were putative adhesins and invasins not previously found in strains of *H. influenzae* (Figure 3). Description of the *Hae* accessory genome will focus on these putative adhesins.

These new *Hae*-specific adhesins include 4 novel fimbrial operons, unique high-molecular-weight (HMW) proteins, and a 10-member family of trimeric autotransporter adhesins (TAAs). Many of these coding sequences are associated with simple sequence repeats (SSRs), indicating that phase variation may confer the potential for antigenic variation and immune response evasion during infection.

The presence of duplicated *hafABCDE* operons (*27*) was confirmed in the F3031 and F3047 genomes. We also identified 4 more *Hae*-specific fimbrial gene clusters, *aef1–aef4* (Figure 4). Clusters *aef1–aef3* were present in both strains, although not identical (55%–100% similarity on gene-by-gene comparison), but *aef4* was not present in *Hae*BPF F3031. Each *aef* operon encodes 4–6 proteins and has modest sequence identity to products of corresponding *haf* genes (38%–57%) and to F17 fimbrial adhesins (25%–64%) produced by pathogenic *Escherichia coli* associated with septicemic diarrheal diseases. Three clusters (*aef1*,

Table 1. *Haemophilus influenzae* strains included in pan-genome comparison*

Strain	Disease	Serotype	Genome size, Mb	G+C content, %	Identified CDSs	Sequencing location
F3031	Brazilian purpuric fever	Nontypeable	1.99	38.2	1,892	WTSI, Imperial College
F3047	Conjunctivitis	Nontypeable	2.0	38.2	1,896	WTSI, Imperial College
Rd KW20	Laboratory strain	d, capsule-deficient	1.83	38.1	1,743	JCVI
86–028NP	Otitis media	Nontypeable	1.91	38.2	1,821	Ohio State University
10810	Meningitis	b	1.98	38.0	1,896	WTSI, Oxford University
R2846	Otitis media	Nontypeable	1.98	37.0	1,691	University of Washington, SBRI
R2866	Meningitis	Nontypeable	1.89	38.0	1,817	University of Washington, SBRI

*CDSs, coding sequences; WTSI, Wellcome Trust Sanger Institute; JCVI, J. Craig Venter Institute; SBRI, Seattle Biomedical Research Institute.

Figure 2. Pair-wise comparisons of genome alignments between 7 *Haemophilus influenzae* strains. Each colored block represents the total number of bases shared between 2 *H. influenzae* genomes. Scale bar indicates percent relatedness.

aef3, *aef4*) are associated with mononucleotide SSRs of 10–17 nt located in the putative promoter region upstream of the *aefA* gene (Figure 4), conferring capacity for phase-variable expression through expansion and contraction of the SSR, altering efficiency of promoter binding.

The *Hae* genomes each encode a much richer repertoire of autotransporter adhesins than is found in other sequenced *Haemophilus* spp. Monomeric (classical) and novel trimeric autotransporter adhesins are present (MAA and TAA, respectively). Of the established *Haemophilus* autotransporter adhesins, the MAA Hap (*Haemophilus* adhesion and penetration protein), widely distributed in *H. influenzae* and proposed as a candidate NTHI vaccine antigen, is present as a pseudogene in F3031 and F3047, as previously reported by Kilian et al. (*28*). IgA1 protease, previously identified in the BPF clone, is also present in conjunctivitis strain F3047. Sequence alignment to other *H. influenzae* demonstrated that IgA1 from F3047 is more closely related to IgA1 from Rd KW20 (88% aa identity) than from F3031 (65% aa identity). Homologs of the HMW adhesins HMW1 and HMW2 (MAAs) and of the TAA *H. influenzae* adhesin Hia are present in F3031 and F3047. In contrast to the many NTHI strains for which substantial sequence information is available, where HMW1/HMW2 or Hia have almost always been alternatives, both are found in these *Hae* strains. HMW1 and HMW2, encoded at loci each consisting of 3 genes (*hmwABC*), were first identified in NTHI strain R2846 as HMW surface-exposed proteins, mediating attachment to human epithelial cells (*29*). More than 75% of NTHI encode HMW1 and HMW2, present at the same chromosomal locations in almost all HMW-containing NTHI isolates examined. *hmw1A* and *hmw2A* encode adhesins with different receptor binding specificities resulting from domains in variable regions comprising amino acid residues 114–237 of mature Hmw1A and 112–236 of mature Hmw2A (*30*). Despite conservation in binding specificity, *hmw1A* or *hmw2A* alleles from different isolates are highly polymorphic in the receptor binding domains (*30*). *Hae* Hmw1A– and Hmw2A–binding domain sequences (deduced from comparison with R2846 sequence) were aligned by using ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2) with those from homologs in other NTHIs, regardless whether they were Hmw1A or Hmw2A, and the alignment was used to construct a phylogenetic tree (Figure 5). The *Hae* HmwA–binding domains are distinct from those in other NTHIs, suggesting that in *Hae* these proteins have diverged separately from other NTHIs.

In *Hae* F3031 and F3047, the HMW clusters are not at the homologous NTHI chromosomal position; they are elsewhere, with a 22-kb bacteriophage insertion directly downstream of *hmw2ABC*. The *hmwA* alleles are further differentiated from those found in other NTHI strains by their associated SSRs. The putative promoter region upstream of the *hmw1A* and *hmw2A* homologs contains the octanucleotide repeat unit 5′-GCATCATC-3′; there are 14 and 15 copies, respectively, in F3031 and 13 and 12 copies, respectively, in F3047. This repeat pattern contrasts with all *hmwA* genes so far sequenced in different NTHI strains, in which 7 basepair SSRs of either 5′-ATCTTTC-3′ or 5′-TGAAAGA-3′ in varying copy numbers are located upstream of the genes (*31*,*32*).

The *Hae* accessory genome includes a 10-member gene family that encodes proteins with the sequence characteristics of TAAs (Table 2). These TAAs are distinct from the

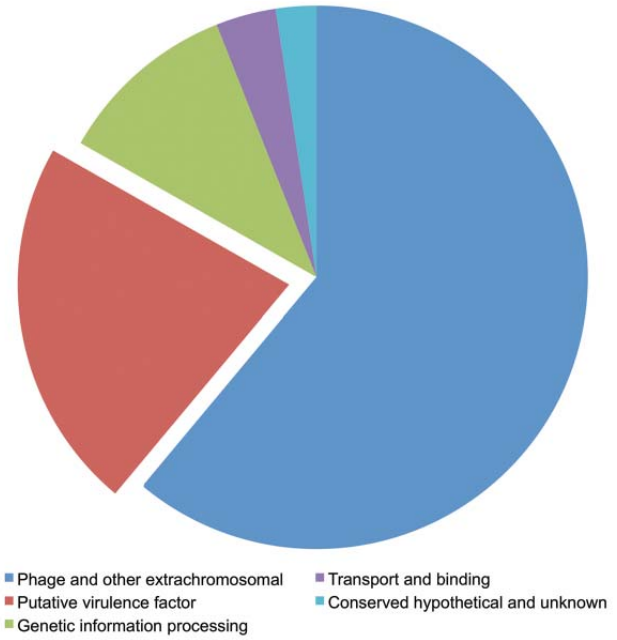

Figure 3. *Haemophilus influenzae* biogroup aegyptius (*Hae*)–specific features (163 coding sequences [CDSs]) determined from the pan-genome comparison. Putative virulence factors (red) accounted for ≈22% (13 CDSs) of all features identified.

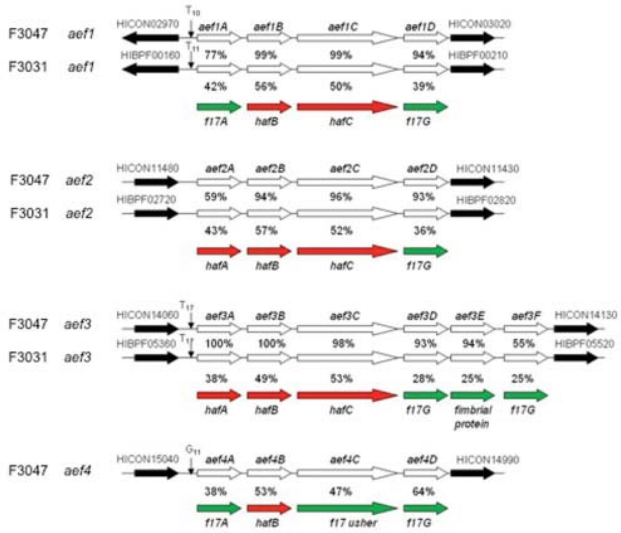

Figure 4. *aef* fimbrial operons in *Haemophilus influenzae* biogroup aegyptius strains F3047 (1–4) and F3031 (1–3). The *aef* fimbrial genes in each putative cluster are indicated by open arrows, and the flanking genes by solid arrows. The genes encode *aefA* (a fimbrial protein), *aefB* (a fimbrial chaperone), *aefC* (a fimbrial usher protein), *aefD* (a fimbrial adhesion), and *aef3E* and *aef3F* (additional fimbrial adhesins). Simple sequence repeats in the promoter region for each gene cluster are shown. Percent sequence identity between the aef genes from F3047 and F3031 is given between respective genes. Percent identity to closest homologue in Hae (red arrows) or other organisms (green arrows) is shown by features below each operon. BPF, Brazilian purpuric fever; CON, conjunctivitis.

Haemophilus TAAs Hsf (*33*), Hia (*34*), or the recently described Cha (*35*). In strains F3031 and F3047, a total of 8 genes (*1–6*,*8*,*9*) are present as homologs, termed *tabA* (for the *Hae*BPF trimeric autotransporter [bpf] alleles) or *tahA* (for the regular *hae* [conjunctivitis] alleles). *tahA7* has no homlog in *Hae*BPF. The tenth gene, *tabA10*, is the recently described adhesin/invasin gene *hadA* (*36*). This gene is found only in *Hae*BPF F3031; F3047 has no corresponding gene. Each gene appears to be locus specific, sharing the same flanking regions, but sequences differ substantially between homologs 1 and 2 in particular. *tabA4/tahA4*, *tabA5* and *tabA9/tahA9* seem to be pseudogenes, carrying frameshift mutations within the coding sequence. All TAAs except *tabA8/tahA8* and *tabA10* (*hadA*) are associated with SSRs located either within the coding sequence or upstream in the putative promoter region, indicating that expression may be modulated by phase variation.

All these TAAs share the characteristic 3-domain structure of N-terminal signal peptide and C-terminal outer membrane translocator domain, separated by an internal passenger domain. However, comparison of orthologous TAAs revealed striking differences between their passenger domains for TabA1/TahA1 and TabA2/TahA2, suggesting different functions of these proteins in the 2 strains (Figure 6). The passenger domains of these proteins vary in the number of binding domains (hemagglutinin and Hep_Hag domains) and in possession of different-sized, low-complexity spacer regions consisting of approximate heptapeptide repeats. TabA1 from F3031 contains 90 copies of tandemly duplicated AASSSAS with occasional T, N, or other substitutions in many copies; TahA1 from F3047 contains 48 copies of tandemly duplicated AETAKAG with occasional R, V, or other substitutions in many copies. In the prototypic TAA YadA, a series of 15-residue repeats appears to have such a spacer function between the protein head and its anchor in the outer membrane (*37*), holding any receptor-binding domains away from the bacterial cell surface.

In the context of the unusual virulence of the *Hae*BPF clone, the *tabA1* locus is particularly intriguing. Comparison with the *tahA1* locus indicates not only the substantial difference between the genes themselves, in the sequence encoding the putative stalk domain, but also (in F3031) an additional gene, HIBPF06250, encoding a conserved hypothetical protein, homologous to an uncharacterized gene product in the *Haemophilus* cryptic genospecies strain 1595 (*35*). In this strain, the gene (tandem duplicated) lies downstream of the TAA Cha. In F3031, HIBPF06250 is interposed between *tabA1* and IS*1016* (Figure 7), and the gene (like the insertion sequence) is absent in F3047. Association of IS*1016*, first described as the *Haemophilus* capsulation locus–associated insertion sequence, with unusual and invasive virulence of NTHI strains has been suggested elsewhere (*17*,*18*), although no specific gene association has been identified.

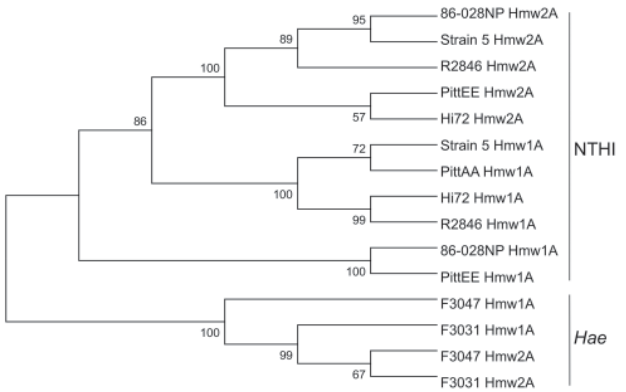

Figure 5. Phylogenetic relatedness of HmwA binding domain. Neighbor-joining tree based on the predicted binding domain of the HmwA adhesins from the indicated nontypeable *Haemophilus influenzae* (NTHI) strains, constructed by using MEGA5.02 (*25*). Bootstrap confidence values are shown at the branches, based on 1,000 replications. The population divides into 2 major clusters; HmwA alleles from nontypeable *H. influenzae* strains are clearly separated from *H. influenzae* biogroup aegyptius (*Hae*).

Table 2. New trimeric autotransporter adhesin proteins identified from genome sequences of *Haemophilus influenzae* biogroup aegyptius strains F3031 and F3047*

Name	Protein	Total length, aa	Molecular weight, kDa	G+C content, %	SSR, promoter CDS/CDS⁻
TabA1	HIBPF06240	1,490	140	47	TA (8) pr
TahA1	HICON14840	1,182	119	43	TA (5) pr
TabA2	HIBPF05270	2,185	211	47	G (13) CDS
TahA2	HICON14020	2,233	206	48	G (20) CDS
TabA3	HIBPF07130	464	41	38	CAAA (14) CDS⁻
TahA3	HICON16690	464	42	39	CAAA (12) CDS⁻
TabA4†	HIBPF07270	857	88	40	T (12) pr
TahA4†	HICON16820	759	77	41	T (10) pr
TabA5†	HIBPF10940	847	88	42	CAAA (15) pr
TahA5	HICON05410	1,016	106	41	CAAA (30) pr
TabA6	HIBPF01360	484	50	41	GCAA (16) CDS⁻
TahA6	HICON03690	484	50	41	GCAA (24) CDS
TahA7	HICON13720	905	95	39	GCAA (23) CDS⁻
TabA8	HIBPF01360	260	28	42	GCAA (3) CDS⁻
TahA8	HICON03690	282	30	43	GCAA (19) CDS⁻
TabA9†	HIBPF08080	232	25	36	NA
TahA9†	HICON17550	232	25	36	NA
TabA10 (HadA)	HIBPF19140	256	27	35	NA

*SSR, simple sequence repeats. Numbers in parentheses indicate the number of mono/di/tetranucluotide repeats found in each SSR; pr, SSR located within predicted promoter (pr) region upstream of the coding sequence (CDS); CDS, SSR located within the CDS; CDS⁻, SSR in CDS results in a frame-shift and an out-of-frame CDS; NA, not applicable.
†Pseudogene.

The *Hae*BPF-specific Accessory Genome

The part of the *Hae* accessory genome unique to *Hae*BPF amounted to 102,304 bases (5.2% of its genome). Ten *Hae*BPF-specific loci ranged in size from 370 to 20,002 bases and in G+C content from 27.9% to 44.5%. Deviation from the *Haemophilus* average of 38% suggests that these are more recently acquired regions. Much of this DNA is located within 5 bacteriophage domains, containing all 219 coding sequences (12 *Hae* specific, 11 *Hae*BPF specific) (Table 3) and including 1 (phage region 1) now termed HP3, similar in size and gene content to *Haemophilus* bacteriophage HP2, found in NTHI strains associated with unusual virulence (*38*). The *Hae*BPF-specific accessory genome comprises these and another 10 coding sequences (Table 4), which remained apparently BPF specific after BLASTP analysis of their deduced amino acid sequences against the nondegenerate public databases (October 2011), which include many more *Haemophilus* sequences from incomplete genome sequencing projects (*19*). The nearest matches to these sequences were mainly homologs in other pathogenic bacterial species that occupy the same ecologic niche. One gene (*hadA* at BPF-specific locus 10) has recently been characterized as encoding an epithelial adhesin/invasin plausibly contributing to *Hae*BPF virulence (*36*), but the function of the others, and any part their products may play in the serum resistance of the *Hae*BPF clone that endows it with pathogenic potential, remains to be established. Eleven genes appear to be phage cargo (Table 3); these are either homologs of conserved hypothetical proteins identified in other organisms or entirely unknown and might represent novel virulence factors. Four F3031-specific gene products do not have homologs in any other bacterial species and cannot be assigned a putative function. Novel genes have generally formed a much larger part of newly sequenced bacterial genomes, and identification of so few unknown genes in *Hae*BPF strain F3031 reflects the current availability of a large amount of *Haemophilus* sequence data, in particular from strains of NTHI.

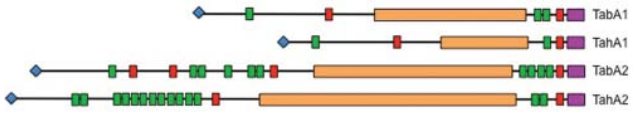

Figure 6. Domain organization of the *Haemophilus influenzae* biogroup aegyptius trimeric autotransporter adhesins TabA1/TahA1 and TabA2/TahA2, showing differences in passenger domain sequence motifs. Purple, C-terminal translocator domain; red, hemagglutinin domains; green, Hap_Hag domains; orange, degenerate repeats; blue, N terminal signal peptide.

Discussion

Although the unique virulence of the BPF clone of *Hae* might result from its acquisition of few (or even just 1) novel gene(s), our analysis indicates that sequence variation and variable gene expression through phase variation plausibly play a major role. Among the 21 *Hae*BPF-specific genes, just 1, *hadA* (*36*), is readily identifiable as a determinant of pathogenic behavior (virulence). This, however, is but 1 member of a new family of *Haemophilus* TAAs, which is unique to *Hae* but (except for *hadA*) shared among conjunctivitis isolates (12 diverse strains probed, unpub. data, the authors) and among members of the BPF clonal lineage (4 examples probed, unpub. data, the authors). Striking differences in sequences within the passenger domains of homologous TAAs indicate the possibility of differences in function, perhaps loss of epithelial

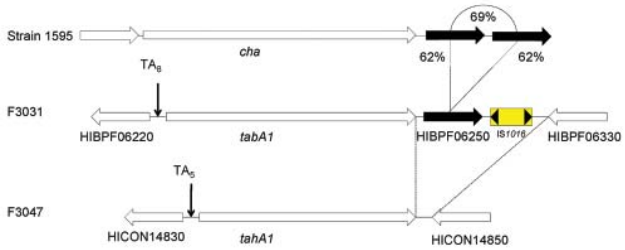

Figure 7. Comparison of the *cha* locus from *Haemophilus* cryptic genospecies strain 1595 to the TabA1 locus in the Brazilian purpuric fever (BPF) clone of *H. influenzae* biogroup aegyptius (*Hae*BPF) F3031 and the TahA1 locus in *H. influenzae* biogroup aegyptius (*Hae*) conjunctivitis (CON) F3047. Strain F3031 includes an additional 2 coding sequences downstream of *tabA1*, HIBPF06250 and IS*1016,* that are absent from strain F3047. HIBPF06250 is a conserved hypothetical protein with homology (62% aa identity) to the 2 coding sequences located directly downstream of *cha* that share 69% aa identity with each other.

localization through alteration of ≥1 of these adhesins in the *Hae*BPF clone. The abundance of other genes encoding putative adhesins, which differentiates *Hae* from other *H. influenzae*, underscores the early observation (*39*) that pilus and nonpilus factors mediate interactions of *Hae* with human cells in vitro. An understanding of expression of these multiple adherence factors will probably provide insights into *Hae* pathogenesis.

Comparison of complete, rather than draft or partially assembled, sequences leads to hypothesis-generating insights, which enable inferences as to possible gene function and clarification of phenotypic observations made before genomic information became available. For example, the pathogen-specific ≈145-kDa phase-variable protein identified by Rubin (*15*) can now be identified with some confidence as the intriguing TAA TabA1 (1 of few *Hae*BPF proteins predicted to be of this size and phase variable as a result of the SSR in the promoter region), enabling future investigations of its role in BPF virulence. The set of iron-regulated proteins identified experimentally by Smoot et al. (*40*) also should be identifiable by using a bioinformatic approach, greatly facilitating future study of this phenotype.

The next challenge is to experimentally test such hypotheses. Functional studies in *Hae*BPF have been hampered by the difficulty of genetically manipulating these strains, a difficulty that genomics does not explain. In silico analysis demonstrated that strains of *Hae* appear to encode all genes and regulatory sites needed for *H. influenzae* competence and transformation. Although small amino acid substitutions are found in most of the proteins when compared with homologs in readily transformable Rd KW20, not enough is known about their individual functions to enable prediction as to whether particular residue changes might affect function.

Our *H. influenzae* pan-genomic analysis demonstrated a close relationship between the *Hae*BPF strain F3031 and the conjunctivitis strain F3047. This finding contrasts with the remote relationship suggested by previous phylogenetic analyses (*8*). Analyzing complete genomes overcomes the limited discriminatory power of typing methods like multilocus sequence typing and, in this instance, supports the proposition that *Hae* strains are closely related and have a gene content that partially reflects their mucosal niche specificity.

The growing number of complete bacterial genomes provides increasing potential for comprehensive pan-genomic comparisons of related strains that vary in pathogenic potential. Such comparisons might reveal strain-specific features involved in virulence, which could lead to development of genotyping methods for tracking emerging pathogens and of new vaccines. Comparison of *Hae* with other strains of *H. influenzae* has detected novel candidate virulence determinants (the families of TAAs and fimbrial adhesions) that plausibly confer selective advantages in adapting to upper respiratory tract and conjunctival mucosae. It is tempting to speculate that alteration through mutation in the specificity of adhesins such as the TAAs might, as with *Hae*BPF, have created a maladaptive phenotype less firmly localized to the mucosal surface and able to invade the bloodstream. To investigate the role that the novel family of TAAs might play in host–pathogen interactions, we are conducting in vitro studies of gene function.

Table 3. Phage loci identified in genome of Brazilian purpuric fever clone of *Haemophilus influenzae* biogroup aegyptius strain F3031*

Phage region	Cluster start	Size, kb	G+C content, %	No. genes	No. *Hae*-specific (*Hae*BPF-specific) genes	Closest phage/gene product homologs
1	85,874	32	40.5	35	5 (5)	*Haemophilus* bacteriophage HP1, HP2, S2
2	325,263	47	41.2	60	0 (0)	Putative phage-related proteins from *H. influenzae, Neisseria meningitidis*
3	418,932	33	40.0	38	2 (2)	Mu-like phage from *H. influenzae*, *Mannheimia haemolytica*
4	857,914	54	39.5	60	5 (3)	Putative phage-related proteins from *H. influenzae, N. meningitidis*
5	1,240,967	30	40.6	26	0 (1)	Mu-like phage from *H. influenzae, H. somnus, H. parasuis*

**Hae, Haemophilus influenzae* biogroup aegyptius; BPF, Brazilian purpuric fever.

Table 4. Coding sequences specific to *Haemophilus influenzae* biogroup aegyptius strain F3031 at 10 loci*

Locus no. and F3031 ID	G+C, %	% Identity	Predicted product	Species harboring closest homologue
1, phage region 1†				
HIBPF00881	41.2	60	Conserved hypothetical protein	*Neisseria meningitidis*
HIBPF00900	37.6	68	Plasmid maintenance system killer	*Haemophilus parasuis*
HIBPF00910	40.2	82	Plasmid maintenance system antidote protein	*Neisseria gonorrhoeae*
HIBPF01110	40.2	70	Conserved hypothetical protein	*H. parasuis*
HIBPF01260	38.5	NA	Unknown protein, no known homologs	NA
2, phage region 3†				
HIBPF04833	40.1	NA	Unknown protein, no known homologs	NA
HIBPF04834	37.6	54	Conserved hypothetical protein	*H. parasuis*
3				
HIBPF05360	27.5	NA	Unknown protein, no known homologs	NA
4, phage region 4†				
HIBPF09220	41.2	55	Conserved hypothetical protein	*Haemophilus haemolyticus*
HIBPF09642	31.2	NA	Unknown protein, no known homologs	NA
HIBPF09722	31.9	80	Conserved hypothetical protein	*H. parasuis*
5, phage region 5†				
HIBPF13250	44.1	75	Conserved hypothetical protein	*H. parasuis*
6				
HIBPF16620	37.5	65	Adenine-specific methyltransferase (pseudo)	*Mannheimia haemolytica*
HIBPF16630	38.1	71	HNH endonuclease	*M. haemolytica*
7				
HIBPF17711	32.6	53	Conserved hypothetical protein	*Escherichia coli*
HIBPF17712	26.5	49	Conserved hypothetical protein	*N. meningitidis*
8				
HIBPF18000	28.1	52	DNA methyltransferase	*Macrococcus caseolyticus*
HIBPF18010	26.7	56	DNA methyltransferase	*M. caseolyticus*
HIBPF18040	30.3	44	Restriction endonuclease	*M. caseolyticus*
9				
HIBPF19140	35.3	100	HadA trimeric autotransporter adhesin	Previously identified in *Hae*BPF
HIBPF20030	36.1	77	Antibiotic biosynthesis monooxygenase	*Aggregatibacter aphrophilus*

*Putative product based on closest homologue in public databases, shown by percent amino acid identity. Percentage G+C content given for each coding sequence. ID, identification; NA, not applicable.
†Genes occur within regions of bacteriophage.

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Using Genotyping and Geospatial Scanning to Estimate Recent *Mycobacterium tuberculosis* Transmission, United States

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To determine the proportion of reported tuberculosis (TB) cases due to recent transmission in the United States, we conducted a cross-sectional study to examine culture-positive TB cases, with complete genotype results (spoligotyping and 12-locus mycobacterial interspersed repetitive unit–variable-number tandem repeat typing), reported during January 2005–December 2009. Recently transmitted cases were defined as cases with matching results reported within statistically significant geospatial zones (identified by a spatial span statistic within a sliding 3-year window). Approximately 1 in 4 TB cases reported in the United States may be attributed to recent transmission. Groups at greatest risk for recent transmission appear to be men, persons born in the United States, members of a minority race or ethnic group, persons who abuse substances, and the homeless. Understanding transmission dynamics and establishing strategies for rapidly detecting recent transmission among these populations are essential for TB elimination in the United States.

Molecular characterization of *Mycobacterium tuberculosis* complex has been available for >2 decades in the United States. As a tool to enhance programmatic activities, tuberculosis (TB) genotyping is a useful adjunct to epidemiologic field investigations by defining outbreaks (*1*,*2*), discerning episodes of reactivation and relapse (*3*,*4*), confirming suspected laboratory contamination (*5*,*6*), and evaluating and monitoring TB control program performance (*7*). TB genotyping results, when combined with epidemiologic data, help identify persons with TB disease who are involved in the same chain of recent transmission (*8*). Previous analytic studies have used TB genotyping data in conjunction with epidemiologic data to assess correlates of recent TB transmission within localized populations (*9*–*15*). A basic assumption of this approach is that recent TB transmission is localized in place and time, that is, progression to TB disease from an infection acquired within the past few years and in the same jurisdiction.

Population-based molecular epidemiologic studies are often subject to several biases and methodologic limitations that impede the ability of investigators to make valid statements about recent TB transmission events in the absence of direct data regarding interpersonal contacts (*16*). Estimating recent TB transmission is often limited by abbreviated study periods, convenience isolate sampling, and ambiguous geographic boundaries defined for jurisdictional or geopolitical reasons (*17*,*18*). TB transmission is not likely to be bound by these artifacts, however. Spatial scanning to detect disease clusters has been successfully applied in multiple settings and for various diseases (*19*). Using this method in a multiyear, nationally representative database of both genotype and routinely collected TB surveillance data may offer a better solution for accurately defining recent TB transmission.

In 2004, the US Centers for Disease Control and Prevention (CDC) offered universal access to TB genotyping through the National Tuberculosis Genotyping Service (NTGS) to routinely characterize at least 1 *M. tuberculosis* complex isolate from every TB case-patient in the United States (*20*). Although the intent of this system is to support local TB programs for public health action,

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data collected from this system offer a unique opportunity to explore and describe the molecular epidemiology of TB and establish comprehensive molecular TB surveillance in the United States. In this analysis, our goals were to estimate the proportion of TB in the United States attributable to recent transmission and to assess clinical, demographic, and epidemiologic factors associated with recent TB transmission.

Methods

Study Population

This study includes verified cases of TB reported to the US National Tuberculosis Surveillance System (NTSS) by the 50 states and the District of Columbia. Clinical, demographic, and epidemiologic variables for each case-patient are collected for surveillance purposes and are described elsewhere (*21*). *M. tuberculosis* complex isolates were characterized by using a standardized protocol for spacer oligonucleotide typing (spoligotyping) and 12-locus mycobacterial interspersed repetitive unit–variable-number tandem repeats (MIRU-VNTRs) (*22*). NTGS results for each submitted isolate were linked to NTSS case records by state and local TB control programs; a standardized case identification number and a unique laboratory accession number were used to form discrete individual isolate-case records (*20*). When multiple isolates were genotyped for the same person in the same surveillance year, case-patients with discordant genotyping results were excluded from analysis for clustering assignment and risk factor analysis. The final study population included all persons with verified culture-positive TB cases reported during January 2005–December 2009 with a complete spoligotype and 12-locus MIRU-VNTR result.

Four major phylogenetic lineages for *M. tuberculosis*, along with speciation of *M. africanum* and *M. bovis,* were identified by using spoligotyping motifs that referred to an international standard (*23*). Substance abuse was defined by using previously published methods (*24*). Persons with TB who received a positive HIV test result at the time of TB diagnosis were classified as TB/HIV case-patients. Persons with TB and negative HIV results or unknown HIV status were classified as having non-HIV TB.

Genotype and Geospatial Clustering

Genotype clusters were defined as cases with matching spoligotype and 12-locus MIRU-VNTR results (i.e., exact match on all loci) reported within statistically significant geospatial zones determined by a spatial scan statistic (*25*). SaTScan version 9.1.0 (*26*) was employed to identify geographic areas with a larger-than-expected rate of discrete genotype clustering, and all other culture-positive TB cases counted during the study were considered as the background rate. In brief, all cases were aggregated by genotype according to residential ZIP code where they were reported. Each genotype was then scanned separately, applying a purely spatial analysis, in which the number of events in an area was assumed to be Poisson-distributed to generate circular zones of various sizes up to a maximum radius of 50 km. An evaluation of outbreak investigations conducted by CDC demonstrated no difference in cluster membership when 50-km and 100-km SaTScan search radii were used to identify known epidemiologically linked genotype cases (CDC, unpub. data).

A log-likelihood ratio was calculated for each zone in comparison with all possible zones, with the maximum likelihood ratio representing the zone most likely to identify spatial clustering for each genotype. A Monte Carlo simulation with 999 repetitions was used to determine the distribution of the scan statistic under the null hypothesis of spatial randomness; significant spatial clusters were chosen at an α of $p<0.05$. Three scans comprised of 3-year overlapping intervals (scan A, 2005–2007; scan B, 2006–2008; scan C, 2007–2009) were performed to identify spatial clusters occurring within a 3-year period. If cases were identified as a member of a statistically significant spatial cluster in any of the 3 periods, they were considered clustered. No duplicative case counting occurred. The purpose of this spatial scan was to characterize each case for a dichotomous outcome: clustered or not clustered. Cases that were both genotypically and spatially clustered were considered recent TB transmission for the purposes of this study. All cases that were not genotypically and spatially clustered were considered reactivation of remotely acquired TB infection, or reactivation TB. For comparative purposes, national-, state- and county-level clustering definitions were created. National-level clustering was defined as $\geq$2 culture-positive cases with identical genotypes reported anywhere in the United States during 2005–2009. State-level clustering was defined as $\geq$2 culture-positive cases with identical genotypes reported from the same state during 2005–2009. County-level clustering was defined as $\geq$2 culture-positive cases with identical genotypes reported from same county during 2005–2009.

Statistical Analyses

A predictive logistic regression model was used to determine potential associations between clinical (e.g., sputum-smear status, known HIV positivity, site of disease and cavitation on chest radiograph, and previous TB diagnosis) and demographic and risk characteristic variables (e.g., race/ethnicity, age, country of birth, homelessness, substance abuse, incarceration at time of diagnosis, and residence at long-term care facility at diagnosis) and the outcome of interest: geospatial and genotype clustering as a proxy for recent TB transmission. Univariate analysis of

the categorical independent variables was done by using Pearson χ^2. Any variable with a significance value of ≤0.20 was included in a best subset, multivariate logistic regression model. We built our final model using backward elimination of nonsignificant independent variables (p>0.01). The log-likelihood ratio was used to assess the overall significance of the final models, and the Hosmer-Lemeshow statistic was used to evaluate the fit of each of the final models. To test the hypothesis that factors associated with recent TB transmission events varied by geographic region of the United States, an additional 4 independent models were created following the same process but subset to western, midwestern, northeastern, and southern states, respectively (*27*).

Results

TB Case Population

During 2005–2009, a total 65,529 verified cases of TB were reported to CDC. Of these, 51,015 (77.9%) were culture-positive (Figure 1). During this period, the overall incidence of TB in the United States declined from 4.8 to 3.8 per 100,000 persons, representing a decline of 20.1% in the overall case count (*21*).

TB Isolates and Genotype Clusters

During 2005–2009, a total of 45,188 isolates were submitted to NTGS for molecular characterization; 39,474 (87.4%) were successfully matched to a case-patient with reported TB. Two hundred seventy isolates (0.7%) had incomplete results on spoligotype, MIRU-VNTR, or both; 344 case-patients (0.9%) had multiple isolates with discordant genotyping results and were excluded from the analysis. The total number of genotyped TB cases available for analysis was 36,860, representing 72.3% of all reported culture-positive cases. The proportion of reported case-patients for whom complete genotype results were available increased over time, with 6,863 (62.7%) of 10,953 in 2005 and 7,845 (88.4%) of 8,876 in 2009. The number of individual genotype strains (i.e., distinct spoligotype and 12-locus MIRU-VNTR combinations) identified over the study period was 11,722. The proportion of new strains identified per year gradually decreased over time. In 2006, 40.7% of strains identified were new; this percentage was reduced to 14.2% in 2009 (data not shown).

Of the 36,860 cases for which genotyping had been performed, 8,499 (23.1%) were considered clustered by both genotype and spatial concentration and therefore were thought to be members of a putative recent TB transmission event. The average number of spatially concentrated genotype clusters identified per 3-year scanning period was 1,039 (range 970–1,128). Nationally, the overall mean cluster size was 5.7 members (range 2–173 members) (Figure 2). The median cluster size was 3 members, and almost half (46.1%) of the clusters had only 2 members. Other clustering definitions that use geopolitical boundaries had higher average clustering percentages when the same 3-year window periods were used (national-level, 77.3%; state-level, 57.1%; county-level, 38.7%) (Figure 1).

Characteristics of Members with Putative Recent TB Transmission

Cluster members were more likely to be male (66.3% vs. 61.7%), to have been born in the United States (57.4% versus 34.4%), to abuse substances (28.4% versus 14.8%), and to have a history of homelessness (11.1% versus 5.0%) than those thought to have reactivation TB (Table 1). The proportion of cluster members also varied by race compared with those with cases due to reactivation TB: Asian, non-Hispanic (17.6% vs. 29.8%); black, non-Hispanic (39.7% versus 21.6%); Hispanic (25.5% versus 28.5%); and white, non-Hispanic (15.0% versus 17.9%).

Cluster members with recent TB transmission events were also more likely to have reported HIV-positive results (8.7% versus 5.5%), pulmonary disease exclusively (78.4% versus 72.2%), and positive sputum smear results (61.5% versus 55.3%) and to have had a cavitary chest radiograph at time of diagnosis (36.8% versus 32.2%) than those thought to have reactivation TB. Of the 8,499 persons with cases believed to be caused by recent TB transmission, only 2.1% and 4.4% resided in a long-term care or correctional facility at the time of diagnosis, respectively.

Genotype Lineage and Recent TB Transmission Events

The proportions of isolates in each phylogenetic lineage were as follows: Euro-American, 64.2%; Indo-

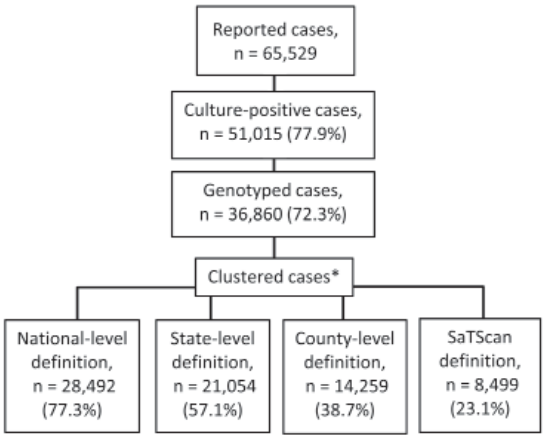

Figure 1. Number of reported cases of tuberculosis, including culture-positive cases, genotyped cases, and genotype clusters, Unites States, 2005–2009. *Indicates ≥2 cases with *Mycobacterium tuberculosis* isolates with identical spoligotype and 12-locus mycobacterial interspersed repetitive unit–variable-number tandem repeat analysis results.

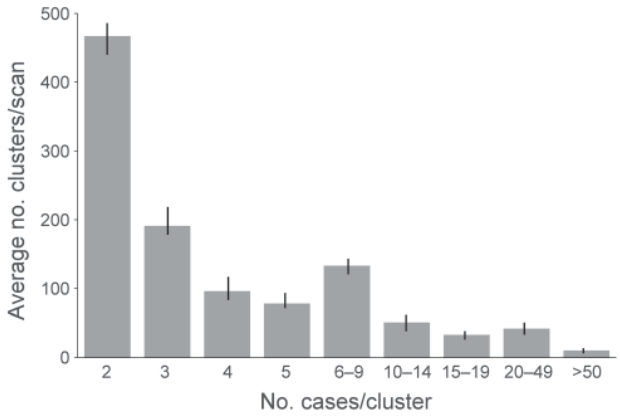

Figure 2. Frequency of genotype clusters of tuberculosis, by cluster size (mean 5.68, median 3, range: 2–173), United States, 2005–2009. Frequency was determined by using SaTScan version 9.1.0 (*26*) on the basis of 3 consecutive, overlapping years: scan A, 2005–2007 (n = 970); scan B, 2006–2008 (n = 1,019); scan C, 2007–2009 (n = 1,128). Error bars indicate upper and lower limits of clusters identified between scan periods.

Oceanic, 15.4%; East-Asian, 13.5%; East-African/Indian, 4.3%. *M. bovis* isolates accounted for 1.6% of reported cases of TB. Seventy-two percent of reported case-patients with *M. bovis* isolates were non–US-born. *M. africanum* isolates were identified among 179 patients (0.5%), with 88.6% non–US-born. Among members with recent TB transmission events, 69.2% had TB isolates with Euro-American lineage; 14.9% had isolates with East-Asian lineage, 11.8% had isolates with Indo-Oceanic lineage, 2.3% had isolates of East-African/Indian lineage, 1.5% had *M. bovis* isolates, and 0.3% had *M. africanum* isolates.

Factors Associated with Putative Recent TB Transmission Events

In our final adjusted model, the following odds ratios were noted for variables significantly associated with a higher odds of having a case attributed to putative recent TB transmission (Table 1): age (0–4 years of age: adjusted odds ratio [aOR] 3.1, 99% CI 1.4–6.8); black, non-Hispanic (aOR 2.4, 99% CI 2.2–2.7); Hispanic (aOR 1.7, 99% CI 1.5–2.0); Native Hawaiian/Pacific Islander (aOR 2.6, 99% CI 1.5–4.4); US-born (aOR 2.4, 99% CI 2.1–2.7); homeless persons (aOR 1.4, 99% CI 1.2–1.6); persons who abuse substances (aOR 1.4, 99% CI 1.3–1.7); East-Asian lineage (aOR 1.9, 99% CI 1.5–2.6); and Indo-Oceanic lineage (aOR 1.7, 99% CI 1.3–2.3).

Geographic Variation Associated with Recent TB Transmission

Best-fit models to predict those with recent TB transmission were conducted for each of the 4 US geographic regions. Many of the main effects associated with recent TB transmission remained constant (US-born, substance abuse, homeless), although factors varied in both magnitude and risk factor across the United States (Table 2).

Ethnic disparities for recent TB transmission were found among black, non-Hispanic persons living in midwestern and southern states (aOR 2.1, 99% CI 1.7–2.6; aOR 3.6, 99% CI 1.5–8.6), whereas Hispanic persons had the highest odds among those living in northeastern (aOR 2.3, 99% CI 1.7–8.8) and western states (aOR 2.1, 99% CI 1.5–3.0).

Phylogenetic lineage also varied among the different regions. Euro-American lineage (aOR 2.2, 99%,CI 1.1–4.3) had the strongest association for recent transmission in the south, whereas the East-Asian lineage was most strongly associated with recent transmission in western (aOR 2.4, 99% CI 1.4–4.2) and northeastern states (aOR 2.0, 99% CI 1.1–3.6).

Discussion

According to these findings, ≈1 in 4 TB cases reported in the United States may be attributed to recent TB transmission; this increases to 1 in 3 among US-born persons (Table 1). Our approach to identifying the proportion of reported TB attributable to recent transmission is based on the concept that epidemiologically related organisms share indistinguishable genotypes, whereas unrelated organisms differ at some genetic loci (*8*). TB cases that occur in spatial clusters and share indistinguishable genotypes are thought to be caused by recently transmitted TB infection; those with nonclustered genotypes are thought to result from progression from an infection acquired >3 years in the past. In the absence of detailed data about interpersonal contact between persons, relying on genotype and on place and time data routinely collected during surveillance activities becomes imperative to assessing recent transmission at a national level. This goal was achieved by using the established infrastructure of NTSS and TB genotyping, universally accessible to TB programs through NTGS, to capture 72% of all cases with culture-positive results over a 5-year period.

Spatial scanning provides a new insight into TB transmission that is independent of jurisdictional or geopolitical boundaries. This nationally representative study incorporated spatial concentration as a core element for defining recent TB transmission. Previous studies were limited to clustering definitions confined to a single jurisdiction (*9–11*,*14*,*15*), state, or province (*28*,*29*), or incomplete sampling of an entire nation (*13*,*30*). The proportion of cases representing recent TB transmission varied considerably by cluster definitions based on geopolitical borders. If a national clustering definition was used, up to 80% of culture-positive cases would be attributed to recent TB transmission. If a state-based definition or county-based definition was used, up to 57% and 39% of culture-positive cases, respectively, would be attributed to

Table 1. Demographic, behavioral, and clinical characteristics of persons involved in putative recent TB transmission events, United States, 2005–2009*

Variable	No. (%) clustered†	Total no. available	Crude OR (99% CI)	Adjusted OR (99% CI)‡
Sex, n = 36,852				
M	5,634 (66.3)	23,136	**1.2 (1.1–1.3)**	1.1 (1.0–1.2)
F	2,862 (33.7)	13,716		
Age group, y, n = 36,860				
0–4	206 (2.4)	395	**3.5 (2.7–4.6)**	**3.1 (1.4–6.8)**
5–14	99 (1.2)	333	**1.4 (1.0–1.9)**	1.2 (0.8–1.9)
15–24	1,131 (13.3)	4,488	1.1 (1.0–1.2)	1.1 (1.0–1.3)
25–44	2,975 (35.0)	12,688	Referent	
45–64	3,025 (35.6)	11,247	**1.0 (1.1–1.3)**	1.0 (0.9–1.0)
≥65	1,063 (12.5)	7,709	**0.5 (0.5–0.6)**	**0.5 (0.4–0.6)**
Race/ethnicity, n = 36,761				
American Indian/Alaska Native	111 (1.3)	498	**1.4 (0.9–1.5)**	0.8 (0.6–1.2)
Asian, non-Hispanic	1,496 (17.6)	9,922	**0.7 (0.6–0.8)**	**1.5 (1.3–1.8)**
Black, non-Hispanic	3,368 (39.7)	9,471	**2.2 (2.0–2.4)**	**2.4 (2.2–2.7)**
Hispanic/Latino	2,165 (25.5)	10,238	1.1 (1.0–1.2)	**1.7 (1.5–2.0)**
Native Hawaiian/Pacific Islander	59 (0.7)	190	**1.8 (1.2–2.7)**	**2.6 (1.5–4.4)**
White, non-Hispanic	1,272 (15.0)	6,335	Referent	
Other	16 (0.2)	107	0.7 (0.3–1.4)	0.9 (0.4–2.2)
Country of birth, n = 36,745				
United States	4,871 (57.4)	14,594	**2.6 (2.4–2.7)**	**2.4 (2.1–2.7)**
Non–US-born	3,611 (42.6)	22,151		
In US <2 y	1,100 (30.5)	7,675	Referent	§
In US 2–5 y	531 (14.7)	3,326	1.1 (1.0–1.3)	
In US >5 y	1,980 (54.8)	11,150	**1.3 (1.2–1.4)**	
Homelessness within past 12 mo, n = 36,558				
Yes	946 (11.2)	2,361	**2.4 (2.1–2.7)**	**1.4 (1.2–1.6)**
No	7,488 (88.8)	34,197		
Substance abuse within past 12 mo, n = 36,860				
Yes	2,415 (28.4)	6,623	**2.3 (2.1–2.5)**	**1.4 (1.3–1.7)**
No	6,082 (71.6)	30,237		
Residence at correctional facility at time of diagnosis, n = 36,815				
Yes	369 (04.3)	1,400	1.2 (1.0–1.4)	0.8 (0.7–1.0)
No	8,121 (95.7)	35,415		
Residence at a long-term care facility at time of diagnosis, n = 36,801				
Yes	180 (2.1)	800	1.0 (0.8–1.2)	NS
No	8,304 (97.9)	36,001		
Reported HIV status, n = 36,860				
Positive	741 (8.7)	2,290	**1.7 (1.5–1.9)**	1.1 (1.0–1.3)
Not positive	7,756 (91.3)	34,570		
Clinical presentation of TB, n = 36,779				
Pulmonary only	6,653 (78.4)	27,083	**1.6 (1.5–1.8)**	1.2 (1.0–1.4)
Extrapulmonary only	992 (11.7)	5,973	Referent	
Pulmonary and extrapulmonary	841 (09.9)	3,723	**1.5 (1.3–1.7)**	1.0 (0.8–1.3)
Sputum smear positivity, n = 31,625				
Yes	4,640 (61.5)	17,934	**1.3 (1.2, 1.4)**	1.1 (1.0–1.2)
No	2,903 (38.5)	13,691		
Cavitary chest radiograph, n = 31,382				
Yes	2,782 (36.8)	10,411	**1.2 (1.2–1.3)**	1.0 (0.9–1.1)
No	4,771 (63.2)	20,971		
Previous TB diagnosis, n = 36,544				
Yes	426 (5.0)	1,680	1.1 (1.0–1.3)	NS
No	8,010 (95.0)	34,864		
Mycobacterium tuberculosis or other species spoligotype-based lineage, n = 36,458				
East African Indian	197 (2.3)	1,582	Referent	
East Asian	1,259 (14.9)	4,924	**2.4 (1.9–3.0)**	**1.9 (1.5–2.6)**
Euro-American	5,857 (69.2)	23,441	**2.3 (1.9–2.8)**	**1.5 (1.2–2.0)**
Indo-Oceanic	1,000 (11.8)	5,760	**1.5 (1.2–1.8)**	**1.7 (1.3–2.3)**
M. africanum	25 (0.3)	179	1.1 (0.6–2.0)	0.8 (0.4–1.7)
M. bovis	127 (1.5)	572	**2.0 (1.4–2.8)**	**2.0 (1.3–3.2)**

*n values indicate number of persons for whom information in category was available. TB, tuberculosis; OR, odds ratio; NS, not significant. **Boldface** indicates significance at $\alpha = 0.01$.
†Genotype clustering: ≥2 cases with identical spoligotype and 12-loci mycobacterial interspersed repetitive unit–variable-number tandem repeat genotypes occurring within a geospatially concentrated area identified by SaTScan (*20*). Cases must have clustered at least once in 1 of 3 consecutive, overlapping years (scan A, 2005–2007; scan B, 2006–2008; scan C, 2007–2009) (scan windows spatially limited to 50 km).
‡Final model, n = 31,382.
§Not included in the model to include both US-born and non–US-born case-patients.

recent TB transmission. Although which definition most accurately represents recent TB transmission is unclear, a clustering definition based on geospatial concentration appears to be the most conservative and is not subject to the potential misclassification of political boundaries. The limitation of using these boundaries can be best exemplified by known inter-jurisdictional TB outbreaks that crossed geopolitical borders (*31*). Because the proportion of recent TB transmission may be a reflection of the success of control measures, accurately assessing this quantity is of considerable public health importance.

Estimating recent TB transmission also depends on the duration of the study period (*16*). Other studies have shown increasing clustering proportions as the duration of the study increases, with a plateau effect after 3 years (*12*,*13*,*17*,*32*,*33*). The annual proportion of isolates with a new strain identified in the United States during this study period did plateau (data not shown), suggesting a similar phenomenon and potential influencing factor in the long-term estimation of TB genotype clustering nationwide. Using consecutive, overlapping scanning windows that incorporate 3-year intervals maximizes the probability that spatial and temporal clustering represent localized, recent TB transmission within this large and comprehensive dataset. As NTGS continues to mature and grow over time, adjusting for temporal clustering will become essential when estimating recent TB transmission.

Consistent with other published reports from countries with a low incidence of TB, the characteristics of local birth, male sex, minority race, substance abuse, and homelessness were associated with recent TB transmission (*17*,*18*,*33*). These findings highlight the fact that TB may be harder to eliminate among populations characterized by these factors (*34*). The large proportion of cases attributable to recent TB transmission among minorities, persons who abuse substances, and those who are homeless suggests that limited access to routine health screenings, resulting in delayed diagnoses, may extend infectious periods and rates of TB transmission. Indeed, TB patients who use illicit substances and abuse alcohol have been found to be more contagious (*24*).

In low-incidence, high-resource countries, efforts to control recent TB transmission are based largely on contact investigation, yet for many reasons, contact investigations may not be sufficiently intensive or comprehensive, even in successful TB control programs (*35*). Every case of TB began when a person came into contact with a person with contagious TB. Therefore, it follows that clusters of case-patients representing recent TB transmission could be averted through improved contact investigation efforts. Contact investigations are multistep processes in which exposed contacts are systematically evaluated on the basis of the amount of time spent with an infectious person, the environmental conditions of exposure venue, and the contact's intrinsic predisposition for infection or disease (*36*). Numerous studies have demonstrated that eliciting names of contacts is neither optimally effective nor sufficient to interrupt TB transmission among high-risk groups, such as the homeless and persons who abuse substances (*1*,*24*,*37*,*38*). The potential for uninterrupted TB transmission is further exacerbated by the poor yield of name-based contact investigations among these

Table 2. Demographic, behavioral, and clinical characteristics of persons involved in putative recent TB transmission events, by location, United States, 2005–2009*

US Census region, no. (%) persons,† and main characteristics	Odds ratio (99% CI)	Wald p value
West, 11,550 (31.3)		
Mycobacterium bovis TB	4.4 (2.2–8.8)	<0.0001
US-born	2.4 (4.3–4.7)	<0.0001
East-Asian phylogenetic lineage TB	2.4 (1.4–4.2)	<0.0001
Hispanic	2.3 (1.7–3.0)	<0.0001
Homeless‡	1.9 (1.5–2.9)	<0.0001
Midwest, 10,502 (28.5)		
US-born	2.5 (2.1–3.1)	<0.0001
Black, non-Hispanic	2.1 (1.7–2.6)	<0.0001
Substance abuser§	1.4 (1.3–1.6)	<0.0001
Northeast, 6,090 (16.5)		
Hispanic	2.1 (1.5–3.0)	<0.0001
East-Asian phylogenetic lineage TB	2.0 (1.1–3.6)	0.001
US-born	1.6 (1.2–2.1)	<0.0001
Substance abuser§	1.6 (1.2–2.1)	<0.0001
South 8,718 (23.7)		
Black, non-Hispanic	3.6 (1.5–8.6)	<0.0001
US-born	3.2 (2.5–4.2)	<0.0001
Euro-American phylogenetic lineage TB	2.2 (1.1, 4.3)	0.004
Substance abuser§	1.5 (1.1–4.3)	<0.0001
Total, 36,860		

*TB, tuberculosis.
†Column percentage.
‡Self-reported homelessness within 12 mo preceding TB diagnosis.
§Self-reported excess alcohol consumption, injection drug use, or noninjection drug use within 12 mo preceding TB diagnosis.

populations. Locations are as important as named contacts when investigating recent transmission. A recent study found that 81% of case-patients involved in a multiyear TB outbreak lived in close geographic proximity (*38*). Spatial scanning methods may assist with identification of specific clusters representing ongoing transmission that could benefit from targeted location-based interventions. Using spatial scanning methods to determine locations with high concentrations of both spatial and genotype clustering may be an effective way to prioritize resources to intervene in populations with high rates of TB transmission.

This study does have limitations. First, isolate submission for TB genotyping is not universal; thus, the database, although large, did not contain all reported case-patients with culture-positive TB during the study period. Clinical, demographic, and epidemiologic characteristics of patients without TB genotyping data did not differ statistically from those with TB genotyping data (data not shown). Second, spatial and genotype clustering serves only as a proxy for recent TB transmission in the absence of detailed data on interpersonal connections between case-patients. Because of dynamic migration patterns within the United States, these methods may fail to ascertain cases that are due to recent transmission when a putative source case-patient moves or if exposure occurred outside the range of spatial scanning. Increased global migration has influenced the epidemiology of TB in the United States as well. Recent immigrants who became infected with a particular genotype elsewhere may resettle in the same neighborhood and, when TB develops after resettlement, it may falsely be considered recent TB transmission. Third, although spoligotyping and 12-locus MIRU-VNTR have good discriminatory power, these methods may not provide the resolution necessary to differentiate evolutionarily close strains (*39*,*40*). The introduction of an expanded panel of 24 MIRU-VNTR loci in 2009 to NTGS may reduce this misclassification in the future (*40*). It is also critical to note that TB transmission dynamics are multifactorial. TB genotype clustering may overestimate transmission. Consideration of patient characteristics, transmission venues, and temporality may better clarify recent transmission.

The integration of NTGS into routine public health practice and surveillance has led to the establishment of molecular surveillance of *M. tuberculosis* in the United States (*20*). With improved access to and rapid dissemination of genotyping information, it may be possible to more effectively identify some cases of TB transmission. Yet, TB genotyping, and likely future molecular advancements do not alter real-time public health action. Rather recent transmission can only be prevented by implementing thorough contact investigation and ensuring that subsequent preventive treatment is completed among those identified at highest risk of undergoing a progression from infection to TB disease. If such practices had been successfully followed, as many as one third of all reported TB cases in US-born patients may have been prevented, especially among high-risk populations, such as persons with substance abuse disorders, those experiencing homelessness, or both. Greater attention and resources are needed to develop, implement, and evaluate interventions to control and prevent transmission among these populations. As the United States continues toward TB elimination, understanding transmission dynamics among high-risk populations and establishing new strategies for rapidly detecting and effectively responding to these transmission events will enhance the progress toward achieving this target.

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Poultry Culling and Campylobacteriosis Reduction among Humans, the Netherlands

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In the Netherlands in 2003, an outbreak of avian influenza in poultry resulted in extensive culling, especially of layer hens. Concurrently, human campylobacteriosis cases decreased, particularly in the culling area. These observations raise the hypothesis that *Campylobacter* spp. dissemination from poultry farms or slaughterhouses might contribute to human campylobacteriosis.

In the Netherlands during March–May 2003, an outbreak of avian influenza (H7N7) virus among poultry led to the culling of >30 million birds (*1*). The outbreak, and thus the culling, was confined to a relatively small area of 50 × 30 km in the center of the country (*2*). A few years after the avian influenza outbreak, it became apparent that the incidence of campylobacteriosis among humans had decreased during 2003 and that the extent of this decrease varied by region. Because the avian influenza outbreak strongly affected the poultry industry in 2003, a link was suspected.

The Study

In the Netherlands, the laboratory surveillance network for gastroenteric pathogens was started in 1987 and now consists of 15 regional public health laboratories. In April 1995, *Campylobacter* spp. were included in surveillance. Each laboratory reported the number of all first isolates of pathogens weekly to the Department of Epidemiology and Surveillance at the National Institute for Public Health and the Environment (RIVM). For 2002 through 2004, prospective weekly estimates of the expected frequency of campylobacteriosis cases and 99.5% tolerance levels were calculated by using the Farrington algorithm, based on weekly surveillance data for the preceding 5 years, and linear interpolation of the observed frequencies per year (1995–2008) (*3*,*4*). Incidence rates were calculated by taking the area covered by the surveillance network into account (*4*).

Campylobacteriosis incidence in the Netherlands decreased from 46.4 patients per 100,000 inhabitants in 1996 to 38.7 in 1999 and increased thereafter to 44.3 in 2001 and 40.8 in 2002. In 2003, incidence decreased to 33.3 per 100,000 inhabitants, and during 2004–2008, it increased again to 40.0–43.8.

In March 2003, a 30% reduction of reported campylobacteriosis cases in the Netherlands was noted. In December 2003, a 19% reduction was noted (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/3/11-1024-FA1.htm). From March through December 2003, levels of reduction varied markedly among public health laboratories, 10%–70%; the largest reduction occurred in the central region of the country, where the culling took place (Figures 1, 2) (*2*). Overall, the percentages of cases reported by the laboratories in culling areas were 44%–50% less than expected during May–December 2003.

In the poultry culling area, 1 large slaughterhouse (2 locations) and 1 smaller slaughterhouse, which together accounted for 15% of the national slaughter capacity for

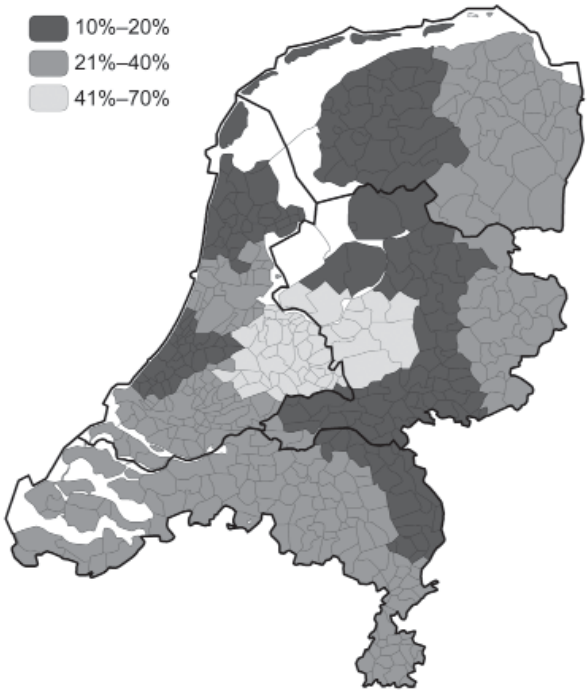

Figure 1. Regional reduction of campylobacteriosis (March–December 2003) following the Public Health Laboratory regions borders in the Netherlands, with the outlines of the 4 clusters of provinces.

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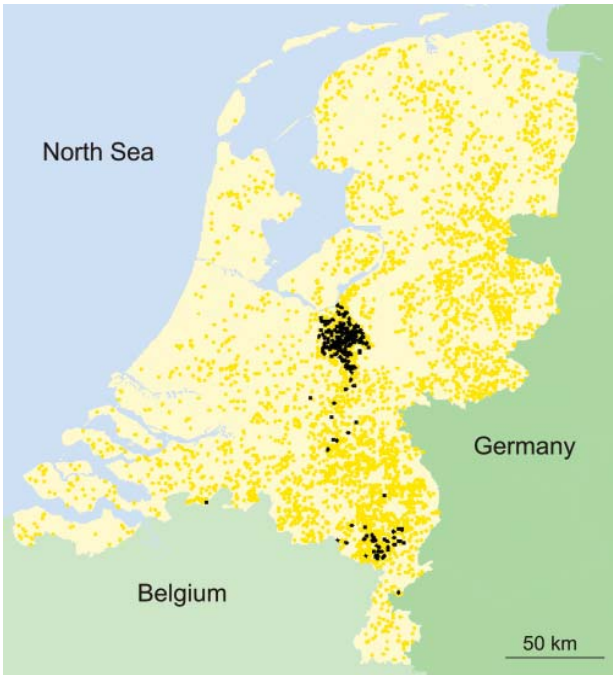

Figure 2. Locations of all 5,360 commercial poultry farms in the Netherlands (*2*). Black dots indicate farms that were infected during the 2003 epidemic of avian influenza; yellow dots indicate farms that were not infected.

broiler chickens, had to be closed during the culling (March–June). Information about poultry purchases was gathered through registration of the food products bought by and interviews with a random sample of 6,000 households, comprising ≈13,400 persons, by GfK Panel Services Benelux (Dongen, the Netherlands) (*5*). The Product Boards for Livestock, Meat and Eggs provided sales data for poultry meat on the national level and stratified by 4 regions.

Comparison of broiler meat purchases during 2002–2003 (Table) indicated a national reduction in sales during March–October 2003; the reduction was greatest during May–June (–9%). The regional reduction never exceeded –12% and was largest in areas roughly overlapping or near the culling area. By 2004, sales had returned to normal (85,165 kg, data not shown).

Conclusions

Consumption of poultry and direct contact with poultry are generally accepted as dominant risk factors for sporadic *Campylobacter* spp. infections among humans (*6*,*7*). In the Netherlands, the strongest reduction in campylobacteriosis cases occurred in the laboratory service areas overlapping the culling area and the areas where the slaughterhouses were closed. Also, sales of poultry meat dropped most in these areas, although not proportional to the reduction in campylobacteriosis, and recovered quickly after June; the reduction in campylobacteriosis occurred at least up to the end of the year. Moreover, culling was mainly among layer hens (54%) and only 8% among broilers. In the Netherlands, meat from spent hens (layer hens that are no longer economically productive) is not consumed as fresh meat.

Environmental pathways of human *Campylobacter* spp. infection remain less understood (*7*) and might play a major role in rural areas (*8*,*9*). These pathways remain to be clarified, although some studies have implicated aerosols and flies as vectors for environmental transmission (*10*–*12*). *Campylobacter* spp. have been detected in the air up to 30 m downwind of and in puddles near broiler houses (*13*). A US study among chicken catchers and poultry plant workers at 1 plant found colonization with *Campylobacter* spp. among 41% and 63% of these persons, respectively (*14*). Surprisingly, 9 community members who lived near, but did not work at, the US plant had positive *Campylobacter* spp. test results.

In Belgium in 1999, the availability of poultry meat was greatly reduced because of dioxin-contaminated feed components (*15*). All poultry meat and eggs from Belgium were withdrawn from the market, which resulted in a 40% decrease in campylobacteriosis cases for 2 weeks after the withdrawal. Two weeks after sale of these products resumed, incidence returned to previous rates, although poultry production took 7 weeks longer to regain levels similar to those of the year before. In the Netherlands, the reduction in campylobacteriosis cases lasted longer. The situations in the Netherlands and Belgium also differed at other points. In the Netherlands, culling was conducted in a relatively small area, at farms under strict biosecurity measures, and was followed by intensive cleaning and

Table. Changes in broiler meat sales, by region, the Netherlands, 2002–2003

Region*	Sales × 1,000 kg, 2002/2003	Change, %					
		Jan–Feb	Mar–Apr	May–Jun	Jul–Aug	Sep–Oct	Nov–Dec
Entire country	84,128/81,137	1	–6	–9	–5	–2	4
Mideastern region†	17,435/16,582	–3	–7	–12	–5	–2	0
Western + middle regions‡	40,546/38,351	–2	–6	–11	–6	–4	–3
Northeastern region§	7,022/7,135	3	–3	–8	0	6	12
Southern region¶	19,125/19,068	–2	–2	–2	2	2	2

*Most culling was conducted in Gelderland and Utrecht Provinces.
†Flevoland, Gelderland, Overijssel Provinces.
‡Noord Holland, Zuid Holland, and Utrecht Provinces.
§Groningen, Friesland, and Drenthe Provinces.
¶Zeeland, Noord Brabant, and Limburg Provinces.

disinfection of the farms and an extended period when farms were empty. In Belgium, the poultry came from farms throughout the country and were slaughtered according to routine procedures before disposal of carcasses or processed meat. Furthermore, in the Netherlands, sales of broiler meat decreased by ≤12%, whereas in Belgium, 100% of broiler meat was withdrawn from the market.

In this retrospective study, measures of environmental dissemination of *Campylobacter* spp. were lacking. The use of aggregated data makes it impossible to prove a causal link between the culling of poultry and the decrease in campylobacteriosis incidence. Nevertheless, on the basis of the combined information, we hypothesize a relationship between the reduced environmental contamination by poultry farms and slaughterhouses and the reduced number of campylobacteriosis cases in humans in the same region. Because slaughterhouses were closed and disinfected farms were empty or closed for everyone except attendants under strict hygiene measures, a temporal, lower environmental load of *Campylobacter* spp. was probably achieved. We are not aware of any other events in this period that might explain the regional and temporary decrease in campylobacteriosis incidence. However, unobserved effects, such as improved kitchen hygiene resulting from regional consumers' awareness of a link between poultry meat and infectious diseases, are also possible explanations.

Our hypothesis of secondary exposure to *Campylobacter* spp. through dissemination from poultry farms or slaughterhouses has public health implications. Even if poultry meat at retail is free of *Campylobacter* spp., campylobacteriosis could occur earlier through exposure during production; thus, control should start at this step of the food chain. More research, including microbiological, analytical, and risk assessment studies, needs to be done to prove or disprove the role of dissemination in the spread of *Campylobacter* spp. and to clarify the possible mechanisms of environmental transmission.

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Novel Orthobunyavirus in Cattle, Europe, 2011

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In 2011, an unidentified disease in cattle was reported in Germany and the Netherlands. Clinical signs included fever, decreased milk production, and diarrhea. Metagenomic analysis identified a novel orthobunyavirus, which subsequently was isolated from blood of affected animals. Surveillance was initiated to test malformed newborn animals in the affected region.

In summer and autumn 2011, farmers and veterinarians in North Rhine-Westphalia, Germany, and in the Netherlands reported to the animal health services, local diagnostic laboratories, and national research institutes an unidentified disease in dairy cattle with a short period of clear clinical signs, including fever, decreased milk production, and diarrhea. All classical endemic and emerging viruses, such as pestiviruses, bovine herpesvirus type 1, foot-and-mouth disease virus, bluetongue virus, epizootic hemorrhagic disease virus, Rift Valley fever virus, and bovine ephemeral fever virus, could be excluded as the causative agent. To identify the cause of the disease, we analyzed blood samples from affected cattle.

The Study

On a farm near the city of Schmallenberg (North Rhine-Westphalia, Germany; Figure 1), 3 blood samples obtained in October 2011 from dairy cows that had clinical signs at sampling (Table, BH 80/11) were pooled and analyzed by using metagenomics. We also investigated a blood sample from a healthy animal from a different farm (Table, BH 81/11). For metagenomic analysis, 4 sequencing libraries (Table) were prepared and sequenced by using the 454 Genome Sequencer FLX (Roche, Mannheim, Germany). Two libraries each were generated from DNA and RNA isolated from plasma samples (Table). By using a combination of BLAST (*1*) and sequence mapping with the 454 reference mapper application (version 2.6; Roche), reads were classified into different superkingdoms (Table). In addition to the anticipated high number of host sequences, we detected in some samples a considerable portion of reads representing diverse bacterial species. These bacteria most likely grew in the samples during the prolonged storage before extraction of the nucleic acids used to prepare the sequencing libraries. Seven orthobunyavirus sequences were detected in the library prepared from pooled RNA from 3 animals of 1 farm (BH 80/11, Table). Repeated sequencing of this library resulted in 22 additional reads of orthobunyavirus-specific sequences. We assembled the reads of all 3 genome segments into contigs by using the Newbler Assembler

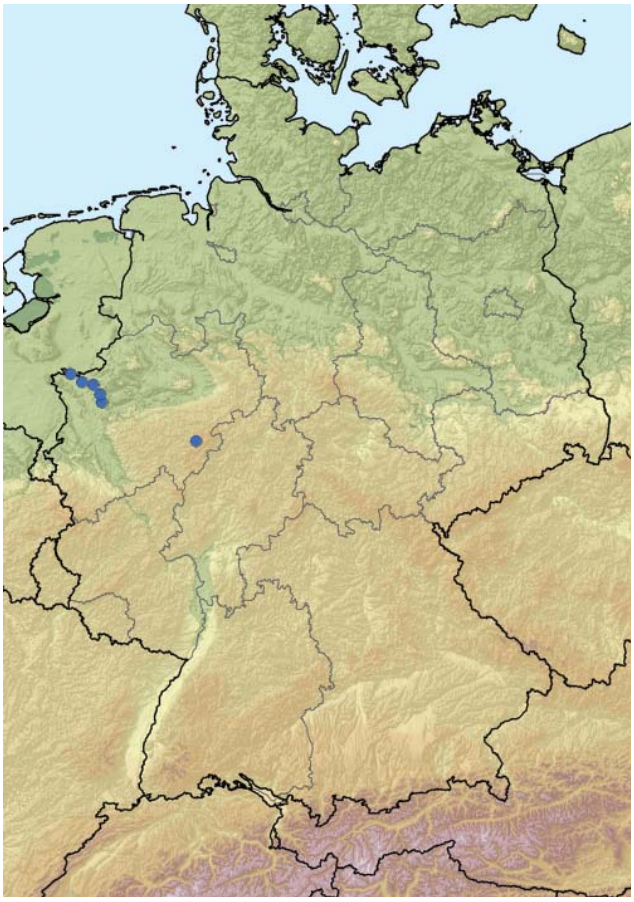

Figure 1. Location of farms with PCR-positive cattle (blue dots) in North Rhine-Westphalia, Germany.

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Table. Output of raw sequence data for the sequencing libraries in the analysis of a novel orthobunyavirus in cattle, Europe, 2011

Sample	Total no. reads	No. reads classified into superkingdom					No. unclassified reads
		Eukaryota	Archaea	Bacteria	Viruses	Root	
BH 80/11 RNA (3 pooled samples)	27,413	12,296	4	13,363	55 (Myoviridae, Siphoviridae, Podoviridae, Bunyaviridae, Retroviridae, Papillomaviridae)	377	1,318
BH 81/11 RNA	16,125	10,220	2	4,821	57 (Myoviridae, Siphoviridae, Podoviridae, Retroviridae)	19	1,006
BH 80/11 DNA (3 pooled samples)	77,929	59,308	3	95	3 (Herpesviridae, Mimiviridae, unclassified virus)	9,181	9,339
BH 81/11 DNA	89,728	79,742	9	44	1 (Retroviridae)	3	9,929

(version 2.6; Roche). A few sequence gaps were filled by Sanger sequencing and by next-generation sequencing of the cell culture isolate. The resulting full-length sequences for the small (S; 830 nt), medium (M; 4,415 nt), and large (L; 6, 865 nt) segments are available from the International Nucleotide Sequence Database Collaboration (www.insdc.org) databases (International Nucleotide Sequence Database Collaboration accession numbers HE649912–HE649914).

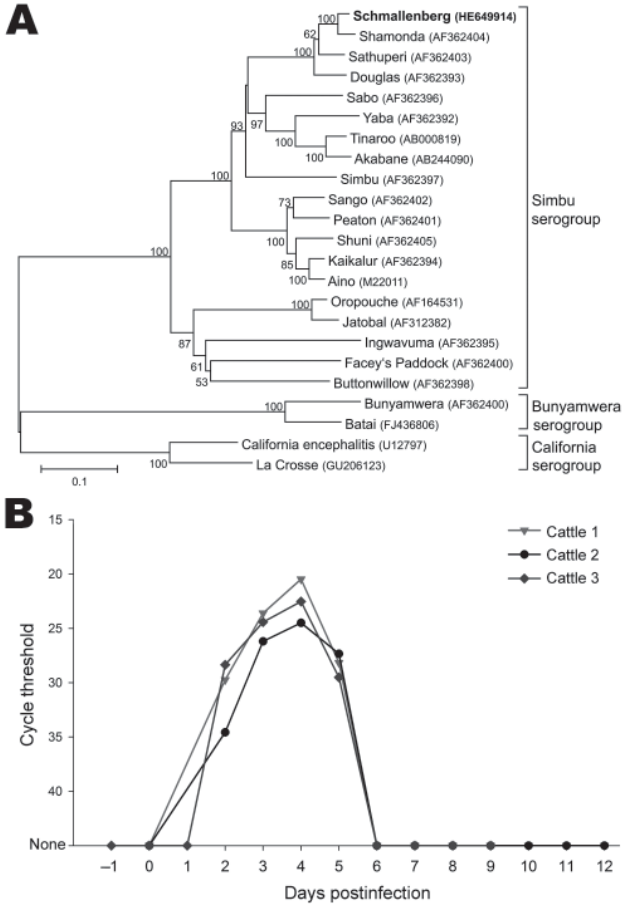

Figure 2. A) Phylogenetic relationship between Schmallenberg virus and orthobunyaviruses of the Simbu, Bunyamwera, and California serogroups. International Nucleotide Sequence Database Collaboration accession numbers of the sequences in the analysis are indicated in the tree. The neighbor-joining tree is based on the nucleocapsid gene of the small segment (702 nt). Numbers at nodes represent the percentage of 1,000 bootstrap replicates (values <50 are not shown). Scale bar indicates the estimated number of nt substitutions per site. B) Detection of Schmallenberg virus genome in the blood of experimentally infected calves. The highest genome copy number was detected on postinoculation day 4.

Sequence comparisons were done with BLAST (*1*). The most similar sequences were from a Shamonda virus detected in cattle in Japan (S segment; INSDC accession no. AB183278; 97% identity) (*2*), an Aino virus discovered in cattle in Japan (M segment; accession no. AB542971; 71% identity) (*3*), and an Akabane virus found in cattle in Japan (L segment; accession no. AB190458; 69% identity) (*4*). This inconsistency might have resulted from the lack of published Shamonda virus M and L segment sequences and does not necessarily indicate that the novel orthobunyavirus is a reassortant. Nevertheless, only future studies that include M and L segment sequences of other members of the Simbu serogroup will enable a final classification.

Because of the paucity of information, only S segment sequences were used for phylogenetic analysis. The S segment sequence encoding the nucleocapsid protein region (702 nt) was aligned with sequences of the Simbu, Bunyamwera, and California serogroups by using ClustalW (www.clustal.org) for codons. Phylogenetic relationship was assessed by using the neighbor-joining method based on a Tamura 3-parameter model and bootstrap analysis (1,000 replicates) as implemented in MEGA5 (*5*). The phylogenetic tree (Figure 2, panel A) shows that the S segment sequence is distinct but clusters closely with Shamonda viruses within the Simbu serogroup, which suggests that the novel virus is a Shamonda-like virus within the genus *Orthobunyavirus*.

Members of this genus within the family *Bunyaviridae* are widely distributed in Asia, Africa, and Oceania; transmission occurs predominantly through biting midges, mainly *Culicoides* spp. and mosquitoes. Especially the Simbu serogroup, which includes Akabane, Aino, and Shamonda viruses, can play a role as pathogens of ruminants. However, to our knowledge, viruses of this serogroup have not previously been detected in Europe (*6*). Because of the origin of the first positive samples, the virus was provisionally named Schmallenberg virus.

A newly developed real-time quantitative reverse transcription PCR (RT-qPCR) (primers and probes are

available on request) was used to test additional samples from affected cattle farms. Twelve samples, mainly from adult cattle from 6 different farms, were positive for the novel virus, with cycle threshold (C_t) values of 24–35. All farms with cattle that tested positive were sampled in September, October, or November and are located within the federal state of North Rhine-Westphalia. Most farms are in close proximity to the border with the Netherlands (Figure 1). The latest case from December is from a stillborn twin calf. Abdominal fluid was PCR positive for the novel virus, with a C_t value of ≈27.

The virus was isolated from the blood of a diseased cow from the farm in Schmallenberg. *Culicoides variipennis* larvae cells (KC cells) (*7*) (Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Greifswald–Insel Riems, Germany) were incubated for 10 days with ultrasonically disrupted blood diluted in Schneider's media. The cells were then lysed by freezing and thawing. A monolayer of baby hamster kidney-21 cells was inoculated with the lysate. The inoculum was removed after 1 h and replaced by Eagle minimal essential medium. A strong cytopathic effect was visible after 5 days, and the culture supernatant tested positive for the novel virus, with a C_t value of ≈14 in the specific RT-qPCR.

In a first animal trial (permit no. LALLF-7221.3–2.5–011/11) 3 calves, ≈9 months of age, were inoculated directly with blood that was PCR positive for the novel virus from 4 different cattle (1 animal was inoculated intravenously with 4 × 1 mL, 1 animal subcutaneously with 4 × 1 mL) or with the initial KC cell isolate described above (1 mL subcutaneously and 4 mL intravenously). All inoculated animals became infected and had positive PCR results 2–5 days postinoculation (dpi), with the lowest C_t values, ≈21, occurring at 4 dpi (Figure 2, panel B). Fever (temperature 40.5°C) developed in 1 animal 4 dpi, and 1 animal (inoculated with the KC cell isolate) had mucous diarrhea for several days. A first serum neutralization assay resulted in titers of ≈15 for serum collected 21 dpi.

Conclusions

The detection of a novel orthobunyavirus in cattle in Germany (Schmallenberg virus) demonstrates the power of a metagenomic approach to discovering emerging pathogens. Specific and sensitive RT-qPCRs could be developed quickly and used in analyzing infected herds.

The role of the virus in the disease needs to be further investigated. However, the clinical signs in 2 of the inoculated animals, together with virus detection in samples of diseased animals in Germany and the Netherlands (*8*) and in the brain of malformed lambs in the Netherlands (*8*), strongly indicate that Schmallenberg virus caused the clinical illness. In further investigations, we will use serology to analyze distribution in the field and will sequence the complete genomes of other members of the Simbu serogroup to better understand the phylogenetic background of Schmallenberg virus.

Concern exists about the congenital defects the virus might induce in newborn calves, goats, and lambs during the next months. Therefore, surveillance has been initiated to test all malformed animals in the affected region. Some members of the Simbu serogroup, e.g., Oropouche virus, are zoonotic. However, because of the close relationship to Shamonda virus and the absence of reports of clinical signs in humans, the risk to humans currently is assessed as very low to negligible. Nevertheless, clinical and serologic surveillance in humans should be conducted in regions with infected animals to update the risk assessments.

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Escherichia coli O104:H4 Infections and International Travel

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We analyzed travel-associated clinical isolates of *Escherichia coli* O104:H4, including 1 from the 2011 German outbreak and 1 from a patient who returned from the Philippines in 2010, by genome sequencing and optical mapping. Despite extensive genomic similarity between these strains, key differences included the distribution of toxin and antimicrobial drug–resistance determinants.

In May 2011, officials in northern Germany reported a sudden surge in illness due to Shiga-toxigenic *Escherichia coli* (STEC). Symptoms of infection ranged from self-limiting episodes of diarrhea to life-threatening hemolytic-uremic syndrome (HUS). As of July 21, 2011, >4,075 persons in 16 countries had become ill. The outbreak was associated with an unprecedented rate of HUS (908 [22.2%] of 4,075 STEC-infected persons), and 50 persons died (*1*).

STEC are foodborne and waterborne pathogens. Human illness is most often associated with *E. coli* O157:H7, but non-O157 serogroups are also being recognized as key agents of STEC disease (*2*–*5*). The recent German outbreak was caused by *E. coli* O104:H4. Unlike *E. coli* O157:H7, which has a characteristic, sorbitol nonfermenting phenotype that is readily detected by routine laboratory testing, non-O157 *E. coli* strains are difficult to distinguish from the nonpathogenic *E. coli* strains commonly found in stool specimens, and frontline laboratories in Canada do not routinely screen for them.

This study describes 2 cases of *E. coli* O104:H4 infection that were imported to Canada. One case was caused by a 2011 isolate associated with the recent German outbreak. The second isolate was identified in 2010. Phenotypic and genotypic features of these 2 strains are described.

The Study

On June 1, 2011, a 67-year-old Canadian man sought treatment at an Ontario hospital with a 3-day history of bloody diarrhea. He had returned from Germany on May 27. He had no signs of HUS, and *E. coli* O157:H7 was not detected by routine testing. Clinical specimens from this patient were referred to the Public Health Ontario Laboratories for testing. Shiga toxin was detected by enzyme immunoassay (Meridian Biosciences, Inc., Cincinnati, OH, USA), and real–time PCR confirmed that the strain, named ON-2011, was positive for the *stx2* gene and negative for the *eae* gene (*3*,*6*). Biochemical and serologic testing confirmed that the isolate was *E. coli* serogroup O104:H4. The patient recovered uneventfully (*4*).

Before the May 2011 outbreak in Germany, a single isolate of *E. coli* O104:H4 had been identified in Ontario. That isolate, ON-2010, was recovered in June 2010 from a 10-month-old boy who had returned from the Philippines 2 days earlier. He was brought to the hospital with a 1-day history of vomiting and nonbloody diarrhea. A sorbitol-nonfermenting colony was recovered from a stool specimen, but it did not react with *E. coli* O157 antiserum. The specimen was referred to the Canadian National Microbiology Laboratory, which confirmed *E. coli* O104:H4. Retrospective PCR-based testing showed that this isolate was negative for *stx* and *eae*. The infant made a full and uneventful recovery.

Etest (AB BIODISK, Solna, Sweden) susceptibility testing was performed by using a standard inoculum (0.5–McFarland standard); agar dilution indicated that ON-2010 was pansusceptible, whereas ON-2011 was resistant to amikacin, tetracycline, trimethoprim/sulfamethoxazole, and extended-spectrum β–lactams. PCR results for CTX–M–15 and TEM–1 were negative for ON-2010 and positive for ON-2011 (*7*). The gene for tellurite resistance was also absent in ON-2010, but present in ON-2011. Although both isolates were of multilocus sequence type 678, pulsed-field gel electrophoresis profiles were distinct (Figure, panel A) (*8*). Patterns for ON-2011 (ENXAI.0024/ENBNI.0022, PulseNet Canada designations) were identical with those reported for the current outbreak strain from Germany, whereas the ON-2010 profiles (ECXAI.2585/ECBNI.0922) were distinct (*9*).

Circular, highresolution *Nco*I restriction maps of the ON-2010 (≈5.1 Mbp) and ON-2011 (≈5.25 Mbp)

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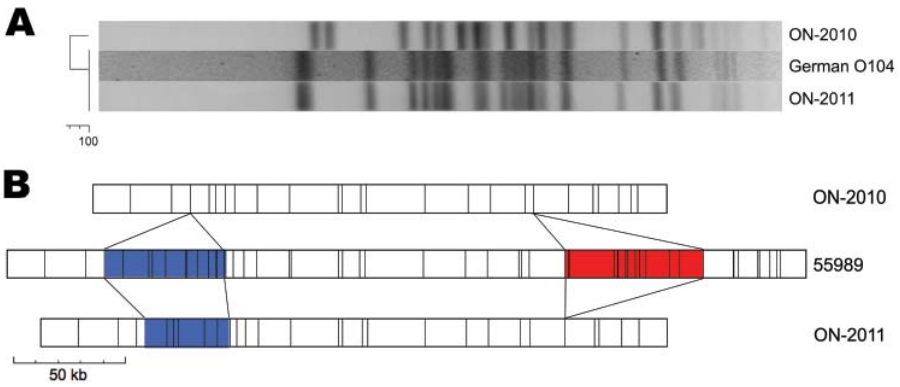

Figure. Comparison of *Escherichia coli* O104:H4 isolates from Ontario. A) The *Xba*I pulsed-field gel electrophoresis profile of ON-2010 is distinct from those of ON-2011 and the outbreak strain from Germany. B) Optical mapping (*Nco*I) patterns reveal genomic similarities and differences between ON-2010, ON-2011, and the O104:H4 strain 55989. Blue, heterogeneity in siderophore biosynthesis region; red, strain-specific insertion of TetR-containing prophage.

genomes were generated by optical mapping (Argus Optical Mapper, OpGen, Inc, Gaithersburg, MD, USA). These were compared with an in silico map of *E. coli* 55989, an O104:H4 strain that was isolated in Africa and sequenced in France (*10*). The extensive synteny observed and the absence of large-scale genomic rearrangements (e.g., inversions, translocations) suggest that ON-2010 and ON-2011 descended from an *E. coli* 55989–like ancestor (Figure, panel B).

The evolutionary relationship of the 2 isolates from Ontario was further assessed by whole-genome sequencing (Roche GS–FLX Titanium; Roche Diagnostics, Laval, QC, Canada). This confirmed the genomic similarity of ON-2010 to *E. coli* 55989 and uncovered a 72-kb plasmid, pON-2010. Virulence genes ON-2010 and ON-2011 were compared by Bielaszewska et al. (*11*) (Table) on the basis of virulence factor analysis of the 2011 outbreak strain of *E. coli* O104:H4 from Germany. The plasmid exhibits >99% identity with p55989 and encodes the aggregative adhesion fimbriae cluster that is a defining features of enteroaggregative *E. coli* (EAEC) (*12*). In contrast, ON-2011 contains 3 plasmids. Reference-based mapping of raw sequencing reads against publicly available genome and plasmid scaffolds (HPA, BGI) confirmed that ON-2011 is a German outbreak clone (*13*). Except for a small number of single nucleotide polymorphisms, the clone isolated in Canada is virtually identical to those from Germany and the United Kingdom.

The O104:H4 outbreak strain from Germany exhibits key features of classic EAEC, but also contains numerous horizontally acquired virulence factors. An *stx2*-encoding prophage is responsible for the toxigenic properties of this strain, and multidrug-resistance loci are present, including a chromosomal tetR loci, and the plasmid-encoded CTX-M-15, TEM-1, and tellurite resistance–encoding loci (*ter*) (*14*). In contrast, the ON-2010 strain does not encode Shiga toxin, and no resistance genes have been identified. Even the tetracycline-resistance genes, independently acquired by both ON-2011 and strain 55989, are absent (Figure, panel B). However, the plasmid-encoding aggregative adhesion fimbriae cluster is present. Preliminary comparison of the 3 genomes has also shown some small-scale rearrangement events. One of these rearrangements eliminates siderophore biosynthesis genes from ON-2010 and may compromise bacterial iron acquisition. However, ON-2010 does have a strain-specific insertion that contains the hydroxyphenylacetate (*hpa*) operon, which may provide a nutritional advantage by enabling the organism to catabolize the phenolic and aromatic compounds abundant in the gut. This insertion is absent from the other O104:H4 genomes (*15*).

Conclusions

Since 2010, 2 cases of travel-associated *E. coli* O104:H4 infection have been identified in Ontario, Canada. Our analysis of these isolates could inform genomic studies on the emergence and evolution of the O104 clone first observed in Europe. The relationship of these strains was assessed by a combination of traditional, molecular, and genomic approaches. Optical mapping and sequencing indicate that ON-2010 and ON-2011 exhibit extensive synteny and are derived from a common EAEC ancestor. However, a series of horizontal gene transfer events has contributed to genotypic and phenotypic divergence. The outbreak clone from Germany, ON-2011, is an antimicrobial drug–resistant STEC isolate, whereas ON-2010 is pansusceptible and nontoxigenic.

These differences are clinically important but are not detected by diagnostic strategies that use serotype as a proxy for pathogenic capacity. Given the potential for severe clinical sequelae of STEC infections, clinical testing should use improved and affordable methods for identification of Shiga toxin that can be easily integrated into routine clinical microbiology laboratories (*4*,*9*). Future capacity to detect additional virulence factors, such as aggregative adhesion fimbriae, would enable expanded diagnostic capacity to detect pathogenic *E. coli* subtypes that cause human disease.

Table. Virulence loci of ON-2011, ON-2010, and EAEC strain 55989*

Gene product	Function	Presence in ON-2011	Presence in ON-2010	Presence in EAEC 55989
STEC				
stx$_1$	Shiga toxin 1	–	–-	–
stx$_2$	Shiga toxin 2	+	–	–
EHEC-*hlyA*	EHEC hemolysin	–	–	–
cdt(I–V)	Cytolethal distending toxin	–	–	–
subAB	Subtilase cytotoxin	–	–	–
espP	Serine protease EspP	–	–	–
eae	Intimin	–	–	–
iha	Iha (IrgA homolog adhesin)	+	Partial (5′ end 21%)	+
lpfA$_{O26}$	Structural subunit of LPF of STEC O26	+	+	+
lpfA$_{O113}$	Structural subunit of LPF of STEC O113	+	+	+
lpfA$_{O157–OI141}$	Structural subunit of LPF of STEC O157:H7 (encoded on O island 141)	–	–	–
lpfA$_{O157–OI154}$	Structural subunit of LPF of STEC O157:H7 (encoded on O island 154)	–	–	–
saa	Saa (STEC autoagglutinating adhesin)	–	–	–
sfpA	Structural subunit of Sfp fimbriae	–	–	–
ter cluster	Tellurite resistance	+	–	–
irp2	Component of iron uptake system on HPI	+	+	+
fyuA	Component of iron uptake system on HPI	+	+	+
EAEC				
aatA	EAEC virulence plasmid (pAA)	+	+	+
aggA	Pilin subunit of aggregative adherence fimbriae I (AAF/I)	+	–	–
agg3A	Pilin subunit of AAF/III	–	+	+
aggR	Transcriptional regulator AggR	+	+	+
aap	Dispersin	+	+	+
set1	*Shigella* enterotoxin 1	+	+	+
pic	Pic (protein involved in intestinal colonization)	+	+	+
astA	EAEC heat-stable enterotoxin 1 (EAST1)	–	+	+
EPEC				
bfpA	Bundle-forming pili	–	–	–
ETEC				
elt	Heat-labile enterotoxin (LT)	–	–	–
estIa	Heat-stable enterotoxin (STIa)	–	–	–
estIb	Heat-stable enterotoxin (STIb)	–	–	–
EIEC				
ial	Invasive plasmid (pInv)	–	–	–

*Derived from (*11*). EAEC, enteroaggregative *Escherichia coli;* STEC, Shiga-toxigenic *E. coli*; –, negative; +, positive; EHEC, enterohemorrhagic *E. coli*; LPF, long polar fimbriae; HPI, high-pathogencity island; EPEC, enteropathogenic *E. coli;* ETEC, enterotoxigenic *E. coli*; EIEC, enteroinvasive *E. coli.*

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We thank the Public Health Agency of Canada/Public Health Ontario *E. coli* O104 Research Group; the clinical and research staff at the Public Health Ontario Laboratories who were involved in the investigation of STEC; Linda Chui for her advice and support; and National Microbiology Laboratory personnel, including Celine Nadon and Michael Mulvey, for their contributions and expertise.

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Laboratory Practices and Incidence of Non-O157 Shiga Toxin–producing *Escherichia coli* Infections

Kathleen A. Stigi, J. Kathryn MacDonald, Anthony A. Tellez-Marfin, and Kathryn H. Lofy

We surveyed laboratories in Washington State, USA, and found that increased use of Shiga toxin assays correlated with increased reported incidence of non-O157 Shiga toxin–producing *Escherichia coli* (STEC) infections during 2005–2010. Despite increased assay use, only half of processed stool specimens underwent Shiga toxin testing during 2010, suggesting substantial underdetection of non-O157 STEC infections.

Strains of Shiga toxin (Stx)–producing *Escherichia coli* (STEC) are differentiated by the O antigen on their outer membrane and are broadly classified as O157 or non-O157 STEC (*1*–*3*). The ability to produce Stx is a key virulence trait of STEC (*1*,*3*,*4*). STEC infections in humans often cause a self-limited diarrheal illness but can be complicated by hemorrhagic colitis or hemolytic uremic syndrome (*1*).

Unlike other *E. coli* strains, serogroup O157 isolates do not ferment sorbitol and are readily identified by culture, appearing colorless on sorbitol MacConkey agar (*1*,*2*,*4*). Both O157 and non-O157 STEC can be identified by detecting Stx with nonculture assays that became commercially available in the United States in 1995 (*2*,*4*). The Centers for Disease Control and Prevention (CDC) published formal STEC testing recommendations for clinical laboratories in 2009, advocating that all stool specimens submitted for routine bacterial pathogen testing be simultaneously cultured for O157 STEC and tested with a nonculture assay to detect Stx. Use of this testing protocol ensures timely identification of all STEC infections (*2*,*5*). Exclusive testing for Stx delays specific identification of O157 STEC and may impede prompt detection of common-source outbreaks (*2*–*4*).

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Non-O157 STEC infection has been a nationally notifiable condition since 2000 (*5*). Although studies have documented the increased incidence of reported non-O157 STEC infections over the past decade, few have determined the proportion of laboratories that routinely test all submitted stool specimens for Stx and, to our knowledge, no study has quantified STEC testing practices by proportion of stool specimens processed for bacterial culture. Our objectives, therefore, were to quantify statewide STEC testing practice by proportion of stool specimens processed for bacterial culture and to determine the contribution of enhanced STEC testing practice to increased reported incidence of non-O157 STEC infections.

The Study

Data for all confirmed STEC infections reported to the Washington State Department of Health (DOH) with illness onset during 2005–2010 were reviewed to determine incidence trends. Confirmed STEC is defined as the isolation of *E. coli* O157:H7 or an Stx-producing *E. coli* isolate from a clinical specimen. Of 945 cases reported to DOH during 2005–2010 (average annual incidence: 2.4 cases/100,000 population), 781 (83%) cases were O157 STEC and 164 (17%) cases were non-O157 STEC infections. The incidence of non-O157 STEC infections increased dramatically during the 6-year period, from 0.13/100,000 population and 6% of all reported STEC infections in 2005 to 1.13/100,000 population and 41% of all reported STEC infections in 2010 (Figure 1). Four serogroups accounted for >80% of non-O157 STEC cases: O26 (48%), O103 (18%), O121 (12%), and O111 (5%).

Using data from the Washington State DOH Office of Laboratory Quality Assurance, we identified 74 clinical microbiology laboratories in the state. To assess statewide STEC laboratory testing practices, we developed an online survey and distributed it to microbiology laboratory

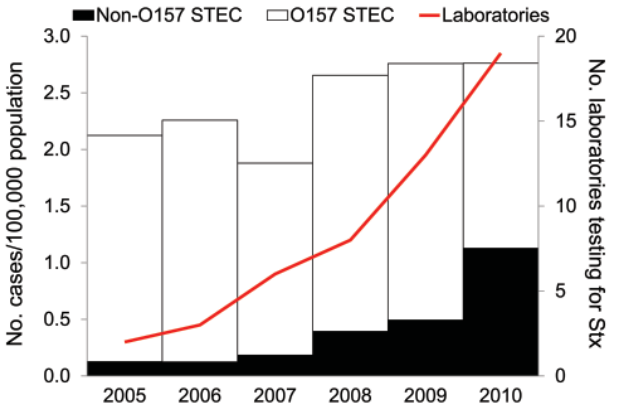

Figure 1. Rate of reported O157 and non-O157 Shiga toxin (Stx)–producing *Escherichia coli* (STEC) infections and number of laboratories performing Stx testing by year, Washington State, USA, 2005–2010.

supervisors at these 74 laboratories during January 2011. Seventeen laboratories reported that all stool specimens are forwarded to a reference laboratory for testing. These 17 laboratories were excluded from the survey sample; however, all indicated reference laboratories were within the state and among the remaining sample (n = 57). Follow-up was completed by email and telephone until a 100% response rate was achieved. The survey requested data on annual number of stool specimens processed for bacterial culture, current protocol for processing stool specimens submitted for routine enteric pathogen testing, and motivations and barriers toward the implementation of Stx testing. If Stx testing was reported, we requested implementation date. Laboratory supervisors were asked to indicate a range for the number of stool specimens processed for bacterial culture at their laboratory during 2010. The 32 laboratories (56%) that reported ≥300 specimens were asked to specify the quantity. For the 25 laboratories that reported <300 specimens, a midpoint of the range was assigned. All data were analyzed by using SAS for Windows, version 9.2 (SAS Institute, Inc., Cary, NC, USA).

Fifty-seven laboratories in Washington State collectively processed an estimated 71,000 stool specimens for bacterial culture in 2010; the number of specimens ranged from 61 to 6,017 specimens per laboratory (median 570). The 10 (18%) largest laboratories processed 51% of the total annual specimens, while the 25 (44%) smallest laboratories processed only 5% of the total annual specimens.

The following results quantify reported routine enteric pathogen testing protocols for all stool specimen submissions. Of 57 laboratories, 56 (98%) performed routine STEC testing on all submitted stool specimens, either by culture, by detecting the presence of Stx with nonculture assays, or both. Fifteen (26%) reported simultaneous culture for O157 STEC and Stx testing, 37 (65%) cultured for O157 STEC exclusively, and 4 (7%) tested for Stx exclusively (Figure 2, panel A). Combining the number of processed specimens and testing protocol for each laboratory, we estimated that 40% of stool specimens in Washington State were cultured for O157 STEC and tested for Stx, 47% were cultured for O157 STEC exclusively, and 13% were tested for Stx exclusively (Figure 2, panel B).

Of the 19 laboratories that tested for Stx, 11 (58%) implemented testing in 2009 or 2010 (Figure 1). When asked about motivations to implement Stx testing, laboratories most commonly reported CDC recommendations and the desire to detect non-O157 STEC infections. The most commonly reported barriers were cost, procedural change, and staffing constraints.

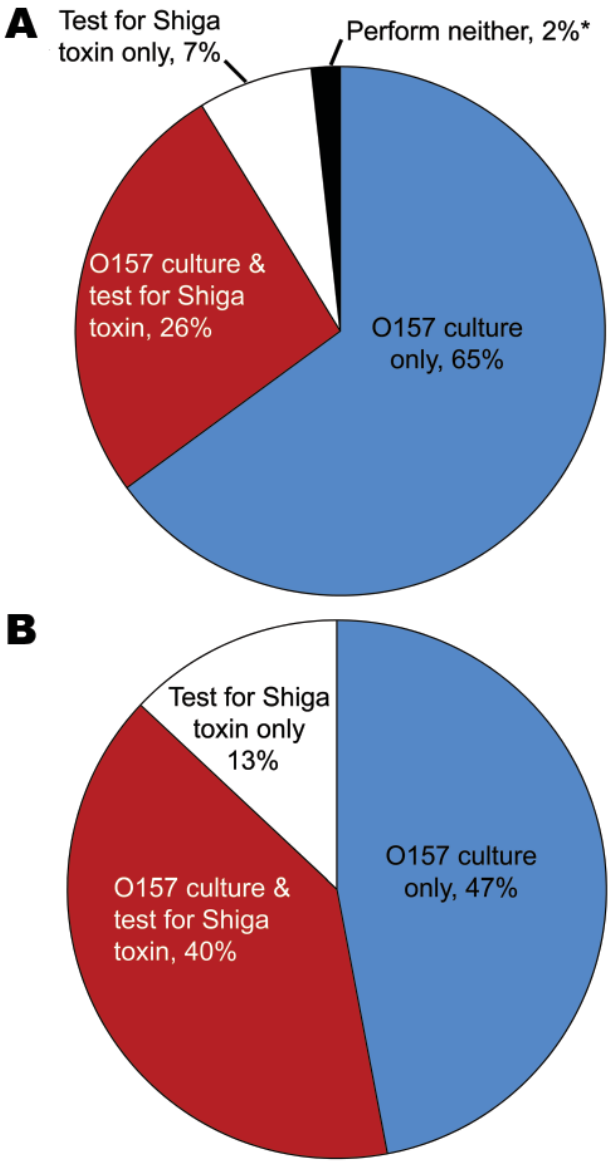

Figure 2. Routine clinical laboratory practice to detect Shiga toxin (Stx)–producing *Escherichia coli* (STEC) by proportion of laboratories (A) and proportion of annually processed stool specimens (B), Washington, USA, 2010. *One laboratory reported use of neither method but represented <0.02% of annually processed specimens.

Conclusions

During 2005 through 2010, the number of laboratories in Washington State that tested for Stx increased from 2 (4%) in 2005 to 19 (33%) in 2010, and the incidence of reported non-O157 STEC infections increased from 8 cases (0.13/100,000 population) in 2005 to 76 cases (1.13/100,000) in 2010. The most dramatic increase in reported non-O157 STEC infections occurred between 2008 and 2010, during which time incidence increased

nearly 3-fold, from 26 cases (0.39/100,000) in 2008 to 76 cases (1.13/100,000) in 2010. This increase in reported incidence occurred at the same time during which most laboratories that test for Stx (11, or 58%) implemented testing (Figure 1). This suggests the increase in the reported incidence of non-O157 STEC is likely caused by changes in testing practice.

Despite the increased use of Stx testing, 37 (65%) of the laboratories in Washington State that processed nearly half (47%) of the stool cultures in the state during 2010 cultured for O157 STEC exclusively and, therefore, could not detect non-O157 STEC. Had all specimens been tested for both O157 and non-O157 STEC, we estimate that the incidence of non-O157 STEC would have been 2.12/100,000 population (60% of all reported STEC infections) in 2010, rather than the reported 1.13/100,000 (40% of all reported STEC infections).

Enhanced detection and reporting of STEC infections will likely increase workloads for local communicable disease investigators and public health laboratories during a time when funding for public health is limited. At the local level, every reported STEC infection requires an epidemiologic investigation, while additional detection of Stx at clinical laboratories will increase submission volume at public health laboratories.

National studies have found that non-O157 STEC infections are clinically indistinguishable from O157 STEC infections, with comparable hemolytic uremic syndrome attack rates (*5*–*7*). The potential virulence of non-O157 STEC infections underscores the need for enhanced laboratory testing and epidemiologic research. To encourage adherence to STEC testing recommendations, healthcare providers should request Stx testing if it is not routinely performed at their laboratory. Public health professionals and epidemiologists are encouraged to assess STEC testing practices to correctly interpret incidence trends and make clinical comparisons.

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Highly Pathogenic Avian Influenza (H5N1) Outbreaks in Wild Birds and Poultry, South Korea

Hye-Ryoung Kim, Youn-Jeong Lee, Choi-Kyu Park, Jae-Ku Oem, O-Soo Lee, Hyun-Mi Kang, Jun-Gu Choi, and You-Chan Bae

Highly pathogenic avian influenza (H5N1) among wild birds emerged simultaneously with outbreaks in domestic poultry in South Korea during November 2010–May 2011. Phylogenetic analysis showed that these viruses belonged to clade 2.3.2, as did viruses found in Mongolia, the People's Republic of China, and Russia in 2009 and 2010.

Since 2003, highly pathogenic influenza (HPAI) virus subtype H5N1 has become enzootic in some countries and continues to cause outbreaks in poultry and sporadic cases of infection in humans, thus posing a persistent potential pandemic threat (*1*). Wild birds, especially waterfowl of the order Anseriformes (ducks, geese, and swans) are the natural reservoir of low pathogenicity avian influenza viruses, and since the HPAI outbreak at Lake Qinghai, People's Republic of China, in 2005, they have been suspected of playing a role as long-distance vectors of HPAI viruses (*2*,*3*).

Three previous outbreaks in South Korea are assumed, on the basis of epidemiologic evidence, to have been caused by HPAI (H5N1) viruses introduced by migratory birds, although a carcass or moribund wild bird infected with these viruses (which would serve as a link to the introduction of infection in domestic poultry) was not found (*4*–*6*). On December 7, 2010, an HPAI (H5N1) virus was isolated from a healthy mallard in South Korea (*7*). After that, subtype H5N1 viruses were frequently detected in wild birds and poultry until May 2011. In this study, we analyzed the epidemiologic features of this outbreak and investigated the characteristics of strains through genetic analysis.

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The Study

From November 26 to December 28, 2010, 6 cases of subtype H5N1 infection were identified in carcasses, feces, and cloacal swab specimens of migratory birds collected throughout South Korea. Subtype H5N1 viruses were found in various bird species (mallard, Baikal teal, mandarin duck, whooper swan, and Eurasian eagle owl) in places such as migratory bird habitats and nearby hills (Figure 1, panel A). Despite the repeated predictions from animal health authorities of poultry outbreaks and the emphasis on protection against contamination, on December 30, 2010, HPAI was confirmed on 2 poultry farms. These farms are located in the middle region of South Korea and are close (distances of 1.3 km and 0.4 km, respectively) to the migratory habitats of birds that have been positive for subtype H5N1 virus (Figure 1, panel B). During the next 2 weeks, the percentage of HPAI-positive birds increased rapidly among clustered poultry farms located in the southern region, and poultry on 23 farms and 13 wild birds were confirmed to be infected with HPAI virus (Figure 1, panel C). The HPAI outbreak spread more slowly until May 16, 2011, and an additional 30 poultry farms and 7 wild birds were confirmed to be infected with subtype H5N1 virus (Figure 1, panel D).

Fourteen bird species were found to be positive for subtype H5N1 (Table). The affected poultry included species of the order Galliformes (chickens, quail, etc.), which exhibited sudden death with severe clinical signs, and domestic ducks (order Anseriformes), which died suddenly or exhibited a decrease in egg production, depending on age. Most infected birds of species that belonged to the orders Anseriformes, Falconiformes, and Strigiformes were found dead, but a few infections were detected in swab specimens from healthy mallards and feces of wild birds. Moreover, species of Anseriformes, such as the Mandarin duck, were dominant among the wild birds with HPAI until the beginning of January 2011. After that time, many HPAI infections were found in birds of prey, such as the Eurasian eagle owl (Table).

All viruses were isolated by inoculating embryonated chicken eggs with specimens from cloacal swab specimens, feces, and homogenized organs from birds with suspected infections. The hemagglutinin (HA) and neuraminidase (NA) proteins were subtyped as previously described (*6*). We selected 27 viruses, taking into consideration the outbreak period, the region, and the host species (online Appendix Table, wwwnc.cdc.gov/EID/article/18/3/11-1490-TA1.htm) and conducted sequencing and phylogenetic analysis of 8 gene segments. The genome sequences of 27 viruses are available from GenBank under accession numbers JN807892–JN808107.

In the HA phylogenetic tree, all 27 viruses were clustered into clade 2.3.2 HPAI viruses, together with the

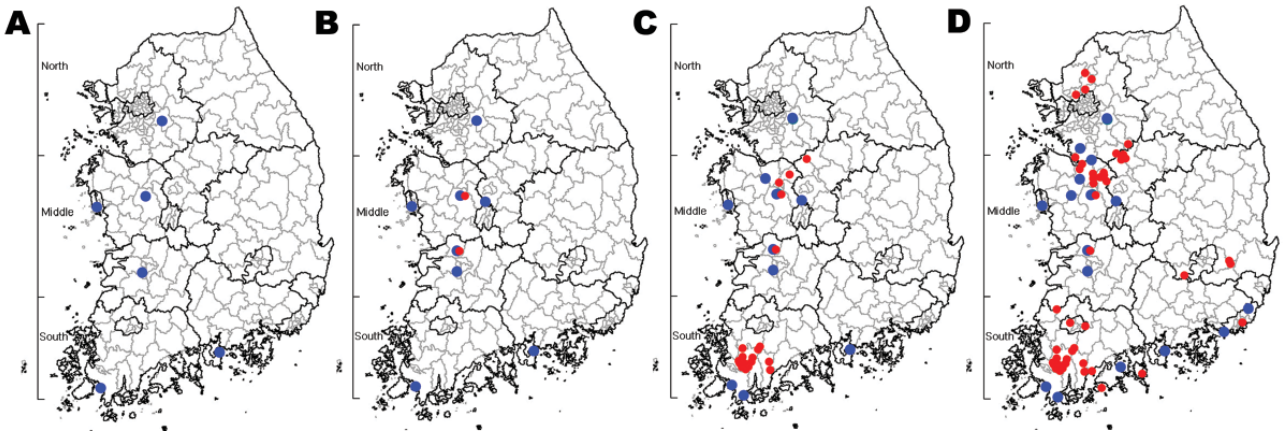

Figure 1. Progress of highly pathogenic avian influenza (HPAI) outbreak by time, South Korea, 2010–2011. A) HPAI-positive cases identified from samples collected November 26–December 28, 2010 (wild birds, 6 cases). B) Cases identified by January 4, 2011 (wild birds, 10 cases; poultry, 2 cases). C) Cases identified by January 11, 2011 (wild birds, 13 cases; poultry, 23 cases). D) Cases identified by May 16, 2011 (wild birds, 20 cases; poultry, 53 cases). Blue circles indicate locations where HPAI viruses were isolated from wild birds; red circles indicate locations where HPAI viruses were isolated from poultry.

subtype H5N1 virus that had been isolated from a healthy mallard (*7*). All isolates showed a high HA homology (>99.5%) (online Appendix Table). Of note, the isolates from poultry fell into 2 sublineages, south-middle and north-middle, which were distinct geographic regions in the HPAI outbreak among poultry, but the isolates from wild birds were not subgrouped in the phylogenetic tree, which displays only topology (Figure 2).

These isolates were different from the HPAI viruses responsible for previous outbreaks in South Korea (A/chicken/Korea/ES/2003[clade 2.5], A/chicken/Korea/IS/2006[clade 2.2] and A/chicken/Korea/Gimje/2008[clade 2.3.2]) and were closely related (>99%) to the subtype H5N1 isolates found in Mongolia, China, and Russia in 2009–2010. The phylogenetic analysis also showed that the NA and other internal genes were closely related to those of subtype H5N1 viruses found in wild birds in Mongolia, China, and Russia in 2009–2010 (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/3/11-1490-FA1.htm; unpub. data).

All 27 viruses characterized were highly pathogenic and had variations in the multibasic cleavage site in the HA molecule (PQRERRRKR) and a 20-aa deletion in the stalk region of NA. They did not have amino acid substitutions that conferred resistance to amantadine or oseltamivir and were associated with the increased virulence of subtype H5N1 viruses in mammalian hosts (*8*).

The intravenous pathogenicity test was conducted by using the A/duck/Korea/Cheonan/2010 virus, the first

Table. Bird species that tested positive for highly pathogenic avian influenza subtype H5N1 in South Korea, 2010–2011

Avian order and species	Scientific name	Sample	No. positive
Galliformes*			
Domestic chicken	*Gallus gallus domesticus*	Carcasses, feces	18
Common quail	*Coturnix coturnix*	Carcasses, feces	1
Common pheasant	*Phasianus colchicus*	Carcasses, feces	1
Domestic turkey	*Meleagris gallopavo*	Carcasses, feces	1
Anseriformes			
Domestic duck*	*Anas platyrhynchos*	Carcasses, feces	32
Mallard	*Anas platyrhynchos*	Cloacal swab specimen,† feces	2
Mandarin duck	*Aix galericulata*	Carcasses, feces	5
Whooper swan	*Cygnus cygnus*	Carcass	1
Baikal teal	*Anas formosa*	Carcasses	2
White-fronted goose	*Anser albifrons*	Carcass	1
Spot-billed duck	*Anas poecilorhyncha*	Carcass	1
Falconiformes			
Eurasian sparrowhawk	*Accipiter nisus*	Carcass	1
Common kestrel	*Falco tinnunculus*	Carcass	1
Strigiformes			
Eurasian eagle owl	*Bubo bubo*	Carcasses	5
Unknown	–	Feces	1
Total			73

*Galliformes and domestic ducks were from poultry farms.
†Source: (*7*).

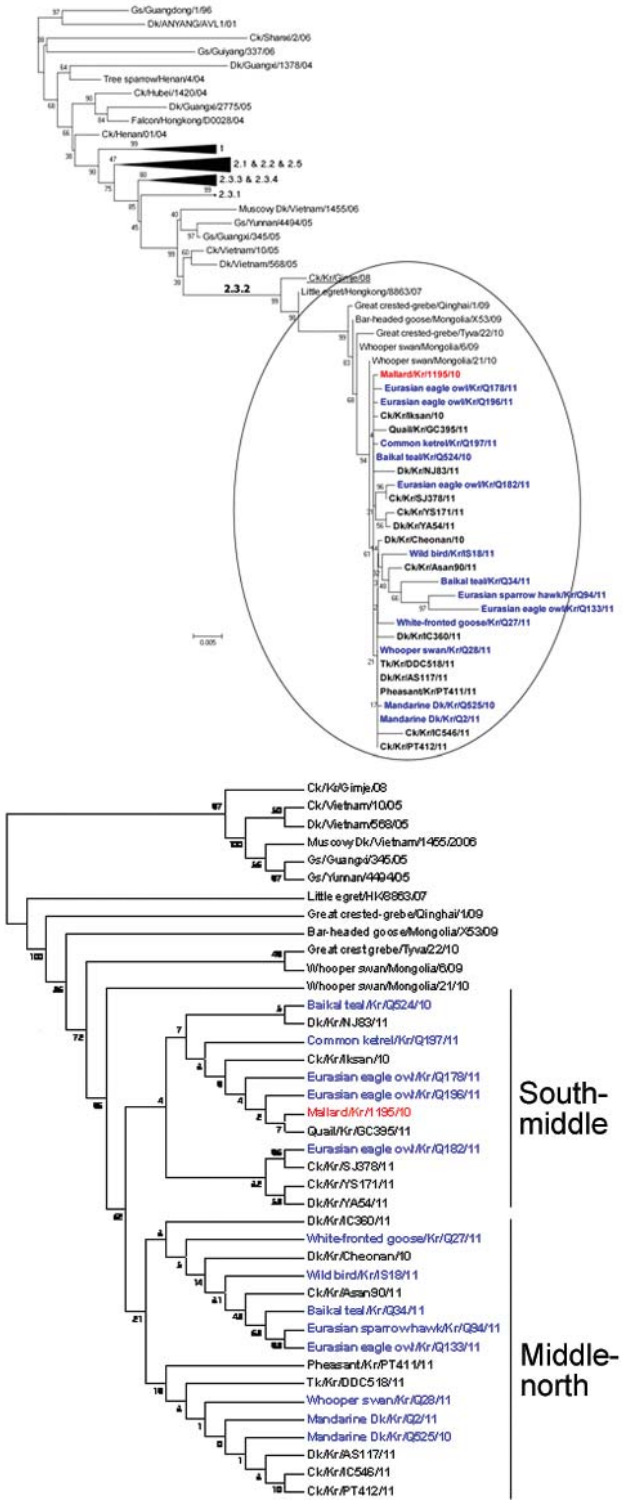

Figure 2. Phylogenetic diagram of hemagglutinin gene of highly pathogenic avian influenza (H5N1) viruses, including viruses isolated in South Korea during 2010–2011. Blue indicates viruses isolated from wild birds, **boldface** indicates isolates from poultry, and red indicates reference virus. Scale bar indicates nucleotide substitutions per site.

isolate from poultry. The intravenous pathogenicity index was 3.0 for chickens.

Conclusion

After the outbreak at Lake Qinghai, China, in 2005, the clade 2.2 viruses spread from Asia to Europe and Africa during 2005–2006 and have been circulating widely in southern Asia, the Middle East, Europe, and Africa for several years. Clade 2.3.2 viruses might spread over an extensive area, similar to clade 2.2 viruses, because clade 2.3.2 viruses are widespread among wild birds and have been continuously evolving in the regions where subtype H5N1 viruses are endemic (*9*). Clade 2.3.2 viruses have circulated in Vietnam and southern China since 2005, and clade 2.3.2 viruses that had undergone reassortment with clade 2.3.4 viruses were isolated from wild birds in Hong Kong in 2007. These reassorted viruses caused HPAI (H5N1) outbreaks in Japan, Russia, and South Korea during 2008 (*6*,*10*–*12*). New 2.3.2 viruses, reassortants that possessed a different acidic polymerase gene from the 2.3.2 viruses of 2007–2008, were isolated predominantly from migratory birds in Mongolia and China in 2009–2010 (*13*,*14*). No HPAI outbreaks occurred in South Korea and Japan in 2009, but outbreaks of a similar virus took place in both countries in late 2010 (*15*). The situation was analogous to outbreaks in 2 countries in 2006–2007 by clade 2.2 HPAI viruses that had been detected in China, Mongolia, and Russia in 2005. Thus, the migratory patterns of infected wild birds might be related to these outbreaks.

During the initial stage of the 2010–2011 outbreak, HPAI viruses were detected in several wild birds, and the viruses were assumed to have been introduced into domestic poultry by migratory birds. The detection of HPAI (H5N1) virus in free-ranging migratory bird might predict a poultry outbreak if biosecurity measures in poultry are inadequate, but during 2006, several European countries reported HPAI (H5N1) virus infections in wild birds without concurrent poultry outbreaks. In the 2010-2011 outbreak in South Korea, the subsequent outbreak cases suggest that the subtype H5N1 virus was spread from farm to farm by humans and associated agricultural practices so that strains of poultry were grouped in sublineages by region.

Clade 2.3.2 subtype H5N1 viruses have been circulating in poultry and migratory birds in Asia and have accumulated antigenic mutations. We can conclude that early detection of HPAI outbreaks and a rapid response to them are essential in controlling the introduction of virus from migratory birds to poultry and in preventing farm-to-farm spread.

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Culturing Stool Specimens for *Campylobacter* spp., Pennsylvania, USA

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In 2010, we surveyed 176 clinical laboratories in Pennsylvania regarding stool specimen testing practices for enteropathogens, including *Campylobacter* spp. Most (96.3%) routinely test for *Campylobacter* spp. In 17 (15.7%), a stool antigen test is the sole method for diagnosis. We recommend that laboratory practice guidelines for *Campylobacter* spp. testing be developed.

Clinical microbiology laboratories play a critical role in surveillance for infectious diseases, including recognition of outbreaks and clarification of disease trends over time (*1*). Few studies have examined laboratory testing practices for common enteric pathogens, particularly *Campylobacter* spp., fastidious organisms that can be difficult to detect because of specimen transport and specific culture requirements (*2*–*4*). With the exception of geographic locations included in the Centers for Disease Control and Prevention's (CDC's) Foodborne Diseases Active Surveillance Network (*5*), surveillance for *Campylobacter* spp. is largely based on passive reporting without additional confirmation by public health laboratories.

Surveillance for *Campylobacter* spp. in Pennsylvania is limited, and only a fraction of isolates are submitted to the state public health laboratory because there is no regulatory requirement to do so. Because testing practices for enteric infections are not standardized (and largely unknown in Pennsylvania), understanding the methods used to diagnose enteric diseases in clinical laboratories is essential if surveillance programs are to be strengthened. We describe the results obtained from a survey conducted among clinical microbiology laboratories in Pennsylvania to assess laboratory testing practices for enteric pathogens, with an emphasis on *Campylobacter* diagnostics.

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The Study

In November 2010, the Pennsylvania Bureau of Laboratories used an automated laboratory information system to send, by fax, a standardized questionnaire to 176 (86.6%) of the 203 clinical microbiology laboratories in Pennsylvania. The questionnaire assessed selected characteristics of stool-testing practices in calendar year 2009, the type of testing for routine stool specimen workup, use of transport media for stool samples, specimen-processing time, and specific laboratory testing practices for *Campylobacter* spp.

One hundred forty-nine (84.7%) laboratories responded to the survey; 144 were hospital based, 3 were reference laboratories, and 2 were public health laboratories. Hospital laboratories had, on average, 5.7 (range 0–43) full-time equivalent employees, of which 5.4 were certified by a credentialing agency, such as the American Society for Clinical Pathology, to perform clinical microbiology testing. Of the 149 responding laboratories, 107 (71.8%) tested stool specimens for enteric pathogens in house.

In Pennsylvania, all 107 laboratories included *Salmonella* and *Shigella* spp. in the routine testing protocol for enteric pathogens, and 104 (97.2%) routinely included testing for *Campylobacter* spp. (Table 1). Sixty-one (57.0%) laboratories included either *Escherichia coli*

Table 1. General laboratory practices for 107 Pennsylvania laboratories performing testing of stool specimens, 2009*

Laboratory practice/method	No. (%) laboratories
Routine stool culture includes the following pathogens	
Salmonella spp.	107 (100)
Shigella spp.	107 (100)
Campylobacter spp.	104 (97.2)
Aeromonas spp.	62 (57.9)
Plesiomonas spp.	59 (55.1)
Vibrio spp.	24 (22.2)
Yersinia spp.	38 (35.5)
Escherichia coli O157 and STEC stool testing	
Routine *E. coli* O157culture	44 (41.1)
Culture plus Shiga toxin antigen testing	17 (15.8)
Special request *E. coli* O157 culture	47 (43.9)
Special request Shiga-toxin antigen	52 (48.6)
Special request stool culture for the following pathogens	
Aeromonas spp.	35 (32.7)
Plesiomonas spp.	34 (31.8)
Vibrio spp.	75 (70.0)
Yersinia spp.	65 (60.7)
Fecal white cell analysis	99 (92.5)
Transport medium†	47 (43.9)
Medium used	
Cary-Blair	41 (87.0)
Not specified	6 (13.0)
No medium used	59 (55.1)
No response	1 (<1.0)
Average time to plating stool specimen after receipt, h	
<4	81 (75.7)
4–8	23 (21.5)
>8	3 (2.8)

*STEC, Shiga toxin–producing *E. coli.*
†Laboratories received specimens in transport media >75% of the time.

O157 cultures or culture plus stool toxin testing. Testing for *Aeromonas* and *Plesiomonas* spp. was included as routine by 57.9% and 55.1% of laboratories, respectively. Most (75.7%) stool specimens were processed within 4 hours after receipt in the laboratory, but only 43.9% of laboratories received specimens in transport media, such as Cary-Blair. Although we did not assess the time from collection of the sample to delivery in the laboratory, given the fastidious nature of *Campylobacter* spp., delays in stool-specimen processing might affect recovery of the organisms, especially if transport medium was not used. Of 107 laboratories in our survey, 99 (92.5%) performed fecal white cell analysis. Fecal white cell analysis has been promoted by some researchers as a useful test for triaging stool samples for culture and for enabling case management decisions (*6*). However, the evidence for using this test in treatment decisions is weak, at best, and it is not recommended for routine use or for decision making regarding type of pathogen or treatment (*7*).

In a College of American Pathologists Quality Probe (CAP Q-Probe) study conducted in 1996 (*3*), 96% of 601 laboratories that responded to a survey reported including *Campylobacter* spp. as part of the routine stool culture workup. In fact, the data on routine culture workup from the current study look remarkably similar to the data from the CAP Q-Probe survey. The CAP Q-Probe survey also showed that 33.9% of laboratories included cultures for *E. coli* O157. A 1999 CDC survey of stool culture practices by 388 laboratories at 9 FoodNet surveillance sites (*2*) found that most laboratories (97%) included *Campylobacter* spp. in their routine stool culture–testing procedure, but the respondents did not comment on specific laboratory testing protocols. All laboratories in the CDC survey performed cultures for *Salmonella* and *Shigella* spp.; however, only 57% of laboratories routinely tested all stool samples for *E. coli* O157. A CDC survey of 264 clinical laboratories at 5 FoodNet sites during 1996 found several laboratory testing differences in culturing for *Salmonella* spp. (*4*).

Among laboratories in the present survey, some variation occurred in the type of culture media used for *Campylobacter* spp. isolation (Table 2), but most laboratories used either cefoperazone-vancomycin-amphotericin agar or *Campylobacte*r blood agar plates (Campy-BAP). Few studies have evaluated multiple media for isolation of *Campylobacter* spp.; however, Arzate Barbosa et al. (*8*) showed that Campy-BAP was significantly less sensitive to a charcoal-containing formulation, charcoal-cefoperazone-deoxycholate agar, for isolating *Campylobacter* spp. Two of the laboratories in our survey reported using a charcoal-based medium, Campy charcoal-based selective medium. In a comparison of several media, Endtz et al. (*9*) also found that Campy-BAP was particularly insensitive for detecting *C. coli* isolates.

Table 2. *Campylobacter*-specific laboratory practices for 107 Pennsylvania laboratories performing testing of stool specimens, 2009*

Laboratory practice/method	No. (%) laboratories
Included in routine testing	104 (97.2)
Special request culture	1 (0.9)
Culture plus antigen testing	1 (0.9)
Perform culture on positive antigen assay	2 (1.9)
*Campylobacte*r antigen testing only	17 (15.8)
Culture broth enrichment usage	
Yes (9 Campy-Thio, 1 GNB, 4 unspecified)	14 (13.1)
No	89 (83.2)
No response	4 (3.7)
Length of Incubation, h	
24	1 (<1)
48	64 (59.8)
72	33 (30.8)
No response	9 (8.4)
Type of medium used for *Campylobacter* culture	
Campy-BAP	65 (60.7)
CVA	30 (27.8)
Skirrow	2 (1.9)
CSM	2 (1.8)
CCDA or mCCDA	0
Not specified or not cultured	8 (7.5)
Temperature used for culture, °C	
37	3 (2.8)
42	96 (89.7)
Not specified	8 (7.5)
Atmosphere used for culture	
Microaerobic, 5% O_2	96 (89.7)
10% CO_2	1 (0.9)
Both	1(0.9)
Not specified	9 (8.4)
Tests used for identification of *Campylobacter* spp.	
Gram stain	96 (89.7)
Oxidase	92 (86)
Catalase	70 (65.4)
Hippurate hydrolysis	51 (47.7)
Naladixic acid/cephalothin disk identification	21 (19.6)
Indoxyl acetate	7 (6.5)
Send to a reference laboratory	6 (5.6)
Other, not specified	33 (30.8)
Performs susceptibility testing	4 (3.7)

*Campy-Thio, *Campylobacter* thioglycollate broth; GNB, gram-negative broth; Campy-BAP, *Campylobacte*r blood agar plates; CVA, cefoperazone-vancomycin-amphotericin; CSM, charcoal-based selective medium; CCDA, charcoal-cefoperazone-deoxycholate agar; mCCDA, modified CCDA.

Several laboratories in our survey used enrichment media for culturing *Campylobacter* spp., although the value of using enrichment media still needs to be addressed (*10*). One laboratory reported using a CO_2 atmosphere for *Campylobacter* culture rather than microaerobic conditions. Although this usage represents a small proportion of laboratories, suboptimal conditions for isolation of *Campylobacter* spp. will result in false-negative results. Whether this practice is more widespread in laboratories outside Pennsylvania is unknown.

Most laboratories used 42°C for incubating *Campylobacter* cultures, the optimum temperature for the most common campylobacters, mainly *C. jejuni* and *C. coli*. The incubation time before the culture is finalized

was 48 hours for 64 laboratories and 72 hours for 33 laboratories. Of note, 1 laboratory incubates the culture for only 24 hours before it reports the results as negative. We also found that laboratory practices vary in performing assays to identify *Campylobacter* once it is isolated. Most laboratories (89.7%) used the Gram stain and oxidase test identify *Campylobacter* spp., but only 51 laboratories (47.7%) used the hippurate hydrolysis test to identify *C. jejuni*. Hippurate hydrolysis is one of the most useful and simplest methods of identifying *C. jejuni* without additional phenotypic testing (*10*). Disk identification methods were used by 21 (19.6%) laboratories, although the usefulness of these tests is limited (*10*). While resistance to antimicrobial drugs is a concern, particularly to fluoroquinolones (*11*), only 4 (3.7%) laboratories tested *Campylobacter* isolates for susceptibility to drugs used for treatment.

In 2009, 18 (16.8%) Pennsylvania laboratories used commercial stool specimen antigen assays for detecting *Campylobacter* spp., and of particular concern, 17 laboratories used these assays in lieu of culture methods. In a previous CDC survey of 388 laboratories concerning practices of stool specimen analysis during 1999, only 1 laboratory used a stool antigen test as a sole diagnostic test for *Campylobacter* spp. (*2*). Taken together, these data suggest that antigen testing for *Campylobacter* spp. in stool specimens is increasing as a sole method for diagnosing *Campylobacter* infection.

Although these data represent a cross-sectional survey of the practices at the time of the survey, laboratory procedures for identifying enteric pathogens, such as *Campylobacter*, typically tend to be stable unless the advantages to implementing new methods are apparent. Given the trend observed, we can reasonably conclude that more laboratories may adopt antigen-detection methods other than stool culture as a means of diagnosing *Campylobacter* infection. A 2011 CDC study that evaluated several different *Campylobacter* stool antigen assays concluded, however, that the performance of stool antigen assays was insufficient as a sole diagnostic for *Campylobacter* spp. (*12*). An increase in stool antigen testing for *Campylobacter* spp. would affect surveillance data by causing the number of cases to be underestimated because of poor testing sensitivity and may also result in hampering outbreak investigation because of the poor specificity of antigen testing. In some jurisdictions (e.g., Pennsylvania) antigen test results are excluded in criteria for the case definition for *Campylobacter* infections, although other public health jurisdictions include such results. Inconsistencies across states, resulting from conflicting evidence (*12*,*13*), present a challenge in interpreting data on the national level.

Conclusions

Standardized guidelines for testing enteric pathogens by clinical laboratories and submission of isolates to public health laboratories can enhance surveillance. For example, in 2009, CDC provided testing guidelines for clinical laboratories for *E. coli* O157 and Shiga toxin–producing *E. coli*. These guidelines recommend both antigen and culture testing of samples from patients with acute community-acquired diarrhea (*14*). Since then, the Pennsylvania Bureau of Laboratories has observed an increase of 48% in the number of laboratories that perform toxin antigen testing. In 2011, 32 sites submitted positive toxin broths, compared with 15 sites in 2009. The characterization of these isolates by public health laboratories has improved surveillance data in addition to enhancing outbreak investigations.

In the CDC survey of clinical laboratories, investigators noted that because almost all laboratories routinely test stool samples for *Campylobacter* spp., regional differences in the incidence of culture-confirmed illness were unlikely to be related to laboratory practices (*2*). Of all fecal pathogens, *Campylobacter* spp. are probably the most difficult for clinical laboratories to isolate, and we found some variation in laboratory practices for isolating these pathogens. Using different methods for testing stool specimens for *Campylobacter* spp. would most likely affect surveillance results. Variation in testing methods would also suggest differences in practices for handling and processing specimens, which would, in turn, affect recovery and detection of *Campylobacter* spp. We conclude that variation in practices likely influences surveillance-based data; however, the extent is unknown.

This study suggests that variation in laboratory practices is a potential problem in surveillance for *Campylobacter* spp. in Pennsylvania. Yet, the differences in laboratory practices for *Campylobacter* spp. are unlikely to be unique to Pennsylvania. These factors need to be considered when surveillance data are interpreted and laboratory training programs are devised. Our study also suggests that laboratory practice guidelines for *Campylobacter* testing should be developed.

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Clinical Significance of *Escherichia albertii*

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Discriminating *Escherichia albertii* from other *Enterobacteriaceae* is difficult. Systematic analyses showed that *E. albertii* represents a substantial portion of strains currently identified as *eae*-positive *Escherichia coli* and includes Shiga toxin 2f–producing strains. Because *E. albertii* possesses the *eae* gene, many strains might have been misidentified as enterohemorrhagic or enteropathogenic *E. coli.*

Attaching and effacing pathogens possess a locus of enterocyte effacement (LEE)–encoded type III secretion system. They form attaching and effacing lesions on intestinal epithelial cell surfaces by the combined actions of intimin, an *eae* gene–encoded outer membrane protein, and type III secretion system effectors. Attaching and effacing pathogens include enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC, respectively) and *Citrobacter rodentium* (*1*,*2*). *Escherichia albertii* have recently been added to this group (*3*–*5*). However, the clinical significance of *E. albertii* has yet to be fully elucidated, partly because it is difficult to discriminate *E. albertii* from other *Enterobacteriaceae* spp. by using routine bacterial identification systems based on biochemical properties (*6*–*9*). A large number of *E. albertii* strains might have been misidentified as EPEC or EHEC because they possess the *eae* gene.

The Study

We collected 278 *eae*-positive strains that were originally identified by routine diagnostic protocols as EPEC or EHEC. They were isolated from humans, animals, and the environment in Japan, Belgium, Brazil, and Germany during 1993–2009 (Table 1; online Technical Appendix, wwwnc.cdc.gov/pdfs/11-1401-Techapp.pdf). To characterize the strains, we first determined their intimin subtypes by sequencing the *eae* gene as described (online Technical Appendix). Of the 275 strains examined, 267 possessed 1 of the 26 known intimin subtypes (4 subtypes—η, ν, τ, and a subtype unique to *C. rodentium*—were not found). In the remaining 8 strains, we identified 5 new subtypes; each showed <95% nt sequence identity to any known subtype, and they were tentatively named subtypes N1–N5. For subtype N1, 3 variants were identified (N1.1, N1.2, and N1.3, with >95% sequence identity among the 3 variants) (Figure 1, panel A).

To determine the phylogenetic relationships of the strains, we performed multilocus sequencing analysis of 179 strains that were selected from our collection on the basis of intimin subtype and serotype (see online Technical Appendix for selection criteria and analysis protocol). Among the 179 strains, 26 belonged to the *E. albertii* lineage (Figure 2). The 26 *E. albertii* strains were from 14 humans (13 from symptomatic patients), 11 birds, and 1 cat. All of the 5 new intimin subtypes were found in the *E. albertii* strains. Intimin subtypes found in other *E. albertii* strains were also rare subtypes found in *E. coli* (*10*). This finding suggests that more previously unknown intimin subtypes may exist in the *E. albertii* population.

We next analyzed the *pheV*, *selC*, and *pheU* loci of the 26 *E. albertii* strains for the presence of LEE elements as described (online Technical Appendix). These 3 genomic

Table 1. Summary of 275 *eae*-positive strains originally identified by routine diagnostic protocols as EPEC or EHEC*

Origin	No. strains
Human, n = 193	
Symptomatic	154
Asymptomatic	7
No information	32
Animal, n = 76	
Bird	38
Pig	31
Cat	1
Deer	1
Bovid	1
Sheep	1
No information	3
Environment, n = 6	6

*EPEC, enteropathogenic *Escherichia coli*; EHEC, enterohemorrhagic *E. coli*.

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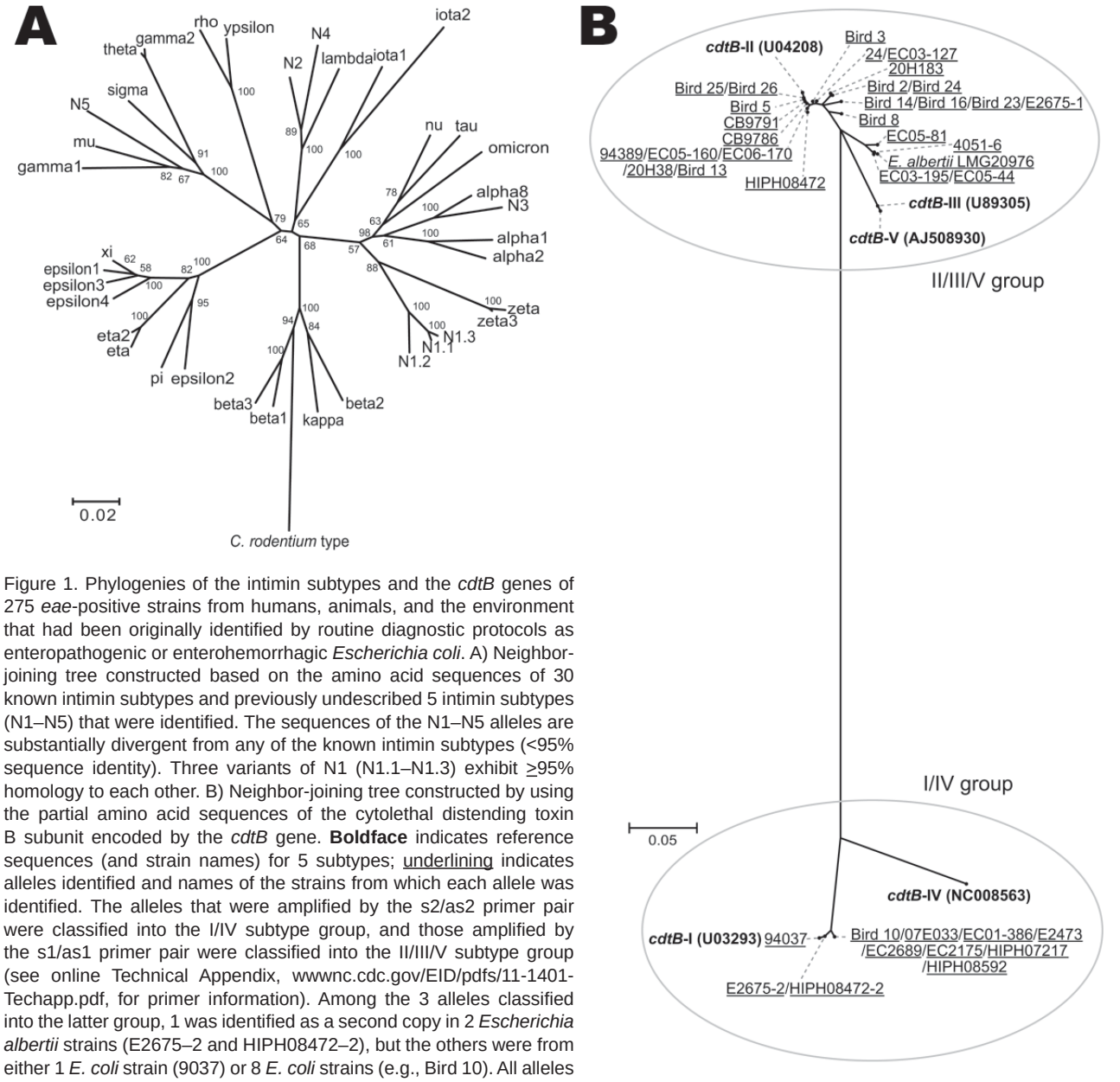

Figure 1. Phylogenies of the intimin subtypes and the *cdtB* genes of 275 *eae*-positive strains from humans, animals, and the environment that had been originally identified by routine diagnostic protocols as enteropathogenic or enterohemorrhagic *Escherichia coli*. A) Neighbor-joining tree constructed based on the amino acid sequences of 30 known intimin subtypes and previously undescribed 5 intimin subtypes (N1–N5) that were identified. The sequences of the N1–N5 alleles are substantially divergent from any of the known intimin subtypes (<95% sequence identity). Three variants of N1 (N1.1–N1.3) exhibit ≥95% homology to each other. B) Neighbor-joining tree constructed by using the partial amino acid sequences of the cytolethal distending toxin B subunit encoded by the *cdtB* gene. **Boldface** indicates reference sequences (and strain names) for 5 subtypes; underlining indicates alleles identified and names of the strains from which each allele was identified. The alleles that were amplified by the s2/as2 primer pair were classified into the I/IV subtype group, and those amplified by the s1/as1 primer pair were classified into the II/III/V subtype group (see online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1401-Techapp.pdf, for primer information). Among the 3 alleles classified into the latter group, 1 was identified as a second copy in 2 *Escherichia albertii* strains (E2675–2 and HIPH08472–2), but the others were from either 1 *E. coli* strain (9037) or 8 *E. coli* strains (e.g., Bird 10). All alleles classified into the II/III/V subtype group were from *E. albertii* strains. Scale bars indicate amino acid substitutions (%) per site.

loci are the known LEE integration sites in *E. coli*. By this analysis, all *E. albertii* strains except 1 (EC05–44) contained the LEE in the *pheU* locus (the integration site in EC05–44 was not identified). This finding indicates that despite the remarkable diversity of intimin subtypes, the LEE elements are preferentially integrated into the *pheU* tRNA gene in *E. albertii*.

Because all *E. albertii* strains isolated so far contained the *cdtB* gene encoding the cytolethal distending toxin B subunit (*8*,*9*), we examined the presence and subtype of the *cdtB* gene as described (online Technical Appendix). This analysis revealed that all *E. albertii* strains except 1 (CB10113) possessed the *cdtB* gene belonging to the II/III/V subtype group (Figure 1, panel B); this finding is consistent with published findings (*9*). In addition, 2 strains (E2675 and HIPH08472) each of which was subtype I , possessed a second *cdtB* gene, (Figure 1, panel B).

We used PCR to further investigate the presence of Shiga toxin genes (*stx*) and their variants (online Technical Appendix) and found that 2 *E. albertii* strains possessed the *stx2f* gene (Figure 2, panel B). Stx2 production by these strains was confirmed by using a reverse-passive latex agglutination

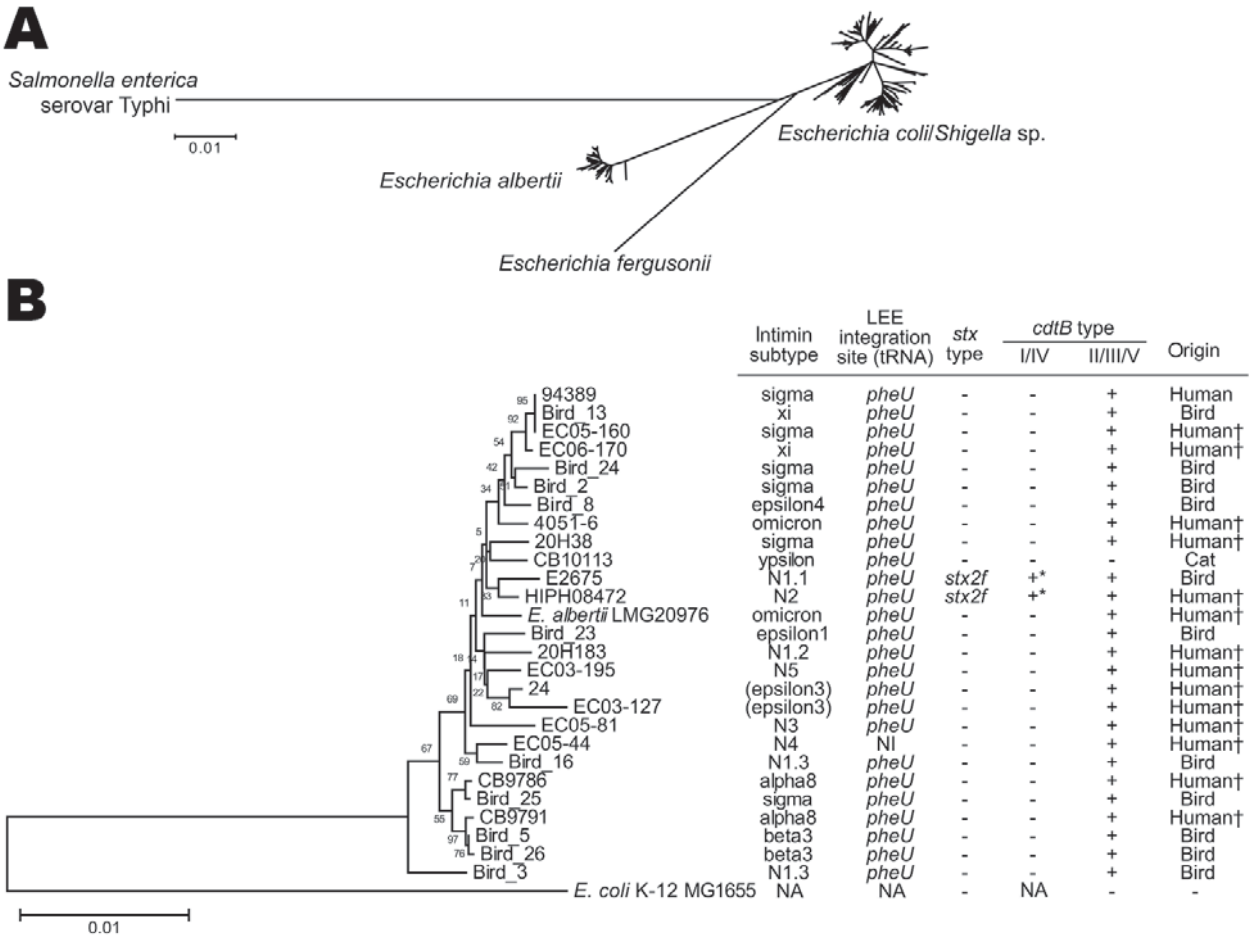

Figure 2. Neighbor-joining tree of 179 *eae*-positive *Escherichia coli* and *Escherichia albertii* strains analyzed by multilocus sequence analysis. The tree was constructed with the concatenated partial nucleotide sequences of 7 housekeeping genes (see online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1401-Techapp.pdf, for protocol details). A) The whole image of the 179 strains examined and 10 reference strains (*E. coli*/*Shigella* sp., *E. fergusonii,* and *Salmonella enterica* serovar Typhi) is shown. B) Enlarged view of the *E. albertii* lineage and the genetic information of the identified *E. albertii* strains. *E. coli* strain MG1655 and *E. albertii* type strain LMG20976 are included as references. There was no phylogenetic correlation between human and animal isolates. The *cdtB* genes indicated by * are classified as subtype I. The strains indicated by † were isolated from patients with signs and symptoms of gastrointestinal infection. LEE, locus of enterocyte effacement; NI, not identified; NA, not applicable. Scale bars indicate amino acid substitutions (%) per site.

kit (online Technical Appendix). The 2 *stx2f*-positive strains were those containing the subtype I *cdtB* gene in addition to the II/III/V subtype group gene: 1 (HIPH08472) was isolated from a patient with diarrhea and the other (E2675) was from a healthy *Corvus* sp. bird (Figure 2).

Last, we examined the phenotypic and biochemical properties of the 26 *E. albertii* strains and compared the results with those obtained in a previous study (*9*) and with those of *E. albertii* type strain LMG20976 (Table 2). To identify features that could discriminate *E. albertii* from *E. coli*, the results were further compared with those of *E. coli* (*11*). Consistent with findings in previous reports (*5*–*7*,*9*), the lack of motility and the inability to ferment xylose and lactose and to produce β-D-glucuronidase were common biochemical properties of *E. albertii* that could be used to discriminate *E. albertii* from *E. coli*, although 1 *E. albertii* strain was positive for lactose fermentation. The inability of *E. albertii* to ferment sucrose has been described as a common feature (*9*); however, a positive reaction to this test was found for 5 (19.2%) *E. albertii* strains. Moreover, approximately half of *E. coli* strains are positive for sucrose fermentation. Thus, the inability to ferment sucrose is not informative. Rather, the inability to ferment dulcitol (all *E. albertii* strains were negative, 60% of *E. coli* strains are positive) may be a useful biochemical property for differentiation.

Conclusions

In the current clinical laboratory setting, a substantial number of *E. albertii* strains are misidentified as EPEC or

Table 2. Comparison of biochemical properties of *Escherichia* spp. strains

Agent or test	26 *E. albertii* strains (this study)†	*E. albertii* LMG20976 (type strain)	*E. albertii* strains (*9*)	*E. coli* (*11*)†
Indole	96.2	–	100	98
Motility	0	–	0	95
Urea	0	–	0	1
ONPG	88.5	+	ND	ND
MUG	0	–	ND	(+)‡
Citrate	0	–	0	1
Acetate	92.3	+	ND	90
Malonate	0	–	ND	0
H_2S on triple sugar iron	0	–	ND	1
Voges-Proskauer	0	–	ND	0
Lysine decarboxylase	100	+	100	90
Ornithine decarboxylase	100	+	100	65
Arginine dihydrolase	0	–	0	17
Glucose, acid	100	+	100	100
Glucose, gas	100	+	100	95
Acid from				
Adonitol	0	–	ND	0
L-arabinose	100	+	100	99
Cellobiose	0	–	ND	2
Dulcitol	0	–	ND	60
Myo-inositol	0	–	ND	1
Lactose	3.9	–	0	95
Maltose	88.5	+	ND	95
Mannitol	100	+	100	100
L-rhamnose	0	–	0	0
Salicin	26.9	–	ND	40
D-sorbitol	57.7	–	V	94
Sucrose	19.2	–	0	50
Trehalose	96.2	+	ND	98
D-xylose	0	–	0	95

*ONPG, ortho-nitrophenyl-β-galactoside; MUG, methylumbelliferyl-β-D-glucuronide; –, negative; +, positive: ND, not determined.
†Average (%) of positive strains.
‡Most *E. coli* strains produce β-D-glucuronidase.

EHEC. Because 13 of the isolates were from patients with signs and symptoms of gastrointestinal infection, *E. albertii* is probably a major enteric human pathogen. In addition, *E. albertii* should be regarded as a potential Stx2f-producing bacterial species, although the clinical significance of Stx2f-producing strains is unknown.

Notable genetic, phenotypic, and biochemical properties of *E. albertii*, which were identified by analyzing the confirmed *E. albertii* strains, are 1) possession of intimin subtypes rarely or previously undescribed in *E. coli*, 2) possession of the II/III/V subtype group *cdtB* gene, 3) LEE integration into the *pheU* tRNA gene, 4) nonmotility, and 5) inability to ferment xylose, lactose, and dulcitol (but not sucrose) and to produce β-D-glucuronidase. These properties could be useful for facilitating identification of *E. albertii* strains in clinical laboratories, which would in turn improve understanding of the clinical significance and the natural host and niche of this newly recognized pathogen. In this regard, however, current knowledge of the genetic and biological properties of *E. albertii* might be biased toward a certain group of *E. albertii* strains because, even with this study, only a limited number of strains have been analyzed. To more precisely understand the properties of *E. albertii* as a species, further analysis of more strains from various sources is necessary.

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Chikungunya Outbreak in Guangdong Province, China, 2010

De Wu, Jie Wu, Qiaoli Zhang, Haojie Zhong, Changwen Ke, Xiaoling Deng, Dawei Guan, Hui Li, Yonghui Zhang, Huiqiong Zhou, Jianfeng He, Linghui Li, and Xingfen Yang

A disease outbreak with dengue-like symptoms was reported in Guangdong Province, China, in October 2010. Testing results confirmed that the pathogen causing the outbreak was chikungunya virus. Phylogenic analysis indicated that this virus was a member of the Indian Ocean clade of the East/Center/South African subgroup of chikungunya virus.

Chikungunya virus (CHIKV) is a mosquito-borne virus that causes fever, headache, rash, nausea, vomiting, myalgia, and arthralgia, and has had a major effect on human health (*1*,*2*). The first human infections caused by CHIKV were reported ≈60 years ago (1952–1953) in eastern Africa (*3*). CHIKV has now become a worldwide public health problem. Although this virus is indigenous to tropical Africa, outbreaks of CHIKV fever have been reported in countries in the Indian Ocean region and Southeast Asia (*4*–*6*). With an increase in global travel, the risk for spreading CHIKV to regions in which the virus is not endemic has increased (*7*).

Multiple sporadic cases of nonindigenous CHIKV infection have been reported in China. In 1987, CHIKV was isolated from the serum of a patient, and antibodies against CHIKV were detected in a second, convalescent-phase patient in Yunnan Province (*8*). Four imported case of CHIKV infection confirmed by reverse transcription PCR (RT-PCR) were detected in Guangzhou and Moming, Guangdong Province, in travelers returning from Sri Lanka and Malaysia in 2008 (*9*,*10*). Another imported case from India was confirmed by using RT-PCR in our laboratory in 2009. We report an outbreak of CHIKV fever that occurred in Guangdong Province, China, in 2010.

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The Study

Guangdong Province is located in a subtropical zone. It has a high relative humidity, an average yearly temperature of 19°C–24°C, and an average yearly rainfall of 1,300–2,500 mm. *Aedes albopictus* mosquitoes are abundant and widespread. However, *Ae*. *aegypti* mosquitoes are found only in western Guangdong Province and not in the region around the city of Dongguan. In the months before the outbreak, the weather in Guangdong Province was particularly rainy.

During September 2010, patients reporting an illness with dengue-like symptoms were recorded by local community clinics in the suburbs of Dongguan, Guangdong Province. For epidemiologic investigation, the Guangdong Center for Disease Control and Prevention defined a clinical case of CHIK fever as a case characterized by sudden onset of fever with arthralgia, maculopapular rash, or myalgia. We identified 173 patients (74 male and 99 female patients) 2–93 years of age in 2 adjacent villages who had similar symptoms. More than 85% of the patients were found in these 2 villages in 97 families (≥2 cases per family in 50 families).

The first patient became ill on September 1, and the number of CHIKV fever cases rapidly increased after September 19 (Figure 1), indicating an outbreak of CHIKV infections in the region. The outbreak spanned 2 months, and the peak occurred at the end of September/early October. None of the patients or any family members reported travel abroad since July 2010. No deaths were reported as a result of the outbreak, and most patients recovered within 1 week after onset of symptoms. No patients were hospitalized; however, several elderly patients reported joint pain after 2 weeks.

Densities of *Ae*. *albopictus* mosquitoes were investigated during the outbreak, and an especially high Breteau index of 77–180 was observed. The abundant rainfall likely resulted in an extremely high mosquito density. To control the outbreak, mosquito control measures were implemented and quarantine of patients with acute disease was enforced.

To identify the pathogen causing the outbreak, we collected 15 serum samples from 12 patients with acute disease and 3 patients with convalescent-phase disease who had dengue-like symptoms. Patient serum was assayed for CHIKV nucleic acid, antibody, and virus. DNA sequence analysis of amplified CHIKV envelope 1 (E1) was performed to infer possible source of transmission. Specimens were tested by real-time RT-PCR for CHIKV (*11*) and dengue virus.

Ten serum samples were positive for CHIKV. Virus-specific IgM and IgG were detected by IgM and IgG capture ELISAs (IBL, Hamburg, Germany). Seven samples were positive for IgM and 1 sample was positive for IgG (Table).

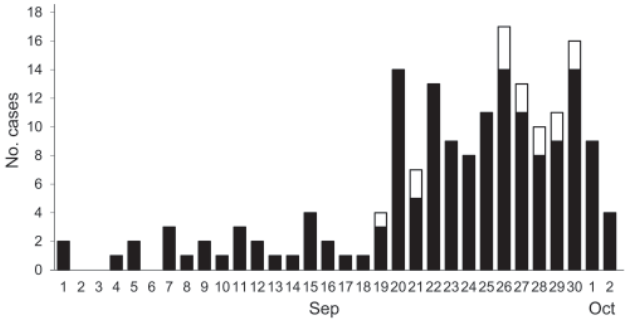

Figure 1. Cases of chikungunya infection in Guangdong, China, September 1–October 1, 2010. Black bar sections indicate clinical cases and white bar sections cases confirmed by molecular analysis.

There were 3 case-patients in whom CHIKV nucleic acid and antibody were found at the same time; 2 of these were in serum samples obtained 3–4 days after these samples were found to be positive for CHIKV IgM. We infer that high cross-reactivity in the ELISAs might contribute to these results.

For phylogenetic analysis, RT-PCR was performed as described (*12*), and 7 amplicons were sequenced. The 10 nucleic acid–positive specimens were placed on C6/36 and BHK-21 cell lines to isolate CHIKV. Serum samples were 2-fold serially diluted 6 times (1:50–1:1,600) in minimal essential medium, and 1 mL of diluted sample was added to each well of a 24-well culture plate. Specimens were incubated at 33°C in an atmosphere of 5% CO_2 and observed daily for ≤7 days for cytopathic effects (CPEs) (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/3/11-0034-FA1.htm). After specimens were incubated for 4–7 days, 3 CPEs were observed on C6/36 and BHK-21 cells. Development of CPEs in C6/36 cells is unusual for CHIKV. However, we observed the effect of C6/36 cell fusion on 3 specimens. We speculate that a virus mutation causes an increase in virulence or changes effects on infected C6/36 cells.

Phylogenetic analysis was performed for partial E1 sequences (7 from this study and 24 from GenBank) by using MEGA5 (*13*). Nucleotide sequences were separated into 3 subgroups corresponding to the 3 globally circulating subgroups (Figure 2). Sequences of the 7 PCR products obtained in this study showed few differences from each other. Paired sequence identity ranged from 98% to 99% at the nucleotide level. Genetic analysis of the 325-nt fragment of E1 genes obtained in this study showed that all 7 sequences clustered in a unique branch within the Indian Ocean clade of the East/Central/South African (ECSA) genotype, and close to Thailand (GQ870312, FJ882911, GU301781), Malaysia (FJ998173), Taiwan (FJ807895), and China (GU199352, GU199353) isolates (98%–99%). The translated E1 gene fragment from 1 of the 7 isolates in this study (China/GD112/2010) had an expected 2-codon deletion. This deletion was also present in the ESCA clade but was not found in the other 6 isolates.

On the basis of sequence analysis, the highest degree of identity was observed with outbreak isolates and the E1 sequence from the Thailand strain (FJ882911) isolated in 2009. Paired identity values were 99% at the nucleotide level and 100% at the amino acid level. Nucleotide substitute analysis showed that a common nucleotide substitution was observed at partial E1 gene site 250 (T′C) in outbreak isolates and FJ882911. This substitution was not observed in other analyzed sequences from GenBank. These results suggested that the virus causing this outbreak was likely transmitted from a source in Southeast Asia and probably evolved from a strain that originated in Thailand.

Table. Characteristics of case-patients and serum sample detection for chikungunya virus, Guangdong, China, 2010*

Case-patient ID no.	Age, y/sex	Date of symptom onset, Sep 2010	Signs and symptoms						Test results		
			Fever	Red face	Headache	Arthralgia	Myalgia	MR	Virus isolation	Real-time RT-PCR/RT-PCR	IgM/IgG
D10112	33/F	27	+	–	–	+	+	+	–	+/+	–/–
D10113	7/M	29	+	+	–	+	+	+	+	+/+	–/–
D10114	62/M	30	+	+	+	+	–	–	+	+/+	–/–
D10115	48/F	30	+	–	–	+	–	+	+	+/+	–/–
D10116	60/M	28	+	–	–	+	+	–	–	+/–	–/–
D10117	39/M	27	+	+	–	+	+	+	–	+/–	+/–
D10118†	59/M	19	+	+	–	+	–	+	ND	–/ND	+/+
D10119	59/F	26	–	+	–	+	–	+	ND	–/ND	–/–
D10120	10/F	26	+	–	+	–	–	+	ND	–/ND	+/–
D10121†	56/F	21	+	+	–	+	–	+	ND	–/ND	+/–
D10122†	24/F	21	+	+	–	+	+	+	ND	–/ND	+/–
D10123	3/F	26	+	–	–	–	–	+	–	+/–	–/–
D10124	60/M	26	+	–	–	+	+	+	–	+/+	–/–
D10125	60/F	29	+	–	–	+	+	+	–	+/+	+/–
D10126	39/M	28	+	–	–	+	+	+	–	+/+	+/–

*All samples were obtained on October 1, 2010. ID, identification; MR, maculopapular rash; RT-PCR, reverse transcription PCR; +, positive; –, negative; ND, not done.
† Convalescent-phase case-patient.

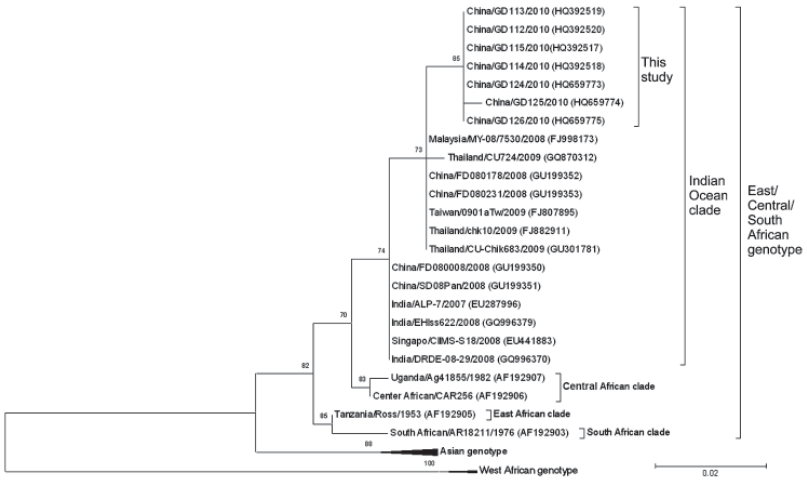

Figure 2. Phylogenetic analysis of partial envelope 1 gene sequences of chikungunya viruses, Guangdong, China, 2010. Numbers along branches indicate bootstrap values. GenBank accession numbers are indicated in parentheses. Scale bar indicates nucleotide substitutions per site.

Conclusions

CHIKV was not endemic to China before 2010. However, in recent years, CHIKV strains from Southeast Asia with the ECSA genotype have been transmitted by infected persons to Guangdong Province. We report an outbreak of CHIKV fever in China. The low severity of the disease and misdiagnosis of dengue fever has likely encouraged widespread transmission of the virus. High-density mosquito populations and an immunologically uninfected population were 2 contributing factors in this outbreak.

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Drug-Resistant Tuberculosis in Zhejiang Province, China, 1999–2008

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To evaluate levels and trends in drug-resistant tuberculosis (TB) in Zhejiang Province, China, we conducted 1 survey in each of 3 years (1999, 2004, and 2008). We found that <5% of new cases were multidrug-resistant TB. The prevalence of multidrug-resistant TB has not increased in new or re-treated cases in this province.

In 2009, China reported results of a nationwide drug resistance survey, which found that 5.7% of new cases of tuberculosis (TB) and 25.6% of re-treated cases were infections with multidrug-resistant TB (MDR TB), i.e., resistance to isoniazid and rifampin (*1*). These results indicated that in 2008 in China, MDR TB developed in ≈100,000 persons, which is ≈25% of the total number of TB cases (440,000) and similar to that in India (*1*).

In China, in addition to the 2008 national survey of TB drug resistance and 10 annual national TB surveys, surveys of TB drug resistance have been conducted in several provinces (*2*–*4*). Zhejiang is one of the few provinces that have conducted a series of cross-sectional surveys from which we can evaluate the scale of the drug-resistance problem at one time point and changes over time.

Data from a sequence of surveys are vital in assessing evolution of resistance to TB drugs in China and ultimately in evaluating the effect of control measures. We report findings of 3 cross-sectional surveys, 1 each of which conducted in Zhejiang in 1999, 2004, and 2008. These surveys included prevalence of MDR TB among TB cases diagnosed in clinics, trends, and risk factors for resistance to isoniazid and rifampin singly and in combination.

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The Study

We would need 784 cases (i.e., $1.96^2_{0.05}0.5(1 - 0.5)/(0.07/2)^2 = 784$) to achieve 95% precision and a margin of error of 7%, and assume no prior knowledge of prevalence of drug resistance, to measure prevalence of any form of drug resistance across the entire province (i.e., not enabling stratification) in each year. Assuming that ≥10% of samples would be lost, we sought to obtain 900 cases. With no prior information for prevalence of drug resistance at different sites, we randomly selected 30 TB treatment centers in 30 counties (among 90 centers in Zhejiang Province) and anticipated that each center would recruit ≥30 sputum smear–positive patients. Three surveys were conducted at the same 30 sites to obtain the same sample size in each year (*5*).

Sputum was collected from persons with suspected TB who came to clinics for a diagnosis. Three sputum samples were obtained (morning, midday, and evening), and patients with ≥2 samples with positive sputum smear results were enrolled in the study. Drug sensitivity tests were performed in provincial reference laboratories by using the percentage method, and results were compared with results for standard drug-resistant strains (*6*). Quality of provincial reference laboratories was ensured by the Republic of Korea Supranational Reference Laboratory (Seoul, South Korea) during 3 surveys, and was evaluated annually by the national reference laboratory in China. Recruitment of consecutive case-patients continued until ≥30 (often more) were enrolled at each site. Each case-patient completed a questionnaire on medical and medication history.

New cases, re-treatment cases, and cases of MDR TB were defined as described by the World Health Organization (*5*). Prevalence of resistance to isoniazid and rifampin or MDR TB was defined as the number of resistant cases in patients who were given a diagnosis of TB in clinics and tested for drug resistance.

Although surveys were not designed a priori to evaluate time trends for prevalence of drug resistance, we investigated trends by using repeated measures analysis of variance and making appropriately cautious conclusions. Logistic regression models were used to investigate factors associated with single drug resistance and MDR TB. Statistical analysis was performed by using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA).

Totals of 1,013, 984, and 938 MDR TB case-patients were recruited from routinely diagnosed new and re-treatment case-patients in 1999, 2004, and 2008, respectively (Table 1). In the 3 surveys, 71%, 74%, and 69% of cases were in men, and 17%, 16%, and 10% were re-treatment cases.

In the 3 surveys, average prevalence of new cases resistant to isoniazid and rifampin and having MDR TB was 10.5% (95% CI 8.4%–12.5%), 5.1% (95% CI 3.6%–6.6%), and 3.3% (95% CI 2.1%–4.5%), respectively. Equivalent

Table 1. Resistance to 2 TB drugs, by patient age, sex, and treatment status in Zhejiang Province, China, 1999, 2004, and 2008*

Year, group	No. (%) patients			
	Total	Isoniazid	Rifampin	MDR
1999				
Total	1,013	138 (13.6)	116 (11.5)	87 (8.6)
Patients 0–14 years of age	12 (1.2)	1 (8.3)	1 (8.3)	1 (8.3)
Patients 15–64 year of age	808 (79.8)	120 (14.9)	97 (12.0)	78 (9.7)
Patients ≥65 years of age	193 (19.1)	17 (8.8)	17 (8.8)	8 (4.2)
Male patients	723 (71.4)	100 (13.8)	81 (11.2)	60 (8.3)
Female patients	290 (28.6)	38 (13.1)	35 (12.1)	27 (9.3)
New cases	841 (83.0)	72 (8.6)	51 (6.1)	35 (4.2)
Re-treatment cases	172 (16.9)	66 (38.4)	65 (37.8)	52 (30.2)
2004				
Total	984	159 (16.2)	94 (9.6)	75 (7.6)
Patients 0–14 years of age	3 (0.3)	1 (33.3)	1 (33.3)	1 (33.3)
Patients 5–64 years of age	764 (77.6)	125 (16.4)	74 (9.7)	58 (7.6)
Patients ≥65 years of age	217 (22.1)	33 (15.2)	19 (8.8)	16 (7.4)
Male patients	730 (74.2)	125 (17.1)	73 (10.0)	61 (8.4)
Female patients	254 (25.8)	34 (13.4)	21 (8.3)	14 (5.5)
New cases	831 (84.5)	102 (12.3)	41 (4.9)	30 (3.6)
Re-treatment cases	153 (15.6)	57 (37.3)	53 (34.6)	45 (29.4)
2008				
Total	938	125 (13.3)	75 (8.0)	56 (6.0)
Patients 0–14 years of age	5 (0.5)	0	1 (20.0)	0
Patients 15–64 years of age	756 (80.6)	103 (13.6)	63 (8.3)	48 (6.4)
Patients ≥65 years of age	177 (18.9)	22 (12.4)	11 (6.2)	8 (4.5)
Male patients	646 (68.9)	91 (14.1)	55 (8.5)	40 (6.2)
Female patients	292 (31.1)	34 (11.6)	20 (6.9)	16 (5.5)
New cases	842 (89.8)	88 (10.5)	43 (5.1)	28 (3.3)
Re-treatment cases	96 (10.2)	37 (38.5)	32 (33.3)	28 (29.2)

*TB, tuberculosis; MDR, multidrug resistant.

percentages among re-treatment cases were 38.5% (95% CI, 28.8%–48.2%), 33.3% (95% CI 23.9%–42.7%), and 29.2% (95% CI 20.1%–38.3%), respectively.

Compared with new cases, re-treatment cases were more likely to be resistant to isoniazid (odds ratio [OR] 1.8, 95% CI 1.2–2.7) and rifampin (OR 6.3, 95% CI 4.2–9.5) or to have MDR TB (OR 9.0, 95% CI 6.4–12.7) (Table 2). Resistance to isoniazid was strongly associated with resistance to rifampin and vice versa (models 1 and 2; OR 19.9, 95% CI 13–31) (Table 2).

Prevalence of resistance to isoniazid and MDR TB tended to be lower in case-patients 15–64 years of age than

Table 2. Factors associated with resistance to tuberculosis drugs, Zhejiang Province, China, 1999–2008*

Characteristic	Coefficient	OR (95% CI)	p value
Model 1: Risk factors associated with resistance to isoniazid			
Constant	–4.10	0.02	
Year	0.23	1.26 (0.87–1.84)	0.221
Rifampin	2.99	19.91 (12.91–30.70)	<0.001
Age 0–14 years	0.89	2.43 (0.20–34.90)	0.51
Age 15–64 years	–0.49	0.61 (0.40–0.94)	<0.05
Sex	0.16	1.17 (0.80–1.71)	0.43
Re-treatment	0.62	1.85 (1.24–2.76)	<0.005
Model 2: Risk factors associated with resistance to rifampin			
Constant	–3.33	0.10	<0.001
Year	–0.53	0.59 (0.38–0.93)	<0.05
Isoniazid	2.99	19.85 (12.92–30.51)	<0.001
Age 0–14 year	–1.74	0.18 (0.02–1.29)	0.09
Age 15–64 year	–0.05	1.02 (0.62–1.66)	0.95
Sex	–0.30	0.73 (0.47–1.16)	0.19
Re-treatment	1.84	6.29 (4.15–9.53)	<0.001
Model 3: Risk factors associated with MDR TB			
Constant	–2.78	0.62	<0.001
Year	–0.16	0.86 (0.61–1.21)	0.384
Age 0–14 year	–0.69	0.52 (0.06–4.36)	0.56
Age 15–64 year	–0.45	0.62 (0.40–0.96)	<0.05
Sex	–0.17	0.86 (0.58–1.23)	0.37
Re-treatment	2.20	9.01 (6.39–12.68)	<0.001

*Reference groups are ≥65 years for age, female for sex, and new for re-treatment. OR, odds ratio; MDR TB, multidrug-resistant tuberculosis.

in those ≥65 years of age (models 1 and 3), but this effect was not shown for rifampin (model 2) (Table 2). There was no significant difference in prevalence of resistance between male and female case-patients across all surveys (models 1–3) (Table 2).

Prevalence of isoniazid and rifampin resistance and MDR TB changed little across the 3 surveys among new and re-treatment cases (Table 1). Time trends for isoniazid prevalence (increase) and MDR TB (decrease) among new cases were marginally significant (F = 3.33, p<0.05, and F = 1.13, p<0.05) but in opposite directions. There were no significant trends in resistance among re-treatment cases or among men or women.

Conclusions

Approximately 25% of the world's MDR TB cases are in China, and it is vital to know whether this number is increasing, decreasing, or stable. There are few data with which to judge trends in drug resistance in China, although a few regions, including Shanghai municipality (*7*), Shenzhen Province (Z. Jia, Y. Yong, unpub. data), and Zhejiang Province (this study), have conducted cross-sectional surveys.

The principal finding of our study is that although drug-resistant TB needs careful management in Zhejiang Province (6% of all TB cases in 2008 were MDR TB and resistance to second-line drugs has also been found in the province; X. Wang, unpub. data), prevalence of isoniazid and rifampin resistance and MDR TB monitored at the same 30 sites changed little during 1999–2008. Although surveys were not designed to detect time trends in drug resistance, prevalence of MDR TB decreased from 8.6% in 1999 to 6.0% in 2008. This decrease in Zhejiang was consistent with changes observed during 2000–2010 in 2 national TB prevalence surveys (*3*,*4*).

Our results contrast with those that MDR TB prevalence increased in nearby Shanghai during 2000–2006. Shen et al. (*7*) reported that introduction of directly observed treatment, short course, and other improved management practices contained spread of drug resistance in Shanghai after 2004, and introduction of similar practices in Zhejiang may also have stopped the increase in MDR TB after 2002. However, the role of improved TB control practices cannot be shown from these data. Nevertheless, possible differences among different sites underline the need for monitoring resistance trends locally and nationally in China. It is also necessary to monitor treatment outcomes, which will be linked to development of drug resistance. In this context, the percentage of patients who sought re-treatment was lower in 2008 than in previous years, which suggested that case management had improved.

The greatest risk factor for resistance to either isoniazid or rifampin in this study was resistance to the other drug, a finding that indicates the high risk for acquiring MDR TB after treatment failure. In this context, and consistent with previous studies (*8*,*9*), prevalence of MDR TB was higher among re-treatment cases than new cases. These results also underscore the need for following good management practices as described by the World Health Organization (*10*).

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Southeast Asian Foot-and-Mouth Disease Viruses in Eastern Asia

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Foot-and-mouth disease (FMD) outbreaks recently affected 2 countries (Japan and South Korea) in eastern Asia that were free of FMD without vaccination. Analysis of viral protein 1 nucleotide sequences indicated that FMD serotype A and O viruses that caused these outbreaks originated in mainland Southeast Asia to which these viruses are endemic.

Foot-and-mouth disease (FMD) is a highly contagious transboundary disease that affects domesticated animals and wildlife in Africa, Asia and parts of South America. Outbreaks of FMD in these disease-endemic regions continuously threaten livestock industries in countries that are free of FMD (with or without vaccination). The causative agent, FMD virus (FMDV), is a small, nonenveloped, picornavirus (genus *Aphthovirus*) that has 7 serotypes. This virus is easily transmitted by movement of infected livestock or animal products, contaminated persons, objects, and aerosols.

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FMDV serotypes O, A, and Asia 1 are endemic to countries in mainland Southeast Asia (Cambodia, Laos, peninsula Malaysia, Myanmar, Thailand, and Vietnam) where regular outbreaks of FMD have been reported (*1*,*2*). Viral protein 1 (VP1) nucleotide sequence data are widely used for phylogenetic analyses (*2*,*3*) and have been used to characterize different FMDV lineages in Southeast Asia and track transboundary movements of the virus. Studies have shown close epidemiologic links between field outbreaks in countries in Southeast Asia (*4*–*6*).

During 2009–2010, the geographic range of 2 FMDV lineages endemic to Southeast Asia (serotypes A and O) expanded into eastern Asia and caused outbreaks in 6 countries in the region. Although outbreaks of FMD caused by serotype Asia 1 have been recently reported (2005–2009) (*7*), there have been no reported outbreaks caused by serotype A in eastern Asia since 1973.

Furthermore, serotype O strains from Southeast Asia have not been detected in countries in eastern Asia since 2004 when samples were sent to the Federal Centre for Animal Health (Vladimir, Russia) from Mongolia (GenBank accession no. JQ070317) (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/3/11-0908-FA1.htm). In 1999–2002, extensive outbreaks caused by the PanAsia strain of serotype O were reported in Japan, South Korea, China, Taiwan, and Russia (*8*–*10*). The purpose of this study was to determine the origins of recent FMD outbreaks in eastern Asia.

The Study

FMDVs characterized by antigen ELISA as serotype A were isolated from samples collected from FMD field outbreaks in Hubei Province, China, and in Gyeonggi-do Province, South Korea. Before FMD cases appeared in China in January 2009, serotype A infections had not occurred in that country China since 1964 (*11*), and until serotype A appeared in South Korea in January 2010, that country had been free of FMD without vaccination (for all FMDV serotypes) since 2002.

Phylogenetic analysis (*12*) showed that VP1 sequences obtained at Lanzhou Veterinary Research Institute (Gansu, China), the National Veterinary Research and Quarantine Service (Gyeonggi Do, South Korea), and the Institute of Animal Health (Pirbright, UK) were genetically similar to sequences obtained during 2008–2009 in Southeast Asia (Thailand and Malaysia), which belonged to the A/ASIA/Sea-97 lineage (online Appendix Figure, panel A) (*6*). Additional FMD outbreaks caused by the same serotype that affected mainly cattle were reported in 6 provinces in China (Shanghai, Jiangsu, Guangxi, Guizhou, Shangdong, and Xinjiang) in 2009; in Beijing in January 2010 (online Appendix Figure, panel A); and in Gyeonggi-do Province, South Korea, in January–March 2010 (6 cases).

Similar outbreaks occurred during 2010 that were caused by an FMD lineage of serotype O that is endemic to Southeast Asia (online Appendix Figure, panel B). As of 2011, outbreaks caused by this serotype continue to occur across a wide region (online Appendix Figure, panel B) and have affected China (including Hong Kong), South Korea, Japan, Mongolia, Russia, and North Korea.

Many FMD outbreaks (n = 292) were reported in Miyazaki Prefecture, Japan (April–June 2010). Fewer outbreaks (n = 18) were reported in China (in Guangdong, Gansu, Shaanxi, Jiangxi, and Guizhou Provinces; and in the autonomous regions of Ningxia, Xinjiang, Uyghur, and Tibet. Three outbreaks were reported in Hong Kong (February–March 2010); thirteen in South Korea (April 2010); nine, including 1 ongoing, in Mongolia (April 2010), and 2 in Russia (July and August 2010).

Japan had been free of FMD (without vaccination) since 2000, and Mongolia and Russia had not reported FMD outbreaks caused by serotype O since 2003 and 2004, respectively. These outbreaks have affected domesticated pigs, cattle, and small ruminants, and have spread to (gazelles, as represented by isolate O/MOG/9/2010) in Domod Province, Mongolia. A new increase in cases of FMD caused by serotype O has recently been reported in South Korea during December 2010 (>100 outbreaks) and in North Korea, and this serotype continues to pose a threat to livestock industries in the region.

VP1 sequences generated in the United Kingdom, China, Japan, South Korea, and Russia and those available in GenBank were analyzed by using by MEGA5 (*12*). Analysis showed that FMDVs causing serotype O outbreaks form 2 genetic clusters related to viruses within the Southeast Asia topotype (O/SEA/Mya-98 lineage), which are usually restricted to mainland Southeast Asia (online Appendix Figure, panel B). There was >97.3% sequence relationship between sequences for FMDVs from China and those from outbreaks in Hong Kong, South Korea, and Japan.

Sequences for viruses collected in Mongolia were distinct (differing by 11.9% nt identity) and more closely related to other viruses collected during 2009 and 2010 in Southeast Asia (Thailand, Vietnam and Malaysia) (*6*). The 2 outbreaks in Russia (July and August 2010), which were caused by viruses from 2 of these sublineages, were located close to the borders with China and Mongolia and separated by ≈250 km. These outbreaks represent 2 distinct introductions of FMD into Russia.

Conclusions

Sequence data implicate regions of mainland Southeast Asia to which FMD is endemic as the source of serotype O and A FMDVs that have caused recent outbreaks in eastern Asia. These events are not unprecedented; a previous instance of spread of FMDV from Southeast Asia into China (Yunnan Province) in 2006 involved the Asia 1 serotype. Furthermore, FMDV O/SEA topotype (Mya-98) was also detected in China in 2003 and in Mongolia in 2004. These findings provide evidence for the porous nature of borders between mainland Southeast Asia and neighboring countries and highlight the continued threat posed by FMD as a transboundary disease in the region. The extent to which viruses have spread into countries that were previously free of FMD (without vaccination) is a cause for concern.

Although VP1 sequence data can be used to characterize the viruses that are causing these outbreaks, further coordination and sharing of sequence data are now urgently required to formally identify transboundary transmission links between affected countries in the region. Complete FMDV genome sequence analyses from these field cases and additional material may provide a suitable approach to reconstruct high-resolution transmission trees and connect clusters of outbreaks (*13*,*14*).

This report describes recent incursion of FMDVs from Southeast Asia into eastern Asia. In vitro vaccine matching data (from the Institue of Animal Health) indicate that currently available vaccine strains (A/May/97and O/Manisa) should protect against representative isolates of these 2 serotypes. However, close monitoring of antigenicity and of the spread of these lineages from Southeast Asia is essential to ensure that risks for further and continued outbreaks can be mitigated.

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Hepatitis E Virus Infection in HIV-infected Persons

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To determine whether hepatitis E virus (HEV) is a cause of hepatitis among HIV-infected persons, we evaluated 1985–2009 data for US military beneficiaries. Evidence of acute or prior HEV infection was detected for 7 (4%) and 5 (3%) of 194 HIV-infected persons, respectively. HEV might be a cause of acute hepatitis among HIV-infected persons.

Among immunosuppressed persons in industrialized countries, hepatitis E virus (HEV) is a cause of sporadic acute viral hepatitis and chronic hepatitis (*1*,*2*). In the United States, liver test results are often abnormal for HIV-infected persons; however, few studies have evaluated whether HEV is a cause of hepatitis in this population (*3*).

The Study

We retrospectively evaluated HIV-infected persons for whom alanine aminotransferase (ALT) levels had increased acutely (≥5 × the upper limit of normal) during the HIV epidemic (1985–2009). Eligible participants were US military beneficiaries (persons entitled to receive care at a military treatment facility) for whom a stored serum specimen, collected from 3 days before ALT increase through 180 days after ALT increase, was available for HEV testing. A case of acute HEV infection was defined as a sample with HEV RNA and/or IgM against HEV or evidence of IgG seroconversion. All samples collected at the time of ALT increase were tested for IgM and IgG against HEV by using commercially available enzyme immunoassays (Diagnostics Systems, Nizhniy Novgorod, Russia) (*4*) and PCR for HEV RNA (*5*). The testing strategy is shown in Figure 1. All positive results were verified by retesting.

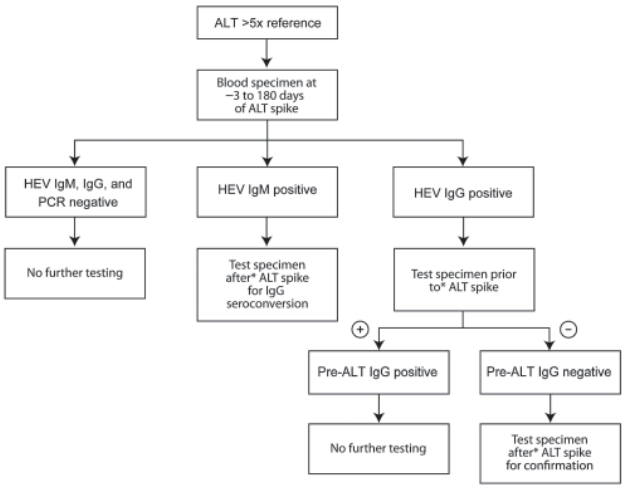

Figure 1. Testing strategy for acute hepatitis E virus (HEV) infection among US military beneficiaries who had had increased alanine aminotransferase (ALT) levels during 1985–2009. +, positive; –, negative.

Statistical analyses included descriptive statistics presented as numbers (percentages) for categorical variables and medians (interquartile ranges [IQRs]) for continuous variables. The percentage of participants with HEV infection was defined as the number with an initial positive result for IgM or IgG against HEV divided by the total number of evaluable study participants. To compare characteristics among those with and without HEV infection, we used Fisher exact testing for categorical variables and rank-sum testing for continuous variables. A multivariate logistic regression model was used to identify factors associated with HEV infection. All analyses were performed by using Stata version 10.0 (StataCorp LP, College Station, TX, USA).

Among 4,410 HIV-infected persons, 458 (10%) had increased ALT levels at least 1 time during 32,468 person-years of follow-up. Among these, serum samples were available for HEV testing for 194 (42%) participants, among whom median age was 34 (IQR 30–40) years, 95% were male, and 42% were white (Table 1). The median ALT level was 440 (IQR 322–812) IU/mL. At the ALT spike, participants had been infected with HIV for a median duration of 5 (IQR 2–9) years; median CD4 cell count was 436 (IQR 239–627) cells/mm^3, median plasma HIV RNA

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[1]Members are listed at the end of this article.

level was 13,581 (IQR 762–71,586) copies/mL, and 28% of participants were receiving antiretroviral therapy.

Samples for HEV testing were available at a median of 27 (IQR 0–104) days after the increase in ALT level. For 13 (6.7%) participants, IgM and/or IgG against HEV were present at the time closest to the ALT increase; antibody prevalence among those with elevated ALT levels did not increase during the HIV epidemic (χ^2 0.76, p = 0.68). The 13 HIV-infected persons who were HEV seropositive (IgM or IgG at ALT spike) were similar to the 181 who were HEV seronegative in terms of demographics, military duty status, laboratory data, and overseas travel (Table 1). HEV-seropositive persons had higher plasma HIV RNA levels (4.7 vs. 4.1 $\log_{10}$ copies/mL, p = 0.04) and the association with lower CD4 cell counts was borderline (median 217 vs. 439 cells/mm^3, p = 0.07). In the multivariate logistic regression model adjusted for age, plasma HIV RNA levels remained significantly associated with HEV seropositivity (odds ratio 1.96 per $\log_{10}$, 95% CI 1.04–3.71, p = 0.04).

Additional testing was conducted for all 13 participants with IgM or IgG against HEV or with HEV RNA at the time of ALT increase (Figure 1). HEV RNA was detected in 1 participant who also seroconverted (IgG) at the time of ALT increase. According to samples collected at or near ALT spike and after ALT spike, 5 more participants seroconverted. One participant had IgM detectable in all 3 samples (at or near ALT spike, after ALT spike, and at follow-up); the participant did not seroconvert, and HEV RNA was not detectable in any sample. In total, 7 (3.6%, 95% CI 1.6%–7.6%) of the 194 HIV-infected persons had

Table 1. Characteristics of 194 HIV-positive US military beneficiaries at time of ALT increase, 1985–2009*

Characteristic†	Total cohort	HEV seropositive,‡ n = 13	HEV seronegative, n = 181	Odds ratio	p value
Demographics					
Age, y	34 (30–40)	35 (32–40)	34 (29–40)	1.01	0.66
Male gender	185 (95)	13 (100)	172 (95)	–	–
Ethnicity					0.4
White	82 (42)	7 (54)	75 (41)	Referent	
African American	77 (40)	4 (31)	73 (40)	0.59	
Hispanic	29 (15)	1 (8)	28 (16)	0.38	
Other	6 (3)	1 (8)	5 (3)	2.14	
Military status					0.32
Active duty	98 (50)	4 (31)	94 (52)	Referent	
Retired	85 (44)	8 (61)	77 (43)	2.44	
Spouse/dependent	11 (6)	1 (8)	10 (5)	2.35	
Overseas travel§	48/127 (38)	1/5 (20)	47/122 (39)	0.4	0.65
Liver function test results					
Timing of blood collection after ALT increase, d	27 (0–104)	31 (7–107)	23 (0–105)	1.0	0.78
ALT level, IU/L	440 (322–812)	367 (241–483)	454 (333–821)	0.99	0.63
AST level, IU/L	262 (183–653)	297 (152–474)	260 (185–693)	1.0	0.66
Clinical conditions					
Gonorrhea§	54 (28%	2 (15)	52 (29)	0.44	0.36
Chlamydia/nonspecific urethritis§	20 (10)	1 (8)	19 (11)	0.7	1.0
Syphilis§	32 (17)	4 (31)	28 (16)	2.38	0.24
Any STI§¶	84 (44)	6 (46)	78 (44)	1.1	1.0
Hepatitis B#					
Prior infection	97 (51)	8 (62)	89 (50)	1.6	0.57
Chronic	30 (15)	3 (23)	27 (15)	1.69	0.43
Hepatitis C#	12 (6)	2 (15)	10 (6)	3.05	0.19
HIV–specific factors					
HIV infection duration, y	5 (1.8–8.8)	5.3 (2.3–10.0)	4.9 (1.7–8.6)	1.01	0.89
CD4 cell count, cells/mm^3	436 (239–627)	217 (9–589)	439 (258–633)	0.79	0.07
<200	40 (21)	6 (46)	34 (19)	Referent	–
200–499	80 (41)	3 (23)	77 (42)	0.22	0.06
≥500	74 (38)	4 (31)	70 (39)	0.32	0.10
Median HIV RNA level, $\log_{10}$ copies/mL§	4.1 (2.9–4.9)	4.7 (3.9–5.4)	4.1 (2.9–4.8)	1.96	0.04
HIV RNA copies/mL					
<1,000	48 (27)	1 (9)	47 (28)	Referent	–
1,000–10,000	36 (20)	2 (18)	34 (20)	2.76	0.57
>10,000	96 (53)	8 (73)	88 (52)	4.27	0.27
Antiretroviral drug use	55 (28)	1 (8)	54 (30)	0.2	0.12

*ALT, alanine aminotransferase; HEV, hepatitis E virus; AST, aspartate aminotransferase; STI, sexually transmitted infection.
†Characteristics are expressed as number (percent) for categorical variables and medians (interquartile range) for continuous variables.
‡IgM and/or IgG against HEV.
§Some data were missing: for overseas travel n = 127; STIs n = 191; HIV RNA level n = 180.
¶Gonorrhea, chlamydial infection, nonspecific urethritis, or syphilis.
#Based on clinical diagnoses; similar results noted when prior hepatitis B virus infection was defined as total positive for hepatitis B virus core antigen, chronic hepatitis B infection as positive for hepatitis B virus surface antigen, and hepatitis C infection as positive for IgG against hepatitis C virus.

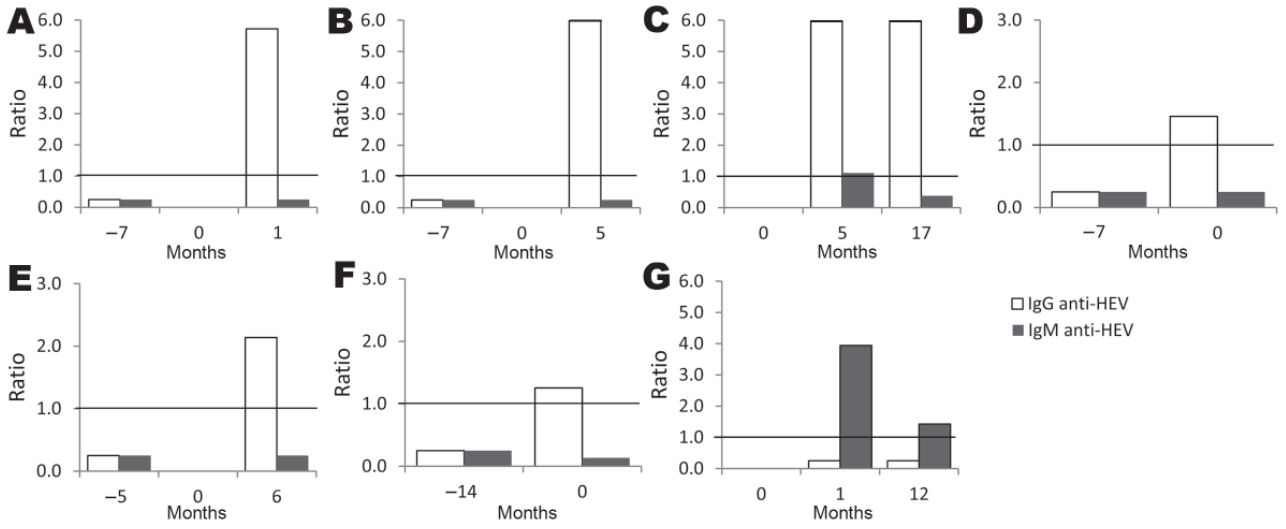

Figure 2. IgM and IgG against hepatitis E virus (HEV) signal/cutoff ratios for 7 HIV-infected US military beneficiaries with acute HEV infection, 1985–2009. Serum specimens were tested for HEV markers before and after alanine aminotransferase spike, indicated by 0.0 on x-axis. Horizontal lines indicate enzyme immunoassay signal/cutoff ratio of 1.0.

evidence of acute HEV infection at time of ALT spike (Figure 2). HEV was deemed not to be the cause of the ALT spike for 5 participants with evidence of prior HEV infection because IgG positivity preceded the ALT increase**.** For 1 participant, IgM was found in only 1 sample; all other samples were negative for IgM, IgG, and HEV RNA. Because of unconfirmed positive follow-up results, this participant was excluded from further analysis.

For the 7 participants with acute HEV infection (Table 2), HEV was not considered during the ALT increase, and HEV testing was not conducted as part of clinical care. No significant differences in clinical or laboratory characteristics were found among the 7 participants with acute HEV infection and those without evidence of HEV infection (data not shown). Chronic HEV infection did not develop in any participant.

Conclusions

HEV infection accounted for 4% of acute liver abnormalities among HIV-infected persons. Overall, HEV was detected in 6% of HIV-infected participants, similar to the 5%–21% reported earlier from the United States (*1*,*6*). Because study participation was limited to persons who had a sample available for HEV testing near the time of ALT increase, we might have missed cases of HEV infection. Overall, on the basis of our study and data from other industrialized countries (*7*,*8*), HEV is a cause of liver abnormalities in HIV-infected persons but does not seem to be more common in this population than in the general population.

HEV seropositivity did not increase over the course of the HIV epidemic. Despite increasing reports of HEV among HIV-infected persons and the general population (*1*,*3*,*7*–*10*), this increase is probably associated with increased recognition and testing. Recent studies in the United States and Europe have shown that HEV seroprevalence is stable or decreasing (*1*,*11*).

HEV infections among HIV-infected persons have been reported (*3*,*7*–*9*,*12*); however, whether this population is at increased risk for HEV infection remains uncertain. Recent studies from Europe suggest that HIV-infected persons or other immunocompromised persons are not at increased risk of acquiring HEV infections (*7*–*9*,*12*). Nonetheless, these groups are at higher risk for chronic HEV infection (*2*,*7*,*13*).

We propose that a diagnosis of HEV infection be considered for persons with viral-like hepatitis. Serologic test results may be negative despite ongoing HEV infection; hence, for HIV-infected persons (especially those with low CD4 cell counts), PCR testing for HEV RNA should be conducted (*7*). Because HEV infection may be fulminant in the presence of underlying liver disease (common among HIV-infected persons) (*14*) and may lead to chronic infection in immunosuppressed persons (*2*,*13*), testing should be considered for these persons as treatment options for HEV infection evolve. Moreover, chronic HEV infection may be averted by reducing the level of immunosuppression (*15*) and use of highly active antiretroviral therapy (*3*,*8*), but more data are needed to support these measures.

HEV infection is a newly defined cause of acute liver dysfunction among HIV-infected persons in the United States. HEV infections do not seem to preferentially occur among HIV-infected persons, suggesting that HIV itself may not be a risk factor for HEV acquisition. HEV infection

Table 2. Characteristics of HIV-positive US military beneficiaries with acute HEV infection at time of ALT increase, 1985–2009*

Patient	1	2	3	4	5	6	7
Age, y	34	35	35	33	44	41	30
Ethnicity	White	African-American	White	African-American	White	African-American	African-American
Duty status	Active	Retired	Active	Retired	Retired	Retired	Retired
Year of ALT increase	2001	1995	2000	2006	1989	1996	1996
Clinical presentation	Nausea, vomiting, abdominal pain, pale stools, dark urine	Fever, malaise, anorexia, diarrhea, dark urine, icterus	Fever, nausea, vomiting, diarrhea, abdominal pain, loss of appetite, malaise	Jaundice	Abdominal pain	Asymp	Asymp
Peak ALT, U/L	489	2,540	282	2,829	229	477	226
AST, U/L	354	988	174	4,273	209	508	130
Alkaline phosphatase, U/L	80	153	99	409	157	125	137
Total bilirubin, mg/dL	3.2	5.0	1.9	5.3	1.6	0.5	1.2
Antibodies against							
Hepatitis B virus core antigen	Neg	Pos	Pos	Pos	Neg	Pos	Pos
Hepatitis B virus surface antigen	Neg	Neg	Neg	Pos	Neg	Pos	Neg
Hepatitis C virus	Neg	Neg	Neg	Neg	Neg	Neg	Pos
History of STI since HIV Infection	None	None	None	Syphilis and chlamydia infections	Gonorrhea	Syphilis	Gonorrhea
Travel overseas	NK	NK	Kuwait	NK	NK	NK	NK
Duration of HIV, y	11	2	<1	13	2	8	9
CD4 count, cells/mm^3	822	517	660	454	753	98	217
HIV RNA level, copies/mL	427	52,929	6,854	52,682	40,000	430,946	8,068
HAART received	Yes	No	No	No	No	No†	No‡
HEV serostatus	IgG sero and HEV RNA	IgG sero	IgM and IgG positivity	IgG sero	IgG sero	IgG sero	IgM with persistent positivity

*All patients were male; none had evidence of acute hepatitis A virus infection or chronic HEV. HEV, hepatitis E infection; ALT, alanine aminotransferase; asymp, asymptomatic; AST, aspartate aminotransferase; neg, negative; pos, positive; STI, sexually transmitted infection; NK, none known; HAART, highly active antiretroviral therapy; sero, seroconversion.
†Patient was receiving monotherapy with zalcitabine.
‡Patient was receiving dual therapy with stavudine and ritonavir.

should be considered among HIV-infected persons with liver abnormalities of unclear etiology.

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Adenovirus-associated Deaths in US Military during Postvaccination Period, 1999–2010

Robert N. Potter, Joyce A. Cantrell, Craig T. Mallak, and Joel C. Gaydos

Adenoviruses are frequent causes of respiratory disease in the US military population. A successful immunization program against adenovirus types 4 and 7 was terminated in 1999. Review of records in the Mortality Surveillance Division, Armed Forces Medical Examiner System, identified 8 deaths attributed to adenovirus infections in service members during 1999–2010.

Adenoviral respiratory disease has been recognized as a frequent cause of illness in the US active duty military population for >5 decades, particularly at basic training installations (*1–5*). A dramatic decrease in adenovirus outbreaks was related to a vaccination program against adenovirus types 4 and 7, which was begun in 1971 (*6,7*). After the only manufacturer of the adenovirus vaccines ended production, adenoviral respiratory disease resurged after the phased cessation and eventual termination of adenovirus vaccinations during 1996–1999 (*3,4,8*). From 1967 through 1998, only 5 adenovirus-associated deaths, all related to types 4 and 7, were reported in active duty military members (*1,9,10*).

The Mortality Surveillance Division, Armed Forces Medical Examiner System (AFMES), has collected perimortem records for active duty service personnel who died since 1998 (*11*). The Mortality Surveillance Division records were evaluated to identify and describe adenovirus-associated deaths in the US military from 1998 through 2010. Case data and information obtained included age, race, sex, branch of military service, training status, year and location of death, adenovirus type, and clinical features.

The Patients

During 1998–2010, AFMES recorded ≈18,500 deaths of active duty personnel for all causes. Of these, ≈14,000 were not attributed to combat or hostile action. Of the noncombat, non–hostile action deaths, 121 (0.9%) were caused by confirmed primary infections, including community acquired acute respiratory infections, meningitis, and chronic viral infections, such as hepatitis. Of these, 8 were attributed to adenovirus respiratory disease as the sole contributor or a co-contributor to death after review of available records by an AFMES pathologist (J.A.C.). For these 8 patients, the mean age was 21.3 years (range 18–32 years). Basic demographic data and adenovirus types are shown in the Table. In addition, most decedents were white (6 patients), 1 was black, and 1 was of unknown race. Brief clinical summaries of each case follow.

Patient A had a respiratory infection with adenovirus type 14, which was confirmed by testing of a nasal wash specimen. Several days later he was hospitalized and required care for multilobar pneumonia and acute respiratory distress syndrome. He died 8 days after admission. The autopsy showed necrotizing pneumonia with diffuse alveolar damage. Postmortem lung tissue was positive for adenovirus 14 by PCR.

Patient B was hospitalized with pneumonia 1 month after receiving a diagnosis of infectious mononucleosis. During a hospitalization of 83 days, her course of illness was complicated by multiple bacterial and fungal infections, acute respiratory distress syndrome, pneumothorax, bilateral deep vein thrombosis of lower extremities, acute renal failure, thrombocytopenia, seizures, acute disseminated encephalomyelitis, and acute hemorrhagic leukoencephalitis. PCR testing of serum on admission was positive for adenovirus. Postmortem lung findings included acute bronchopneumonia changes superimposed on diffuse alveolar damage with interstitial chronic inflammation and fibrosis. Postmortem lung tissue was positive for adenovirus 14 by PCR. This case was previously described as part of an adenovirus 14 outbreak (*12*).

Patient C was hospitalized with a 10-day history of treatment for presumed pyelonephritis, extreme weakness, fever, and nausea. He experienced severe sore throat, shortness of breath, chest pain, and mylagias early in the clinical course. Pericardial effusions and pericarditis were identified on the second hospital day. Results of antemortem microbiologic testing were negative, except for a positive serologic test for adenovirus on hospital day 1 with a serum titer of 128 (normal <8). Eighteen days after admission, he died from progressive respiratory failure. Postmortem findings included diffuse alveolar damage, pleural effusions, and fibrinous pericarditis. No postmortem microbiologic testing was done. The autopsy did not indicate a specific cause of death.

Patient D was treated for pneumonia as an outpatient. Two days later, he was found dead. Results of antemortem

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Table. Demographic characteristics and adenovirus type for US military members who died of adenovirus-associated infections, 1998–2010*

Year	Location	Military branch	Age, y/sex	Recruit	Adenovirus type
2000	Illinois	Navy	21/M	Yes	4, 7
2000	Illinois	Navy	18/M	Yes	ND
2003	Missouri	Army	21/M	Yes	4
2003	Oklahoma	Army	18/M	Yes	4, 7
2003	California	Marine Corps	32/M	Yes	4
2004	Oklahoma	Army	22/M	No	ND
2007	Texas	Air Force	19/F	Yes	14
2009	Texas	Air Force	19/M	Yes	14

*ND, not determined.

microbiologic testing were negative. Postmortem findings included pulmonary edema, congestion, and chronic inflammatory cell infiltrates with pleural effusions. Postmortem lung tissue was positive for adenovirus 4 by PCR.

Patient E was hospitalized and treated for pneumonia and died ≈25 days after admission. The autopsy showed pneumonia with superimposed fungal infection (consistent with aspergillosis) and septic emboli to the heart, brain, liver, spleen, gastrointestinal tract, and kidneys. Autopsy lung tissue was positive by PCR for adenovirus types 4 and 7.

Patient F became unresponsive during training and could not be resuscitated; his temperature was 105.5°F. He had sought treatment 1 week earlier for a presumed viral respiratory illness. The autopsy showed multilobar pneumonia. Lung tissue was PCR positive for adenovirus 4. *Neisseria meningitidis* was also identified by PCR and culture of lung tissue.

Patient G was found unresponsive on day 4 of outpatient treatment for a presumed upper respiratory infection. He was hospitalized and died 11 days later. Antemortem microbiologic testing results were negative. A complete autopsy was not performed, but selected specimens were obtained. A postmortem brain biopsy specimen showed histologic changes consistent with viral encephalitis. Brain and lung tissue were positive by PCR for adenovirus (*13*). Serum specimens had antibodies to adenovirus types 4 and 7 (*13*).

Patient H sought treatment as an outpatient for cough, shortness of breath, and fever of several days' duration. The next day he returned with severe dyspnea, weakness, and a petechial rash. Patient H was noted to be in acute multiorgan failure and died within 12 hours. Antemortem testing was negative for microbiologic agents. The autopsy showed diffuse hemorrhagic pneumonia and diffuse alveolar damage. Results of postmortem viral and bacterial cultures were negative. By report, PCR of postmortem lung tissue was positive for adenovirus. This person also met the criteria for group A streptococcal toxic shock syndrome (*13*).

Of these 8 patients, only 3 had an adenovirus infection identified before death. The remaining 5 patients received a diagnosis on the basis of postmortem tissue examination. None had documentation of adenovirus vaccination.

Conclusions

Eight deaths among members of the US military were attributed to adenoviral respiratory disease by an AFMES pathologist during 1998–2010. All 8 deaths occurred after the adenovirus types 4 and 7 immunization program ended in 1999. Five earlier adenovirus-associated deaths in US military service members have been identified and documented. These occurred in 1967 (1 death), 1972 (3 deaths), and 1974 (1 death) (*1,9,10*). We did not identify any adenovirus-associated deaths in the US military during 1975–1998. Differences in medical surveillance and laboratory capabilities preclude attempting to make meaningful comparisons of the risk for adenovirus-associated death during the prevaccine, vaccine, and postvaccine periods.

The population at greatest risk for adenovirus-associated disease is military recruits. Therefore, the findings shown in the Table are not surprising. Most recruits are young men, and the deaths reported here occurred at the recruits' training centers. Second-generation live oral vaccines against adenovirus types 4 and 7 were approved by the US Food and Drug Administration in March 2011 (*14,15*). After a 12-year absence, the adenovirus vaccination program for military recruits resumed in October 2011. Surveillance of the recruit centers will continue for evaluation of the types 4 and 7 vaccines. The military medical community is hopeful that the protective effect of the vaccines will extend beyond adenovirus types 4 and 7.

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Dr Potter oversees the daily surveillance activities at the Mortality Surveillance Division, Armed Forces Medical Examiner System. His research interests include deaths caused by infectious agents, recruit deaths, and exercise-related deaths.

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Epsilonproteobacteria in Humans, New Zealand

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Using PCR–denaturing gradient gel electrophoresis, we examined 49 fecal samples from healthy volunteers and 128 diarrhea specimens to assess the distribution of Epsilonproteobacteria that might be routinely overlooked. Our results suggest that certain taxa that are not routinely examined for could account for a proportion of diarrhea of previously unknown etiology.

Acute gastrointestinal illness is a major health concern in industrialized countries. In New Zealand, an estimated 4.6 million cases of acute gastrointestinal illness occur every year (*1*). For many known causes of acute gastrointestinal illness, conventional methods of diagnosis are available; yet, ≈80% of diarrhea cases go undiagnosed (*1*,*2*). This lack of data concerning causes of diarrhea hinders the development of intervention strategies.

The class Epsilonproteobacteria is a distinct, diverse bacterial group containing ≈100 taxa (*3*), including *Campylobacter jejuni,* recognized as the most frequent bacterial cause of human gastroenteritis worldwide (*4*,*5*). Many other epsilonproteobacterial species have been associated with diarrhea, but accurate estimates of the prevalence and role of individual species and proof of a primary pathogenic role have been elusive. Methods commonly used for isolating *C. jejuni* are not well suited for many other species, and the complex taxonomy of the group makes identification difficult (*4*). Nevertheless, the body of evidence supporting a causative role for several taxa has grown (*5*–*7*). During September 2007–June 2009, we examined fecal samples from healthy volunteers and from patients with diarrhea in New Zealand by using a PCR–denaturing gradient gel electrophoresis (DGGE) method shown to detect and identify Epsilonproteobacteria (*8*).

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The Study

Healthy volunteers were recruited during 2 separate periods in September 2007 and June 2009. The first recruitment period (18 specimens) did not specifically exclude volunteers who had had gastrointestinal disturbances in the 10 days before sampling. The second (31 specimens) were healthy volunteers who had normal bowel habit, no diarrheal disease for ≥6 weeks, no antimicrobial drug therapy for ≥4 weeks, and no medication except for asthma inhalers or antihypertensive or contraceptive medication. Volunteers defecated into a bottle suspended in the lavatory bowl with tissue paper to prevent it falling into the water. The Upper South A Regional Ethics Committee (Christchurch, New Zealand) and the multi-ethics committee of the Ministry of Health, New Zealand (MEC/08/52/EXP), granted ethics approval for the study.

Diarrhea specimens (submitted without patient details during 2008) were distributed among 3 categories, as follows. First were 32 samples in which no causal agent was found; pathogens were excluded by routine examination with conventional diagnostic techniques for bacteria, parasites, and norovirus at Southern Community Laboratories. Second were 57 samples in which a specific causal agent was not found; samples were examined at the Institute of Environmental Science and Research (ESR, Christchurch, New Zealand) reference laboratory by using conventional methods for a specific pathogen at the request of the submitting laboratory. Third were 39 samples in which a known gastrointestinal pathogen had been detected at ESR.

Samples were refrigerated for 24–48 h before DNA extracts were prepared by using the revised protocol described in the ZR Fecal DNA Kit (Zymo Research, Irvine, CA, USA). Fecal DNA extracts were examined with the PCR-DGGE for Epsilonproteobacteria as described (*8*). After visualization of the PCR-amplified product, individual DNA bands were excised and then DNA was eluted by diffusion into buffer and reexamined by PCR to obtain partial 16S rDNA amplicons for sequencing. Sequences were edited (primer sequences were removed) and subsequently compared with those in GenBank by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Comparisons were made during April 2011. Assignment of sequences to a taxon was based on the E (expect) values obtained and on expert opinion of the taxonomic distance between the most likely matches obtained. BLAST matches yielded E-values ranging from 7.13e-62 to 2.26e-124.

Of 177 samples from the healthy volunteers and patients with diarrhea, 159 contained Epsilonproteobacteria, of which 20 contained >1 taxa (Table). *C. rectus/showae*, *C. sputorum*, *C. upsaliensis*, *Helicobacter pullorum*, and *H. pylori/heilmannii/nemestrinae* were detected in 11 (8.6%) of the 128 diarrhea samples but not in fecal specimens from

Table. Prevalence and distribution of Epsilonproteobacteria taxa in fecal samples from 49 healthy volunteers and 128 persons with diarrhea, New Zealand*

Taxa	SCL	ESR−	ESR+	Vol
Campylobacter jejuni/coli complex	0	3	0	4
C. ureolyticus	3	10	1	12
C. concisus	17	27	16	26
C. curvus	0	1	0	1
C. gracilis	4	10	4	3
C. hominis	4	6	1	8
C. rectus/showae	2	1	2	0
C. sputorum	0	0	1	0
Helicobacter sp.	1	0	0	0
C. upsaliensis/helveticus	0	2	0	0
H. pullorum	0	2	0	0
No Epsiloproteobacterium	6	3	8	1

*Detected by PCR–denaturing gradient gel electrophoresis. SCL, samples examined by Southern Community Laboratories (Christchurch, New Zealand) found negative for all common pathogens; ESR–, diarrhea samples screened for specific pathogens by the Institute of Environmental Science and Research (Christchurch) at the request of the submitting laboratory and found negative; ESR+, diarrhea samples screened for specific pathogens by the Institute of Environmental Science and Research at the request of the submitting laboratory and found positive; vol, samples from volunteers with no known recent history of gastrointestinal illness. Specific pathogens found in ESR+ samples included *Cryptosporidium* spp., *Giardia* spp., norovirus, *Bacillus cereus*, toxigenic *Staphylococcus aureus*, and toxigenic *Clostridium perfringens*.

volunteers. *Cryptosporidium* spp. also were present in 2 diarrhea samples in which *C. rectus/showae* were detected. In addition, norovirus was detected in the *C. sputorum*–positive sample. *C. curvus* and *C. jejuni/coli* were found in diarrhea samples examined previously for specific pathogens only, as well as in 1 and 4 samples, respectively, from human volunteers. Sequences of the *C. concisus* complex, *C. ureolyticus*, *C. hominis*, and *C. gracilis* occurred frequently in samples from all study participants.

We used χ^2 analysis to determine whether the proportion of the 32 diarrhea samples subjected to a complete pathogen screen differed significantly from fecal samples from the second group of 31 volunteers in which these organisms were detected. The pathogen screen contained *C.* (*Bacteroides*) *ureolyticus*, *C. concisus* complex, *C. hominis*, or *C. gracilis*. We found no statistical difference between the proportions detected in these 2 groups of samples.

Conclusions

Although many species belonging to the Epsilonproteobacteria have been associated with gastrointestinal illness for decades, few are proven primary pathogens. By using PCR-DGGE to examine feces from healthy volunteers and patients with diarrhea, we aimed to indicate which taxa might be present as commensal flora and which might have a causal role. *C. upsaliensis/helveticus*, *H. pullorum*, *H. pylori/heilmannii/nemestrinae* were all detected in diarrhea specimens but not in specimens from healthy volunteers; no other pathogen was found in these diarrhea specimens. *C. upsaliensis* is presumed to be pathogenic (*7*). *H. pullorum* is poorly studied but bears sufficient similarity to diarrheogenic *C. jejuni* at the molecular–genetic level (*9*) to support a causative role in gastrointestinal disease, at least for some strains. Poultry harbor *H. pullorum* (*10*), and thus represent a vector for foodborne transmission. Use of the PCR-DGGE method (*8*) on domestic drinking and commercial scald water used in New Zealand chicken production detected *H. pullorum* in 2 of 5 samples tested (data not shown). Although detection of the taxa *H. pylori/heilmannii/nemestrinae* might simply represent gastric carriage (the natural environment for these species), perhaps gastric disturbances result in diarrheal sequalae. Even though *C. rectus/showae* were also detected only in diarrhea samples, 2 specimens also harbored *Cryptosporidium* spp. In addition, norovirus was detected in the diarrhea sample in which *C. sputorum* was found.

We detected *C. jejuni/coli* in 3 samples examined for, but not containing, *E. coli* O157, which indicates that some cases of campylobacteriosis go undiagnosed. To our surprise, we detected *C. jejuni/coli* in several fecal samples from healthy volunteers. This detection might represent asymptomatic carriage of *C. jejuni/coli,* a phenomenon more commonly observed in developing countries where repeated exposure during a prolonged period results in tolerance (*11*). The high incidence of infection in New Zealand makes this hypothesis credible.

C. concisus was the most frequently encountered species in this study and occurred in participants from both groups. Strains identified as *C. concisus* with conventional methods might belong to genetically distinct but phenotypically indistinguishable genomospecies differing in their pathogenic potential (*12*). The PCR-DGGE used here cannot differentiate *C. concisus* genomospecies; thus strains detected in volunteers and strains found in diarrhea samples might represent distinct genomospecies with different pathogenic potentials.

We detected *C. hominis*, *C. gracilis*, and *C. ureolyticus* in fecal samples of healthy volunteers and patients with diarrhea. *C. hominis* has long been considered a commensal (*13*). A molecular study found *C. ureolyticus* in 83 (23.8%) of 349 *Campylobacter* spp.–positive diarrhea samples, but no healthy controls were examined (*14*). Our data suggest these species are unlikely causes of diarrhea.

Our results indicate that certain Epsilonproteobacteria that are not routinely examined for account for a proportion of diarrhea cases of previously unknown etiology. PCR-DGGE is a useful tool to study the prevalence and distribution of these bacteria. *C. concisus* genomospecies are frequently detected in human disease (*5*,*15*; this study); elucidation of their pathogenicity should be considered a public health research issue.

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Nonculture Diagnostic Tests for Enteric Diseases

Timothy F. Jones and Peter Gerner-Smidt

The diagnosis of acute gastroenteritis (AGE) has traditionally been based on culture results of feces from patients with diarrhea. Virtually everything we know about disease and the epidemiology of enteric pathogens, such as *Salmonella* spp., Shiga toxin–producing *Escherichia coli* (STEC), e.g., O157, and *Campylobacter* spp., has been generated from the study of patients with culture-confirmed infections. However, this pattern may be changing because AGE diagnostics are moving away from culture toward rapid nonculture methods. These infections are mainly foodborne and therefore preventable, and it is of paramount importance that public health surveillance for these infections is consistent and reliable.

Reports by Stigi et al. (*1*) and M'ikanatha et al. (*2*) in this issue of the journal on changing laboratory practices for the testing of stool specimens illustrate this point, and raise serious issues for clinicians and the public health community. These 2 studies examined different pathogens, but both highlight the need to adapt policies and practices to keep up with rapid technical changes in the clinical laboratory world.

As Stigi et al. demonstrate, laboratory practices of testing for STEC are changing dramatically (*1*). In their study during 2005–2010, the number of laboratories performing antigen tests for Shiga toxin increased 8-fold. Although more than half of fecal specimens tested in Washington State, USA, were assayed for Shiga toxin, it is worrisome that 13% were tested for toxin alone, without concomitant culture. M'ikanatha et al. similarly reported that in Pennsylvania, USA, the number of laboratories submitting STEC antigen–positive culture broths more than doubled from 2009 through 2011, which indicated a major change in diagnostic practice (*2*).

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For clinical purposes, it is generally sufficient to know that an STEC is present because management of an individual case is seldom dependent on additional subtyping. An unfortunate consequence of the increasing use of nonculture diagnostic tests for AGE is that they do not provide isolates for additional testing by public health laboratories. Public health has traditionally relied upon cultured organisms for further characterization, including subtyping for epidemiologic purposes. For this reason, in 2009 the Centers for Disease Control and Prevention published guidelines for the diagnosis of STEC by clinical laboratories (*3*). These guidelines recommend simultaneous culture for STEC O157 and for detection of Shiga toxin and forwarding of isolates or Shiga toxin–positive broths to public health laboratories for further characterization.

The study by M'ikanatha et al. also examined laboratory practices regarding identification of *Campylobacter* spp. (*2*). In their study, use of nonculture diagnostic tests was substantial: in 17% of laboratories that used commercial fecal antigen tests for detecting *Campylobacter* spp.; all but one used only the antigen assay. As with STEC, such practices result in no isolates being available for additional testing by public health laboratories. For *Campylobacter* spp., this approach may be of somewhat less concern because in many states this pathogen is not reportable, molecular subtyping is not routinely performed, and outbreaks are relatively rare. However, it is emblematic of the overall trend away from culturing in commercial laboratories.

With the inexorable shift away from traditional laboratory methods in the clinical world, public health laboratories will increasingly face the challenge of having to develop the capacity to routinely isolate, characterize, and subtype pathogens from clinical specimens to gather the information on which epidemiologists have become so dependent. For example, if clinical laboratories diagnose STEC without culture results, outbreak detection will be more difficult. Molecular subtyping is now relied on heavily to identify small clusters of potentially related

infections before the number of cases is epidemiologically evident. In many respects, loss of this resource would be a step 2 decades backward to the pre–pulsed-field gel electrophoresis era.

In addition, implementation of nonculture diagnostic methods introduces a bias in surveillance of AGE. For example, public health surveillance for STEC has traditionally focused on *E. coli* O157, and culture confirmation is still required for counting these cases in national data (*4*). In 2000, non-O157 STEC became nationally reportable, but numbers remained low until toxin testing became widely available. The recent rapid increase in reported non-O157 STEC is not unique to the studies reported in this issue (*5*–*7*), and as those cases have increased, the number of reported *E. coli* O157 cases has decreased. It is likely that a substantial proportion of STECs identified only by antigen testing are O157 (50% in 1 study) (*5*). Therefore, it is necessary to take changing diagnostic methods into account if trends in AGE are to be assessed accurately.

The sensitivity, specificity, and associated positive and negative predictive values of antigen tests for enteric pathogens also differ from those of culture, which makes it difficult to include the results of such tests as part of the definition of reportable diseases. Although such concerns are valid, policies must be developed that take into account changes in laboratory practices when evaluating trends in these pathogens. Scientific rigor is needed, but one must remember that clinicians respond to test results that they receive, and they trust that commercially performed tests are reliable. Regardless of how accurate is the testing method, the patient is being notified and treated on the basis of these test results, and public health officials must respond promptly on the basis of the information available. Although it is reasonable to keep data on cases of diseases diagnosed by using culture and nonculture methods separate, these data should be monitored so as not to lose essential information regarding the incidence of these diseases.

The repertoire of methods and targets for fecal testing is rapidly expanding. Molecular diagnostics are increasing; improvements include multiplex and quantitative PCR, fluorescence in situ hybridization, and metagenomic analyses (*8*–*10*). It is likely that many isolate-based methods for serotyping, pulsed-field gel electrophoresis, and antimicrobial drug testing will need to transition to sequence-based techniques to remain epidemiologically useful.

If these challenges are to be overcome, several issues must be addressed. Decisions about implementation of new methods in clinical laboratories are often based mostly on cost and ease of use, whereas parameters such as their sensitivity, specificity, and relevance to public health surveillance are less likely to be emphasized. However, all these aspects should be considered carefully before new diagnostic methods are implemented in clinical laboratories. If this does not happen, surveillance for foodborne AGE is likely to become unreliable and unsuitable for guiding public health actions in the future.

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Escherichia coli Producing CMY-2 β-Lactamase in Retail Chicken, Pittsburgh, Pennsylvania, USA

To the Editor: Rates of resistance to various antimicrobial drugs are rapidly increasing in *Escherichia coli*, not only in health care settings but also in the community. The food supply is suspected as a potential source of antimicrobial-resistant *E. coli* strains, which include cephalosporin-resistant *E. coli* found in retail meat products and other types of food (*1*).

We reported a high prevalence of cephalosporin-resistant *E. coli*, most of which produced CMY-2 β-lactamase, among retail poultry products in Pittsburgh, Pennsylvania, USA during 2006–2007 (*2*). CMY-2 is the most commonly acquired ampicillin C (AmpC)–type β-lactamase found in *E. coli* that cause human infections (*3*). The aim of this study was to investigate whether cephalosporin-resistant *E. coli* are present in retail raw meat and ready-to-eat meat products in our area 5 years after our previous study and define subtypes of concern.

A convenience sampling of 104 raw ground meat products from 3 local grocery stores in Pittsburgh was performed during February–April 2011. Items purchased were samples of all available deli counter ground meat, all fresh sausages prepared in stores at the deli counter, and selected uncooked commercially packaged fresh and frozen sausages. Types of meat items were chicken (n = 22), turkey (n = 10), lamb (n = 2), pork (n = 43), and beef (n = 27).

Approximately 10 g of each sample was excised and suspended in 10 mL of nutrient broth. After being incubated overnight at 37°C, 10 μL of broth was plated on MacConkey agar plates containing 2 mg/L of cefotaxime or ceftazidime, and the plates were incubated overnight at 37°C. Lactose-fermenting colonies were identified as *E. coli* by using standard biochemical methods, which included sulfide indole motility, growth on triple sugar iron medium, oxidase activity testing, and the API20E system (bioMérieux, Durham, NC, USA) as needed.

Antimicrobial drug susceptibility was determined by using the disk diffusion method (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions and interpreted according to the criteria of the Clinical and Laboratory Standards Institute (*4*). Isolates were screened for extended-spectrum β-lactamase (ESBL) production by using the double-disk diffusion method and for acquired *ampC*-type β-lactamase genes by using multiplex PCR (*4,5*).

Phylogenetic groups (A, B1, B2, and D) were determined as reported (*6*). Screening for sequence type (ST) 131 was conducted by using PCR and confirmed by using multilocus sequence typing (*7,8*). Pulsed-field gel electrophoresis was performed to determine clonal relationships by using *Xba*I and the protocol available through the PulseNet (www.cdc.gov/pulsenet/protocols.htm). Banding patterns were analyzed by using BioNumerics software version 6.01 (Applied Maths, Sint-Martens-Latem, Belgium).

Among 104 meat samples, 9 contained cephalosporin-resistant *E. coli*, resulting in an overall prevalence of 8.7% (95% CI 4.0%–15.8%). Cephalosporin-resistant *E. coli* was isolated from 7 (31.8%) of 22 chicken, 1 (10.0%) of 10 turkey, and 1 (2.3%) of 42 pork samples. No cephalosporin-resistant *E. coli* was detected from beef and lamb samples. Incidence of samples with cephalosporin-resistant *E. coli* was lower than in our previous study (*2*). This finding may be caused by different types of samples included in the studies or a true decrease in incidence.

Features of cephalosporin-resistant *E. coli* identified are summarized in the Table. None produced ESBL, but all 9 isolates were positive for the CMY-2 β-lactamase gene and positive results were confirmed by sequencing. CMY-2 is the most commonly observed acquired AmpC β-lactamase in *E. coli* and nontyphoidal *Salmonella* species in meat products (*9*).

As for the phylogenetic groups, 6 (66.7%) of 9 cephalosporin-resistant *E. coli* belonged to group A, which is generally considered to be a commensal phylogenetic group. However, 1 group B2 isolate from a chicken sample was identified as ST131. The CMY-2 gene was located on an IncI1-type plasmid and was transferable to another *E. coli* strain by conjugation for this isolate. Two group D isolates from chicken that belonged to ST117, which has been reported in

Table. Characteristics of 9 cephalosporin-resistant *Escherichia coli* isolates in retail meat, Pittsburgh, Pennsylvania, USA*

Sample no.	Origin	Phylogenetic group	Susceptibility					
			CTX	FOX	FEP	CIP	GEN	TET
FD13	Chicken (ground)	D	R	I	S	S	S	S
FD14	Pork (sausage)	A	R	R	S	S	S	R
FD42	Chicken (sausage)	B2	R	R	S	S	S	S
FD44	Chicken (thigh)	A	R	R	S	S	S	S
FD45	Turkey (sausage)	A	R	R	S	S	R	R
FD56	Chicken (sausage)	D	R	R	S	S	S	S
FD63	Chicken (sausage)	A	R	R	S	S	S	S
FD72	Chicken (sausage)	A	R	R	S	S	S	R
FD95	Chicken (liver)	A	R	I	S	S	S	S

*All isolates were CMY-2 type. CTX, cefotaxime; FOX, cefoxitin; FEP, cefepime; CIP, ciprofloxacin; GEN, gentamicin; TET, tetracycline; R, resistant; I, intermediate; S, susceptible.

ESBL-producing isolates of human and animal origins (*10*); no clonality was observed for the other 7 isolates by pulsed-field gel electrophoresis,

E. coli ST131 has emerged as a worldwide pathogen and causes mainly community-onset extraintestinal infections. Although the pandemic spread of *E. coli* ST131 was first identified in isolates producing CTX-M-15 ESBL, it is increasingly recognized that isolates belonging to this clone may also harbor other drug resistance determinants. Among acquired AmpC β-lactamases, CMY-2 has been most frequently reported in ST131 from human clinical isolates (*3*). Infections caused by CMY-producing *E. coli* are common but underrecognized because of the lack of standardized detection methods (*2*).

Given the rapid global spread of the ST131 clone and the possibility of its transmission from food animals to humans, coupled with an abundance of CMY-2–encoding plasmids in poultry environments, *E. coli* ST131 producing CMY-2 β-lactamase may have potential to spread to humans. Our results also show that *E. coli* producing CMY-2 continues to be found commonly among retail chicken products in our study area.

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Ilheus Virus Infection in Human, Bolivia

To the Editor: Ilheus virus (ILHV) was first isolated from mosquitoes of the genera *Ochlerotatus* and *Psorophora* near Ilheus, Bahia, Brazil, in 1944 (*1*). After its discovery, the virus was also isolated from other mosquito species, including the genera *Culex*, *Sabethes*, *Haemagogus*, and *Trichoprosopon*, and from a variety of birds in different countries in Latin America (*2*). Only a few reports describe isolation of this virus from humans in Central and South America with symptoms ranging from subclinical to severe febrile disease (*2–6*). In mild cases, patients often reported gastrointestinal or respiratory symptoms lasting ≈1 week. In severe cases, either the central nervous or cardiac system can be affected. However, long-term sequelae or deaths have not been described. No epidemics attributed to ILHV have been reported.

In November 2005, a 15-year-old boy (farmer) sought medical attention in a health clinic in Magdalena, Bolivia, after having fever for 5 days. The patient's symptoms included malaise, asthenia, conjunctival

injection, vesicular rash, facial edema, arthralgia, myalgias, bone pain, abdominal pain, headache, and earache. Signs of cardiac, neurologic, or renal damage were not detected. A blood specimen was obtained during the clinic visit, and a convalescent-phase sample was obtained 24 days after onset of symptoms. At that follow-up visit, the patient reported a full recovery from his symptoms. Both samples were sent to the Naval Medical Research Unit No. 6 in Lima, Peru, for processing as part of a clinic-based study to determine the etiology of febrile illnesses in Bolivia (*7*). The study was approved by the Naval Medical Research Unit No. 6 Institutional Review Board (Navy Medical Research Center Detachment 2000.0008) and conducted in collaboration with the Bolivia Ministry of Health.

Serologic analyses showed a 64-fold IgM seroconversion between the acute-phase (<100) and convalescent-phase samples (6,400) by using an IgM ELISA as described (*8*). Samples were also tested by ELISA for the following arboviruses: West Nile virus, dengue virus, Oropouche virus, Guaroa virus, Rocio virus, St. Louis encephalitis virus, yellow fever virus, Venezuelan equine encephalitis virus, and Mayaro virus. All test results were negative for these viruses. Virus isolation was attempted on the acute-phase serum sample by using Vero and C6/36 cells, but the culture did not yield any virus. Attempts to isolate virus by intracranial inoculation in suckling mice were also unsuccessful (University of Texas Medical Branch, Institutional Animal Care and Use Committee protocol 9505045).

Viral RNA was extracted from the acute-phase sample and reverse transcription PCR specific for a portion of the nonstructural protein 5 gene was performed by using a described method (*9*). A 189-bp PCR product was obtained, purified, and sequenced by using flavivirus primers FU1 and cFD2 (*9*) and further analyzed by using BLAST (www.ncbi.nlm.nih.gov/blast), resulting in ≈95% homology to ILHV. Phylogenetic analysis with neighbor-joining and parsimony methods grouped the nucleotide sequence of the ILHV virus from Bolivia with ILHV strains from Ecuador and Peru (Figure).

Magdalena is a tropical city in northern Bolivia that borders Brazil. The city is surrounded by rivers and chestnut fields, and agriculture and fishing are the main sources of employment. Despite having ecoepidemiologic conditions similar to those in other locations with a history of ILHV transmission, the virus had not been detected in the area. The patient had no travel history in the 30 days preceding his illness, indicating that the virus is probably endemic to the area.

Mild unspecific symptoms, a short viremic period, and lack of advanced confirmatory laboratory techniques in situ are some of the barriers impeding the diagnosis of ILHV in disease-endemic areas. High levels of antibody cross-reactivity among flaviviruses, which are also endemic to the area, might render diagnosis even more difficult. The presence of the main ILHV vector, *Psorophora* sp. mosquitoes, in the city suggests that much of the population that labors outdoors may be at risk for ILHV infection.

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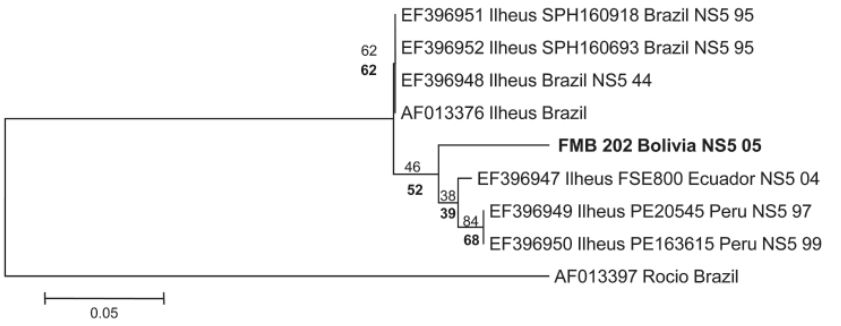

Figure. Phylogenetic analysis of the nonstructural protein 5 (NS5) gene region of 7 Ilheus virus isolates and a 189-bp nt sequence (FMB 202 Bolivia). Alignments were analyzed by using the neighbor-joining method with the Kimura 2-parameter algorithm in MEGA5 (www.megasoftware.net). Variation rate among sites was modeled with a gamma distribution (shape parameter = 1). Bootstrap confidence limits (from 1,000 replicates) are indicated at each node. Values in **boldface** below branches were obtained by parsimony analysis; **boldface** isolate name indicates virus from this study. Rocio virus (GenBank accession no. AF013397) was included as an outgroup on the basis of the phylogram of Kuno and Chang (*10*). Sequence generated in our study was deposited in GenBank under accession no. JN679229. Scale bar indicates nucleotide substitutions per site.

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Shared Human/Rabbit Ligands for Rabbit Hemorrhagic Disease Virus

To the Editor: Rabbit hemorrhagic disease virus (RHDV) is a calicivirus of the genus *Lagovirus* that causes epidemics of an acute disease and mortality rates of 50%–90% among rabbits. The disease, which was first described in 1984, is characterized by hemorrhagic lesions, mainly affecting the liver and lungs 24–72 h after infection (*1*).

Similar to human caliciviruses of the genus *Norovirus*, RHDV binds to histo-blood group antigens (HBGAs), and we recently showed that HBGAs serve as attachment factors (ligands) that facilitate RHDV infection (*2*). HBGAs are polymorphic carbohydrate structures representing terminally exposed portions of larger glycans linked to proteins or glycolipids. In many vertebrate species, they are mainly expressed on epithelial surfaces. Because phylogenetic conservation of receptors is a major risk factor for cross-species transmission (*3*), we analyzed the ability of RHDV strains to recognize human HBGAs expressed on epithelia.

We obtained 38 saliva samples from healthy persons with ABO, Secretor, and Lewis phenotypes, and we selected confirmed *FUT2* (secretor) and *FUT3* (Lewis) genotypes (*4*) to include ABO, secretor, and Lewis phenotypic diversity. Binding capacity of 6 RHDV strains representative of virus diversity (*2*) was tested against human saliva samples by using a method similar to that reported for human norovirus (*5*).

In brief, saliva samples diluted 1:1,000 or B type 2 bovine serum albumin–conjugated tetrasaccharide (positive control) were coated on ELISA plates. After blocking with milk diluted in phosphate-buffered saline, RHDV strains isolated from whole liver extracts of infected animals were incubated on coated plates at dilutions corresponding to 1×10^9 genome copies (0.2 μg/mL capsid protein equivalent) as determined by Nyström et al. (*2*). Monoclonal antibody 2G3, biotinylated anti-mouse IgG, and peroxidase-conjugated avidin were used for RHDV detection; 3,3′,5,5′-tetramethylbenzidine was used as a substrate; and optical density values at 450 nm were measured (*2*).

Binding to the B type 2 epitope was observed for all 6 strains (online Technical Appendix Figure, panel A, wwwnc.cdc/gov/EID/pdfs/11-1402-Techapp.pdf). Human saliva samples were recognized by 5 of 6 RHDV strains. Only G6, an RHDV antigenic variant also known as RHDVa (*6*), did not show binding to saliva. Strains G1 and G2 showed preferential binding to saliva from B secretors over that from O secretors, and A secretors were poorly recognized. Better recognition of A secretor saliva was obtained with the G3 strain. The G4 and G5 strains showed a clear preference for A secretors over B and O secretors, which indicated a shift in specificity toward recognition of the A antigen from the H and B antigens, as reported (*2*). None of the strains recognized nonsecretor saliva, which showed that binding to human saliva required A, B, or H motifs. This finding was confirmed by drastically decreased binding after removal of A, B, and H epitopes from secretor saliva by treatment with specific glycosidases. There was no relationship with the Lewis status.

To determine if human epithelial cells were recognized by RHDV, binding of the G3 strain to human tissue sections was assessed. Human trachea, lung, and gastroduodenal junction samples obtained from organ donors (before current French restrictions of December 1988) were used to prepare tissue microarrays. Tissues from 18 persons were used

and represented the following phenotypes: O secretor Lewis+ (n = 8), A secretor Lewis+ (n = 3), B secretor Lewis+ (n = 2), O secretor Lewis– (n = 1), and O nonsecretor Lewis+ (n = 4). Deparafinated and endogen peroxidase–blocked sections were incubated overnight at 4°C with the G3 strain from an infected liver extract at a concentration of 2×10^9 genome copies/mL.

Binding was detected by using monoclonal antibody 2G3 against RHDV, biotinylated anti-mouse IgG, horseradish peroxidase–conjugated avidin, and 3-amino-9-ethylcarbazole substrate with hemalum counterstaining, as described (*2*). Staining of epithelial cells of stomach or trachea of secretors, but not those of nonsecretors, was observed. This finding indicated that attachment factors for RHDV are present on human cells that constitute potential points of entry for RHDV (online Technical Appendix Figure, panels B–E).

Attachment to HBGAs of human calicivirus strains represents a first step of the infection process (*7*). In this study, we have shown that cross-species recognition of HBGAs in cells that may be likely points of entry of RHDV into human cells. RHDV infection has been shown to be rabbit specific (*8*), which indicates that other molecular elements not shared by rabbits and other mammals restrict its host range. Nevertheless, RHDV RNA was recently isolated from sympatric wild small mammals, which suggested that the species range of RHDV may not be as limited as previously believed (*9*). In addition, recent phylogenetic analysis showed that caliciviruses exhibit high levels of host switching (*10*). Therefore, surveillance of RHDV and studies to decipher molecular mechanisms involved in its extreme pathogenicity are warranted.

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Antimicrobial Drug Resistance in Peru

To the Editor: In Latin American countries, rates of antimicrobial drug resistance among bacterial pathogens are high. Data on these rates in Peru are incomplete (*1*), and no institution in Peru has participated in multinational surveillance studies (*2*–*4*). To document the antimicrobial drug resistance profile of key pathogens, we organized a surveillance network of clinical laboratories from 9 hospitals (public, general, tertiary care, and quaternary care) in Lima, the capital of Peru. Over a 12-month period (April 2008–March 2009), we consecutively collected positive bacterial blood culture isolates (other than coagulase-negative staphylococci) from each of the 9 hospitals. Only the first isolate per patient was included. Patients' age and hospital ward were recorded. Identification and susceptibility testing were performed at the Institute of Tropical Medicine Alexander von Humboldt (Lima, Peru).

Staphylococcus aureus was identified by conventional methods, and susceptibility testing was conducted by oxacillin salt agar screening and disk diffusion (*5*). For gram-negative bacilli, including extended-spectrum β-lactamases (ESBL), identification and susceptibility testing were performed by conventional techniques and by MicroScan NC50 panels (Dade-Behring, West Sacramento, CA, USA) (*5*). American Type Culture Collection strains were used as controls.

During the study period, we collected 1,681 unique isolates. We report the first 934 isolates tested from the more common species collected (375 *Staphylococcus aureus*, 321 *Klebsiella pneumoniae*, 125 *Escherichia coli*, and 113 *Pseudomonas aeruginosa*).

Overall, *S. aureus* was the most frequently recovered species, accounting for 22.0% of organisms. Of 375 *S. aureus* isolates tested, 244 (65.0%) were methicillin resistant (MRSA) and 131 were methicillin susceptible. MRSA frequency was highest among isolates from intensive care units (ICUs) (61 [68.5%] of 89 isolates), but it was also high among isolates from emergency wards (55 [57.3%] of 96 isolates); this difference did not reach statistical significance. Among the 244 MRSA isolates, 170 (69.6%) were also co-resistant to the combination of ciprofloxacin, gentamicin, and clindamycin; rates of co-resistance did not differ significantly among MRSA isolates from patients in the emergency ward (32/55, 58.2%) and those from patients in ICUs and hospital wards (133/184, 72.3%, p = 0.67). Among the 131 methicillin-susceptible isolates, resistance rates were as follows: ciprofloxacin (5.3%), gentamicin (10.7%), clindamycin (14.5%), and erythromycin (14.5%). All *S. aureus* isolates were susceptible to linezolid, teicoplanin, and vancomycin; clindamycin-inducible resistance was found in 10 (38.5%) of 26 isolates resistant to erythromycin and apparently susceptible to clindamycin.

K. pneumoniae was the second most frequently recovered organism, accounting for 19.1% of organisms collected. Among 321 *K. pneumoniae* isolates tested, 241 (75.1%) produced ESBL, 207 (64.5%) showed resistance to ciprofloxacin, and 233 (72.6%) were resistant to trimethoprim-sulfamethoxazole; proportions did not differ among age groups, wards, or hospitals. Of the 241 ESBL-producing isolates, 136 (56.4%) showed co-resistance to ciprofloxacin and gentamicin and 66 (27.4%) were also resistant to amikacin. Of the 80 non–ESBL-producing isolates, 37 (46.3%) were resistant to ciprofloxacin. All *K. pneumoniae* isolates retained susceptibility to imipenem and meropenem. A large proportion of *K. pneumoniae* infections were suspected to have been hospital acquired because most (280/321, 87.2%) were recovered from patients already hospitalized, including one third (96/321, 29.9%) of those from the neonatal ward. Although *K. pneumoniae* occasionally caused microepidemics in neonatal wards, most isolates were recovered randomly over time and from different hospitals.

Among 125 *E. coli* isolates tested, 96 (76.8%) produced ESBL, 107 (85.6%) were resistant to ciprofloxacin, and 108 (86.4%) were resistant to trimethoprim-sulfamethoxazole. The resistance rate to ciprofloxacin was higher among adults than children (90.5% vs. 60.0%, p = 0.002). Of 96 ESBL-positive isolates, 59 (61.5%) were co-resistant to gentamicin and ciprofloxacin but only 9 (9.4%) were resistant to amikacin. Among 29 non–ESBL-producing *E. coli* isolates, 19 (65.5%) were resistant to ciprofloxacin. All isolates were susceptible to imipenem and meropenem. We hypothesized that the high level of *E. coli* resistance to ciprofloxacin may be related to community overuse of fluorquinolones for common infections, such as acute diarrhea.

Among 113 *P. aeruginosa* isolates tested, 62 (54.8%) came from patients in ICUs and 73 (64.0%) were isolated from adults. Multidrug-resistance (defined as resistance to at least 3 of the following: ciprofloxacin, imipenem, amikacin, ceftazidime) was found for 67 (59.3%) of the 133 isolates, more among adults (65.7%) than among children (43.2%, p = 0.024). Overall, 34.5% were resistant to piperacilin-tazobactam.

Our main study limitation was not having complete clinical and epidemiologic information to define which isolates were acquired in the hospital and which were acquired in the community. Overall, rates of antimicrobial drug resistance among common pathogens in hospitals of Lima, Peru, were high.

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Fatal Measles without Rash in Immunocompetent Adult, France

To the Editor: The reemergence of measles in Europe is a reminder of the forgotten risk for severe illness and death associated with this disease in industrialized countries. Since 2008, >20,000 measles cases and 9 measles-associated deaths (in 7 immunocompromised and 2 immunocompetent persons) have been reported to the French Institute for Public Health. Among these cases, the reported causes of death were pneumonia and/or acute respiratory distress syndrome (ARDS) (n = 7) and encephalitis (n = 2). All patients except 1, an immunocompromised patient, had the typical morbillous rash. We report another fatal case of measles, with intractable ARDS but no rash, in an apparently immunocompetent adult.

The patient was a 29-year-old woman in Grenoble, France, who smoked but had no relevant medical history except an episode of depression. In 2011, she sought care for fever, cough, coryza, diarrhea, and a 10-kg weight loss over 10 days. A general practitioner empirically prescribed pristinamycin and oral prednisone (60 mg/d for 5 d) for sinusitis. Five days later, the patient was admitted to the hospital because of persistent signs and symptoms. Physical examination at admission (day 1) detected fever (38.5°C), dyspnea, and a low body mass index of 17.5 kg/m^2. Hematologic tests showed nonregenerative anemia (hemoglobin concentration 9 g/dL) and leukopenia (2.2×10^9 leukocytes/L) with profound lymphopenia (0.2×10^9 lymphocytes/L) and mild thrombocytopenia (135.0×10^9 platelets/L). A chest radiograph showed bilateral diffuse interstitial infiltrates. Antimicrobial therapy with levofloxacin and ceftriaxone was started.

On day 2, several examinations were conducted to explore the possibility of underlying immunosuppressive disease. Body scans showed no adenopathy or lesions suggestive of cancer. HIV test result was negative. General immunologic test results were within normal limits (immunoglobulin quantification, autoantibody testing) or consistent only with an acute viral infection (serum protein electrophoresis). A bone marrow biopsy sample indicated isolated erythroblastopenia with no abnormality of other cell lineages (PCR for parvovirus B19 was negative).

On day 3, because of severe respiratory failure, the patient was

transferred to the intensive care unit, where the diagnosis of ARDS was confirmed and mechanical ventilation was started. Treatment with tazocillin/tazobactam, ciprofloxacin, amphotericin B, and acyclovir was also started. Microbiological findings from bronchoalveolar lavage (BAL) samples were repeatedly negative for bacteria, mycobacteria, fungi, and *Pneumocystis jirovecii*. Cytology of BAL samples showed an acute inflammatory response with atypical epithelial cells, supporting a diagnosis of viral infection. However, none of 14 respiratory viruses or human herpesviruses type 1, 3, 4, 5, or 6 were recovered from BAL samples by PCR. Blood and urine culture results were repeatedly negative, as were serologic test results for *Legionella* spp., *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*.

On day 5, because of refractory ARDS, venoarterial extracorporal membrane oxygenation was started. On day 6, the results of a broad serologic investigation demonstrated isolated IgM against measles virus. The patient was additionally given ribavirin, corticosteroids, and intravenous immunoglobulin. On day 10, the lymphocyte level had returned to reference range and the anemia had become regenerative. However, the patient's respiratory condition did not improve, and after 2 weeks of the oxygenation therapy, the patient died of hemorrhagic shock. Her parents declined an autopsy.

PCR testing of the patient's saliva by the French National Reference Center confirmed the presence of measles virus. Retrospective testing of serum, bone marrow, and BAL specimens collected during days 2–20 of hospitalization demonstrated measles virus RNA. The strain was identified as genotype D4, which is the epidemic strain circulating in France and elsewhere in Europe (*1*). The patient had no history of enanthem (Koplik spots) or morbilliform rash before or after symptom onset and no documented history of measles vaccination.

Deaths from measles with pneumonia or ARDS but without rash have been reported but mostly in patients with deficient cell-mediated immunity (*2*–*6*). Despite all our testing, we found no indications of an underlying immunosuppressive disease in this patient; however, we cannot categorically rule out this possibility, especially that of a primary immunodeficiency. The initial therapy with corticosteroids and the patient's weight loss could also have interfered with her cellular immune response. The diagnosis of ARDS caused by measles was supported by detection of the measles genome in BAL samples and body fluids in the absence of any other pathogen, but pulmonary superinfection with unidentified pathogens could not be ruled out. Detection of the measles genome and isolated erythroblastopenia in the bone marrow biopsy sample is consistent with reports that measles virus can infect erythroid progenitors and interfere indirectly with hematopoiesis (*7*,*8*). Although ribavirin and passive immunotherapy have been reported to aid in recovery from severe measles pneumonia, their clinical efficacy is still unproven (*9*,*10*); and for the patient reported here, they were probably used too late.

This unusual case underscores the need for physicians to consider the diagnosis of measles, even in the absence of classical clinical features, during measles outbreaks. It also reemphasizes the insufficient vaccination coverage against measles in France.

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Carbapenemase-producing *Acinetobacter* spp. in Cattle, France

To the Editor: Multidrug resistance in bacteria isolated from animals is an emerging phenomenon, mirroring what is happening among humans. During the past decade, expanded-spectrum β-lactamases in *Enterobacteriaceae* from humans (*1*) and animals (*2*) worldwide have been reported. Among humans, as a consequence of this high rate, use of carbepenems is increasing selection pressure; carbapenem-resistant gram-negative organisms are increasingly reported, including carbapenemase-producing *Enterobacteriaceae* and *Acinetobacter* spp. (*3*).

The most commonly acquired carbapenemases identified in *Acinetobacter* spp. correspond to carbapenem-hydrolyzing class D β-lactamases (*3*). In particular, the worldwide spread of OXA-23–producing *A. baumannii* is considered a serious threat; those strains are frequently involved in nosocomial outbreaks for which therapeutic options are extremely limited (*3*,*4*). Our study objective was to evaluate the possible occurrence of carbapenemase-producing gram-negative bacteria in dairy cattle in France.

In August 2010, at a dairy farm 30 km from Paris, France, rectal swabs were collected from 50 cows. Samples were precultured in buffered peptone water and incubated for 18 h at 37°C. Cultures were inoculated by streaking 100 μL of the suspensions onto Drigalski agar plates (bioMérieux, Balmes-les-Grottes, France) containing 1 μg/mL of imipenem to select for carbapenem-resistant gram-negative isolates. Of the 50 samples, 9 produced growth on imipenem-containing plates. All colonies tested (10 colonies/sample) by using the API 20 NE (bioMérieux) system were first identified as *A. lwoffii*. Molecular techniques based on sequencing of the *gyrA*, *gyrB*, and *rpoB* genes (*5*) enabled more precise identification and indicated that all isolates belonged to the *Acinetobacter* genomospecies (DNA group) 15TU, which is known to be phylogenetically related to *A. lwoffii* and which has been reportedly isolated from sewage, freshwater aquaculture habitats, trout intestines, and frozen shrimp (*6*).

One colony per sample was retained for further investigation (isolates BY1 to BY9). Susceptibility testing and MIC determinations were performed by disk-diffusion assay (Sanofi-Diagnostic Pasteur, Marnes-la-Coquette, France) and Etest (AB bioMérieux, Solna, Sweden) (Table). All isolates except 1 were resistant to penicillins, combinations of penicillins and β-lactamase inhibitors, and carbapenems but susceptible to cefotaxime and of reduced susceptibility to ceftazidime. Isolate BY1 showed higher MICs for carbapenems (Table). In addition, all isolates were resistant to tetracycline, kanamycin, and fosfomycin and remained susceptible to fluoroquinolones, chloramphenicol, gentamicin, amikacin, tobramycin, and sulfonamides. Susceptibility profiles of 3 *Acinetobacter* genomospecies 15TU reference strains showed that they were fully susceptible to penicillins, carbapenems, tetracycline, and kanamycin.

Clonal diversity between the isolates was assessed by pulsed-field gel electrophoresis (*5*), which showed 6 distinct genotypes. Isolate BY1 corresponded to a single clone (data not shown), which indicated that the occurrence of *Acinetobacter* genomospecies 15TU strains among these animals was not the result of dissemination of a single clone.

PCR detection and sequencing of genes that encode carbapenem-hydrolyzing class D β-lactamases (*5*) showed that the 9 *Acinetobacter* genomospecies 15TU isolates harbored a $bla_{\text{OXA-23}}$ gene, whereas the 3 reference strains remained negative. Sequencing confirmed that all isolates expressed β-lactamase OXA-23, which is known to be widespread in *A. baumannii*.

Mating-out assays and plasmid electroporation assays were performed by using $bla_{\text{OXA-23}}$–positive *Acinetobacter* spp. isolates as donors and rifampin-resistant *A. baumannii* BM4547 isolates as a recipient strain (*5*); however, these assays were unsuccessful. Plasmid DNA analysis (*5*) gave uninterpretable results, with DNA degradations.

The genetic structures surrounding the $bla_{\text{OXA-23}}$ gene were investigated by PCR mapping (*7*), which identified transposon Tn*2008* in isolate BY2 only. Tn*2008* is a major vehicle for the spread of the $bla_{\text{OXA-23}}$ gene in *A. baumannii* in the People's Republic of China (*8*) and the United States (*9*). In the other isolates, the IS*Aba1* element of Tn*2008* had been truncated

Table. Antimicrobial drug MICs for *Acinetobacter* genomospecies 15TU isolates from cows and reference strains, France, August 2010*

	MIC, μg/mL			
	Acinetobacter genomospecies 15TU		Reference strain	
Drug class	BY1	BY2–BY9	NIPH 2171	NIPH 899
Penicillins and combinations				
Amoxicillin	>256	128–256	4	4
Amoxicillin + CLA	>256	128–256	4	4
Cephalosporins				
Cefoxitin	32	16–32	16	16
Cefotaxime	32	16–32	8	6
Ceftazidime	32	16–32	16	16
Cefepime	16	4–16	4	4
Monobactam (aztreonam)	64	32	32	16
Carbapenems				
Meropenem	16	2–4	0.5	0.5
Imipenem	>32	4–6	0.25	0.25
Doripenem	8	2–4	0.5	0.5
Cyclines				
Tetracycline	>256	>256	0.5	0.5
Tigecycline	0.064	0.047–0.064	0.047	0.125
Quinolones (ciprofloxacin)	0.5	0.5	0.25	0.25
Aminoglycosides				
Gentamicin	0.5	0.25–0.5	0.25	0.25
Kanamycin	>256	>256	0.5	0.5
Sulfonamides	4	4	4	>256

*CLA, clavulanic acid (4 μg/mL).

by a novel insertion sequence termed IS*Acsp2* (www-is.biotoul.fr).

The dairy farmer indicated that most animals from which OXA-23 producers had been identified had received antimicrobial drugs in the previous weeks. Although 1 animal had received amoxicillin-clavulanate, most of the others had been given oxytetracycline and neomycin to treat mastitis.

β-lactamase OXA-23 is a common source of carbapenem resistance in *A. baumannii (5)*. Infections with multidrug-resistant OXA-23–producing *A. baumannii* or *A. junii* have been reported from hospitals but not from the community. Our study showed that OXA-23–producers in particular, and carbapenemase producers in general, may be isolated from animals. Among the hypotheses that could explain the selection of this carbapenemase, use of penicillins or penicillin–β-lactamase inhibitor combinations could create selective pressure for β-lactamases because OXA-23 does confer, in addition to decreased susceptibility to carbapenems, a high level of resistance to those compounds. We have previously shown that *A. radioresistens*, an environmental species, was the progenitor of the bla_{OXA-23} gene (*10*). Studies are needed to determine to what extent and at which locations *Acinetobacter* genomospecies 15TU and *A. radioresistens* might co-reside and therefore where the bla_{OXA-23} gene exchange might have occurred.

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Aedes albopictus Mosquitoes, Yucatan Peninsula, Mexico

To the Editor: We collected Asian tiger mosquitoes, *Aedes albopictus* (Skuse), in Cancun in the Yucatan Peninsula of Mexico in September 2011. This mosquito is a nuisance biter of humans and a vector of numerous arboviruses, including those causing dengue, yellow fever, and chikungunya (*1*).

Ae. albopictus mosquitoes, which are native to Southeast Asia, emerged in the continental United States in 1985 and thereafter spread rapidly across the southeastern United States and into northern Mexico (*2,3*). These mosquitoes have also been found in the states of Tamaulipas, Coahuila, and Nuevo Leon in northern Mexico, Chiapas in southern Mexico, and south of Mexico in Guatemala and Belize (*3–9*). These findings are now complemented by our collection of *Ae. albopictus* mosquitoes from Cancun in Quintana Roo State, which with Yucatan and Campeche States compose the Yucatan Peninsula. A previous study of the mosquito fauna of Quintana Roo conducted in 2006 did not report any *Ae. albopictus* mosquitoes (*10*).

During September 2011, *Ae. albopictus* mosquitoes were collected from a cemetery in Cancun, which is located in the eastern part of the Yucatan Peninsula (21°8.53′N, 86°52.79′W) (Figure). The collection location was shaded by trees. Water in containers from which larvae were collected had an average temperature of 24.5°C and a pH of 8.5. The larval collection included ≈30 specimens of different developmental stages that were collected from vases and other artificial containers in the cemetery. The containers were examined as part of routine surveillance activities by Servicios Estatales de Salud de Quintana Roo. Larvae suspected to be those of *Ae. albopictus* mosquitoes were reared to adults for identification, and a colony of *Ae. albopictus* mosquitoes from Cancun was established.

F_0 or F_1 adult specimens were confirmed to be *Ae. albopictus* moszquitoes by species identification at Servicios Estatales de Salud de Quintana Roo (Quintana Roo, Mexico), Universidad Autónoma de Yucatan (Merida, Mexico), and Colorado State University (Fort Collins, CO, USA). The initial mosquito larval collection was composed of 26 *Ae. albopictus*, 3 *Ae. aegypti*, and 1 *Culex* sp. In addition, 6 *Ae. albopictus* female mosquitoes were collected from the cemetery by landing catches.

Finding *Ae. albopictus* mosquitoes in Cancun was not surprising because these mosquitoes have been found in nearby Belize (*9*). Cancun is also a major port for ships carrying tourists and goods that originate in areas to which *Ae. albopictus* mosquitoes are endemic, including Florida and Texas. Nevertheless, the introduction of *Ae. albopictus* mosquitoes into Cancun and the high potential for establishment and spread across the Yucatan Peninsula has major public health implications.

The Yucatan Peninsula is hyperendemic for dengue, with all 4 dengue virus (DENV) serotypes circulating in this region. Should *Ae. albopictus* mosquitoes persist in this region, they may spread and come to play a secondary role to *Ae. aegypti* mosquitoes as local vectors of DENV. *Ae. albopictus* mosquitoes may also change local virus transmission dynamics. For example, DENV transmission may be intensified in rural areas because *Ae. albopictus* mosquitoes are more likely than *Ae. aegypti* mosquitoes to be found in this setting. *Ae. albopictus* and *Ae. aegypti* mosquitoes also may differ in their potential for vertical transmission of DENV, which could

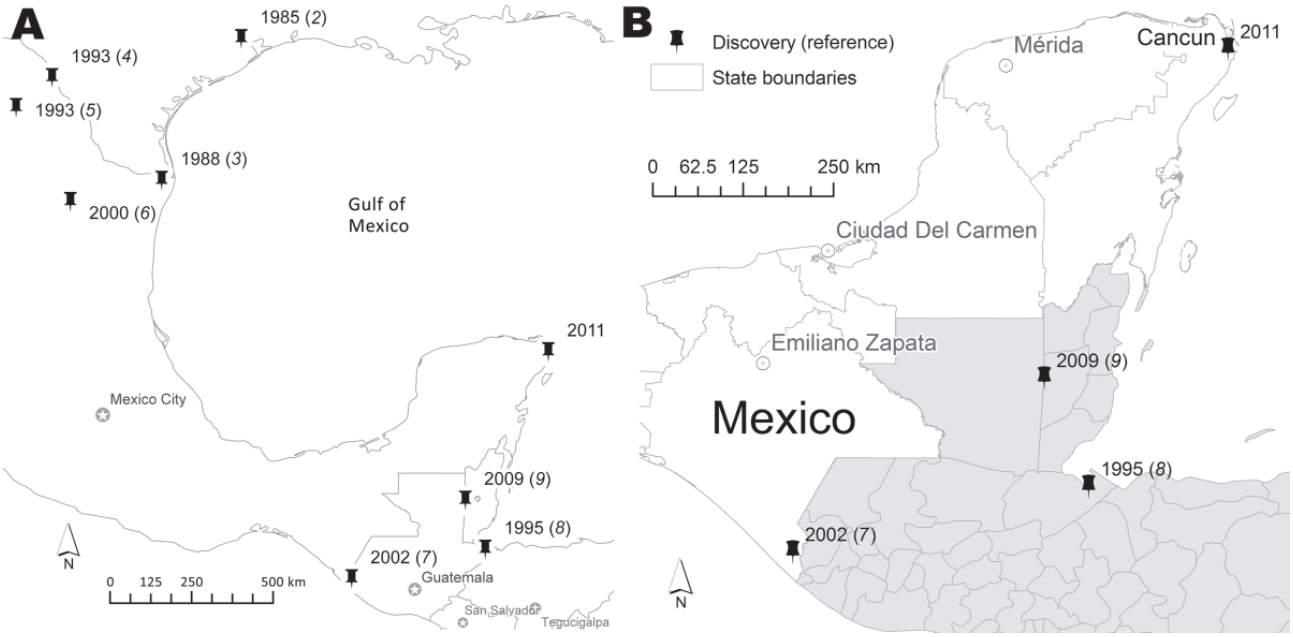

Figure. Notable locations (pushpins) in Mexico, the United States, and Central America where *Aedes albopictus* mosquitoes were collected and year of the first collection (reference) (A), including the current collection in 2011 from Cancun, Quintana Roo State, Mexico (B). Shaded areas indicate countries in Central America (Guatemala, Belize, Honduras, and El Salvador).

affect virus transmission dynamics, especially during interepidemic periods or parts of the year that have low mosquito activity and infrequent human–mosquito contact. Other concerns regarding introduction of *Ae. albopictus* mosquitoes into the Yucatan Peninsula are their role as an aggressive nuisance biter of humans, which may necessitate intensified mosquito control to protect the local tourist industry; and their potential role as a vector of chikungunya virus, which is a major threat to immunologically naive populations in the Americas should the virus emerge there.

Introduction of *Ae. albopictus* mosquitoes into the Yucatan Peninsula requires research on local biology of the mosquito and their potential role as an arbovirus vector in this part of Mexico. Studies are needed to determine how fine-scale spatial segregation of *Ae. albopictus* and *Ae. aegypti* mosquitoes might result from competition for containers that serve as larval development sites, from differential survival related to container type, and from hydrologic microclimates or nutrient conditions. One possible scenario is for *Ae. albopictus* mosquitoes to outcompete and exclude *Ae. aegypti* mosquitoes from certain settings. Other issues include how effectively *Ae. albopictus* mosquitoes can transmit locally circulating DENV strains and, because this species bridges the transitional zone from urban to forested environments and may bite a wide range of mammals, what role it might play in the urban emergence of arboviruses that are currently restricted to sylvatic forest transmission cycles in the Yucatan Peninsula.

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Prevalence of Nontuberculous Mycobacteria Infection, China, 2004–2009

To the Editor: Pulmonary nontuberculous mycobacteria (NTM) diseases share clinical signs with tuberculosis (TB), causing a clinical dilemma with regard to therapy for patients with these diseases (*1*). In the past 30 years (post-AIDS era), NTM have increasingly been associated with pulmonary diseases in humans (*2*). Recent studies in urban areas of the People's Republic of China have shown that the prevalence of NTM (isolation rate of NTM among all mycobacteria) is increasing; for example, prevalence in Shanghai increased from 4.26% in 2005 to 6.38% in 2008 (*3*). To investigate NTM prevalence in rural areas of China, we evaluated the NTM isolation rates, species distribution, and drug-resistance profiles through a population-based TB sentinel surveillance study in Shandong Province, the second largest province in China. The study protocol was approved by the Institutional Review Board of Shandong Provincial Chest Hospital (Jinan, Shandong, China).

Clinical samples were collected through the ongoing sentinel TB surveillance project, which first began in 7 counties in Shandong Province in 2004 and expanded to 13 counties in 2008. Of the total surveillance population, rural populations accounted for ≈80%. Each sample collected in this study was identified only by a unique participant number. Each surveillance site sent sputum samples from all patients with suspected TB to the TB Reference Laboratory of Shandong Provincial Chest Hospital for mycobacterial culture, drug-susceptibility testing, and species identification.

From January 1, 2004, through December 31, 2009, *Mycobacteria* spp. were isolated from sputum specimens from 3,949 patients with suspected pulmonary TB. Of these patients, mean age ± SD was 48.7 ± 20.4 years (range 1–92 years), 74.6% were male, and 300 were being retreated for TB.

Identification of *Mycobacteria* spp. was first conducted by conventional biochemical testing—p-nitrobenzoic acid and 2-thiophene carboxylic acid hydrazide testing—following a standard protocol (*4*). *Mycobacteria* spp. were further identified by 16S rRNA gene sequence analysis (MicroSeq ID Microbial Indentification Software, version 2.0; Applied Biosystems, Foster City, CA, USA) to the species level as described (*5*). Drug-susceptibility testing was performed according to standard procedures recommended by the World Health Organization, and quality control was conducted by inter-laboratory confirmation testing by reference laboratories recognized by the World Health Organization in South Korea and in Hong Kong Special Administrative Region, China (*6*,*7*). The drug panel included 4 first-line anti-TB drugs: isoniazid, rifampin, streptomycin, and ethambutol.

The conventional biochemical testing of the 3,949 *Mycobacteria* spp. strains identified 68 NTM strains, among which the 16s rRNA gene sequence analysis confirmed 64 (1.6%) NTM strains and identified 3 *M. tuberculosis* complex strains and 1 *Nocardia glanders* strain. Among the 64 NTM strains, 52 (81.2%) were *M. intracellulare*, 5 (7.8%) were *M. kansasii*, 3 (4.7%) were *M. fortuitum*, 2 (3.1%) were *M. chelonae*, 1 (1.6%) was *M. gordonae*, and 1 (1.6%) was *M. scrofulaceum*. The first-line anti-TB drug resistance rates of the 64 NTM strains were 100% for isoniazid, 98.4% for streptomycin, 78.1% for rifampin, and 51.6% for ethambutol (Table). Among the 3,949 *Mycobacteria* spp. strains, 163 (4.1%) were resistant to at least isoniazid and

Table. Species and drug-resistance profiles of 64 nontuberculous mycobacteria strains, Shandong Province, People's Republic of China, 2004–2009

Mycobacterium species	Resistant strains, no. (%)				
	Total	Isoniazid	Rifampin	Ethambutol	Streptomycin
M. intracellulare	52 (81.2)	52 (100)	40 (76.9)	27 (51.9)	51 (98.1)
M. kansasii	5 (7.8)	5 (100)	3 (60.0)	0	5 (100)
M. fortuitum	3 (4.7)	3 (100)	3 (100)	2 (66.7)	3 (100)
M. chelonae	2 (3.1)	2 (100)	2 (100)	2 (100)	2 (100)
M. gordonae	1 (1.6)	1 (100)	1 (100)	1 (100)	1 (100)
M. scrofulaceum	1 (1.6)	1 (100)	0	1 (100)	1 (100)
Total	64 (100)	64 (100)	50 (78.1)	33 (51.6)	63 (98.4)

rifampin, of which 50 (30.7%) strains were identified as NTM. Among 300 TB re-treatment cases, 12 (4.0%) were caused by clinically significant NTM infections. Over the 6 study years, NTM isolation rates among the study population did not show a substantial increasing or decreasing trend.

Our data suggest that the NTM isolation rate among patients with suspected pulmonary TB in rural China (1.6%) is relatively lower and more stable than that for urban areas (mean rate 5.09% in Shanghai) and that the *Mycobacterium* spp. differ from those in other areas of China (*3*,*8*–*10*). In China and most other developing countries to which TB is endemic, the decision to initiate pulmonary TB treatment is based only on finding a positive sputum smear by microscopy examination, not on *Mycobacteria* culture, species identification, and drug-resistance testing results. Among our study population, NTM strains showed high drug resistance to first-line anti-TB drugs and accounted for 30.7% of suspected multidrug-resistant TB (MDR-TB) cases and 4.0% of TB re-treatment cases.

These findings suggest that pulmonary NTM infections pose substantial difficulties with regard to clinical management of NTM and MDR-TB diseases in China. Laboratory species identification is imperative before proper treatment can be determined for patients with MDR-TB. Compared with conventional biochemical testing, 16S rRNA gene sequencing analysis can more accurately identify *Mycobacteria* spp.

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Travelers as Sentinels for Chikungunya Fever, Brazil

To the Editor: The reemergence of chikungunya virus (CHIKV) infection recently has been reported in travelers after they returned from affected areas (*1*–*6*). In the Americas, local transmission has not been identified, although imported cases have been reported in travelers returning from Reunion Island to Martinique, French Guiana, and Guadeloupe (*7*). In the United States, CHIKV infections have also been reported in travelers who returned from disease-endemic areas (*8*).

Climate changes in recent decades have affected the dynamics of infectious disease transmission, increasing the incidence, prevalence, and number of outbreaks of mosquito-borne diseases, such as dengue fever. Both CHIKV and dengue virus are transmitted by *Aedes* spp. mosquitoes. *Ae. aegypti* mosquitoes are the most common mosquito involved in dengue transmission, and *Ae. albopictus* mosquitoes have been described as efficient vectors of CHIKV. Recent global expansion of *Ae. albopictus* mosquitoes has been associated with the introduction and dissemination of CHIKV in new areas (*9*).

More than 4,000 cities in Brazil are infested with *Ae. aegypti* mosquitoes, which predominates in urban areas, and such areas have a high incidence of dengue fever and annual outbreaks of this disease. *Ae. albopictus* mosquitoes have been identified in Brazil, where they are more frequently found in rural areas (*10*). The confirmed chikungunya fever cases described here illustrate the risk for introduction and sustained transmission of the disease in Brazil.

In August 2010, a 55-year-old man returned to Brazil from Indonesia, where he had spent 15 days. Seven days after his arrival in Indonesia, a fever (temperature 38.5–39.0°C) developed that lasted for 3 days, along with a facial rash that spread to his neck, trunk, legs, and ankles, followed by desquamation. During the trip, he experienced disabling pain and swelling in the ankles, accompanied by weight loss (5 kg). Four other travelers in his group experienced fever, arthralgia, and malaise. Upon his return to Brazil, the man immediately sought medical attention, and his symptoms were treated with intravenous fluids, parenteral corticosteroids, and nonsteroidal antiinflammatory drugs for 2 weeks. Despite improvement, the arthralgia recurred in the wrists and metacarpal bones. He was referred to the Travel Medicine Outpatient Clinic of the University of São Paulo School of Medicine Hospital das Clínicas. Laboratory tests showed elevated levels of aspartate transaminase (117 U/L), alanine transaminase (179 U/L), and C-reactive protein (27.8 mg/L). Test results for *Plasmodium* spp., dengue virus, cytomegalovirus, and *Toxoplasma* spp. were all negative. Fifty-three days after onset symptom, anti-CHIKV IgM and IgG antibodies were detected by ELISA. By day 60, his IgG titer had risen from 3,200 to 6,400, where it remained 11 months after onset of symptoms.

In October 2010, a 25-year-old woman returned to Brazil from Rajasthan, India, where she had spent 30 days working with a humanitarian aid group. During her return, fever (38.0–39.0°C) and malaise developed. She sought medical attention in the emergency department of the Emílio Ribas Institute of Infectious Diseases, reporting fever, headache, myalgia, fatigue, general malaise, and paresthesia of the hands, as well as severe ankle and foot pain with gait impairment. Physical examination showed dehydration, conjunctival injection, and fever (temperature 38.0°C), as well as skin redness and a faint rash on the trunk. She also had swollen ankles. The fever (temperature 37.8°C) persisted, and she had pain in her ankles and left knee, which made it difficult for her to walk, accompanied

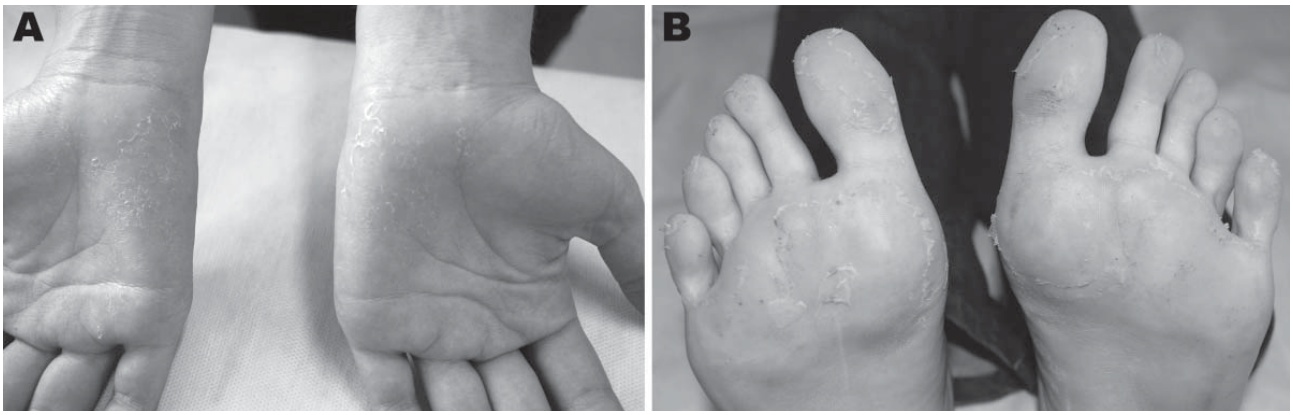

Figure. Clinical features exhibited by patient with chikungunya, Brazil 2010. A) Desquamation of palms after maculopapular rash, 33 days after symptom onset. B) Desquamation of soles after maculopapular rash, 33 days after symptom onset. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/3/11-0838-F1.htm).

by desquamation of palms and soles (Figure). Laboratory tests detected leukopenia and thrombocytopenia. Test results for *Plasmodium* spp. and dengue virus were negative, and blood culture results were negative as well. By using ELISA, anti-CHIKV IgM antibodies were detected 10 days after onset of symptoms, and anti-CHIKV IgG antibodies (titer 25,600) were detected 8 months later.

Both patients were diagnosed after the viremic period; no virus could be isolated or genotyped. Nevertheless, health authorities were alerted and appropriate control measures were taken.

Travelers can serve as sentinels for the introduction of viruses into previously non–disease-endemic areas. Several reports have been made of travelers carrying CHIKV to and from many regions of the world (*2*,*4*–*6*). Recent identification of the expansion of infested areas by *Ae. aegypti* and *Ae. albopictus* mosquitoes, population susceptibility for the virus, and the constant journeying of travelers from affected areas are relevant indications of the risk for introduction and sustained transmission of CHIKV in Brazil.

Health care professionals and public health authorities should be aware of the epidemiologic and clinical aspects of CHIKV infection and diagnoses to adopt prompt control measures to avoid CHIKV transmission in Brazil. Healthcare facilities and epidemiologic surveillance teams have jointly implemented CHIKV prevention and control measures. To date, no autochthonous transmission of CHIKV has been reported in Brazil.

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Enhanced Surveillance for White-Nose Syndrome in Bats

To the Editor: White-nose syndrome (WNS) is an emerging fungal disease in bats that was first described near Albany, New York, USA, in February 2006 (*1*). The causative agent, *Geomyces destructans*, is a psychrophilic (cold-loving) fungus that infects the skin of bats and leads to depletion of their fat stores during hibernation (*2*). WNS has caused dramatic cumulative mortality rates (up to 99%) in some winter hibernacula and has killed millions of

bats among 6 cave-roosting species in 19 central and eastern US states and 4 Canadian provinces (*3*). In addition, the fungus has been identified in 2 additional US states, although bat deaths have not been associated with it. No evidence has been found that WNS is transmitted from bats to humans, although humans may play a role in translocation of the fungus between caves (*4*,*5*).

Current surveillance for WNS is time- and labor-intensive. Wildlife personnel typically enter caves, inspect hibernacula, and collect bats with clinically compatible signs for testing (*4*). In July 2010, the National Park Service (NPS) Office of Public Health proposed an expanded WNS surveillance strategy that involved using opportunistic sampling of bats already submitted to state public health laboratories for rabies testing; the bats submitted include species known to be susceptible to WNS. The pilot study focused on the region around Mammoth Cave National Park, the world's longest known cave system and home to 13 bat species (2 endangered), in south-central Kentucky (*6*). At the time of initial discussions, Kentucky was WNS-free, but the bordering state of Tennessee had recently reported its first WNS cases in spring 2010 in a cave system located <130 km from Mammoth Cave. WNS was first detected in Kentucky in April 2011 in Trigg County (180 km from Mammoth Cave) (*7*).

The goals of this pilot study were to 1) enhance WNS surveillance in counties in and near Mammoth Cave and 2) demonstrate a feasible, cost-effective surveillance system. NPS Office of Public Health staff coordinated meetings in Kentucky and Tennessee with representatives from the state departments of wildlife and health and other partnering organizations. Key representatives at one or both of these meetings included the state epidemiologist, the state public health veterinarian, the public health laboratory director, state wildlife biologists, and NPS and state wildlife veterinarians. Also attending both meetings was a veterinary pathologist from the Southeastern Cooperative Wildlife Disease Study (SCWDS) in Athens, Georgia, USA, one of 3 laboratories that test most samples for WNS in the United States. The surveillance concept was well received in both states, and state-specific protocols were developed for submitting rabies-negative bats to SCWDS only during hibernation months (November–April) when WNS is more likely to be detected (*8*). In Kentucky, a memorandum of understanding was drafted that outlined roles and responsibilities of collaborating agencies. The memorandum was reviewed by legal advisors and signed by public health and wildlife officials.

Both protocols outlined key elements of the submission process, including how laboratory personnel were to submit rabies-negative bats to SCWDS for WNS testing (fungal culture, histopathologic examination, and PCR), how bats were to be stored or destroyed after testing, and the chain of communication for reporting test results. Whenever possible, bats were frozen at –20°C within 48 hours following rabies testing, and their muzzles and forearms were left intact to maximize the yield for *G. destructans* and to facilitate species identification. Protocols included additional criteria to improve testing efficiency (e.g., prioritizing submissions on the basis of known WNS-susceptible species or counties where cave-roosting colonies are located). A project-specific version of the standard SCWDS submission form was completed for all samples. All funding and resources were provided in kind by respective agencies.

In October 2010, the Tennessee State Public Health Laboratory submitted 34 rabies-negative bats (archived during January–April 2010, before pilot study discussions) from 18 counties to SCWDS; all were WNS-negative. Twenty-one additional rabies-negative bats from 9 Tennessee counties collected during November 2010–April 2011 also tested negative for WNS. In Kentucky, 64 rabies-negative bats (from 22 counties) were submitted during November 2011–January 2012; all were WNS-negative except 1 bat tested on January 13, 2012, which was the first known WNS-positive bat from Fayette County, a primarily urban area in northern central Kentucky where little cave-based WNS surveillance is conducted. Overall, although the sample of bats tested to date is modest and likely insufficient as a stand-alone surveillance system, these results supplement other data and can inform the development of interventions, prevention messages, and transmission models.

This pilot study highlights several observations and implications. First, it demonstrates that opportunistic testing of rabies-negative bats for WNS can be facilitated between state departments of wildlife and health through interagency collaboration. Second, the surveillance system is low cost and could potentially be expanded to other states where WNS is likely to emerge and where statewide cave-based surveillance is cost-prohibitive. Last, this project showcases a unique interdisciplinary collaboration in wildlife and human health, disease ecology, and environmental stewardship. Such partnerships are championed by the One Health approach (*9*) and are central to the mission of NPS to protect the health of all species and our environment (*10*).

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NDM-1–producing *Klebsiella pneumoniae*, Croatia

To the Editor: The novel metallo-β-lactamase named New Delhi metallo-β-lactamase (NDM-1) was identified from *Klebsiella pneumoniae* and *Escherichia coli* isolates in Sweden from a patient previously hospitalized in India (*1*). NDM-1 is spreading rapidly worldwide to nonclonally related isolates, many of which are directly or indirectly tracked to the Indian subcontinent (*2*). A carbapenem-resistant *K. pneumoniae* strain, KLZA, was isolated in May 2009 from the culture of a blood sample from of a 40-year-old man on the day after his admission to a surgical intensive care unit of the Clinical Hospital Center in Zagreb, Croatia. The patient had been transferred after 5 days of hospitalization in Bosnia and Herzegovina following a car accident. The clinical history mentioned antimicrobial drug treatment that did not include carbapenems (gentamicin, metronidazole, and ceftriaxone) and no link to the Indian subcontinent. Antimicrobial drug susceptibility testing was performed by Vitek2 (bioMérieux, Marcy-l'Etoile, France) and broth microdilution and interpreted according to the latest documents from the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org/clinical_breakpoints/, version 1.1).

The strain proved resistant to imipenem and meropenem, to all broad-spectrum cephalosporins, and to aminoglycosides and susceptible to ciprofloxacin and tigecycline (Table). We checked for bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{GIM}, bla_{SIM}, and bla_{NDM} resistance genes by using PCR. A PCR product was obtained only with the NDM primers, after being purified (QIAquick PCR Purification Kit, QIAGEN, Hilden, Germany), its sequence showed 100% identity with bla_{NDM-1}.

Strain genotyping was performed by multilocus sequence typing to determine the sequence type (ST) of the isolate and to establish a comparison with previously reported NDM-1–producing isolates. Allelic numbers were obtained on the basis of sequences of 7 housekeeping genes at www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.

Table. MIC of the KLZA strain of *Klebsiella pneumoniae* and its transconjugant and recipient

Antimicrobial drug	MIC, mg/L		
	K. pneumoniae KLZA	*Escherichia coli* J53	*E. coli* T1
Imipenem	8	<0.06	4
Meropenem	8	<0.06	4
Ertapenem	16	<0.06	8
Ceftazidime	>128	<0.06	128
Cefotaxime	>128	<0.06	32
Cefepime	32	<0.06	64
Aztreonam	>128	0.25	>128
Ciprofloxacin	0.5	<0.06	0.12
Gentamicin	8	0.25	0.25
Amikacin	16	0.5	0.5
Tigecycline	1	0.25	0.25
Colistin	<0.5	<0.5	<0.5

html. Multilocus sequence typing identified *K. pneumoniae* KLZA as an ST25 strain, which significantly differs from the ST14 type found in the index NDM-1–producing strain and from other isolates originating from India (*1*) and then in other countries. ST25 *K. pneumoniae* was also found in *K. pneumoniae* isolates in Geneva (*3*). Other *K. pneumoniae* STs harboring NDM-1 were ST15, ST16, and ST147 (*4*–*7*).

Resistance was transferred by conjugation to *E. coli* J53, with selection based on growth on agar in the presence of ceftazidime (10 mg/L) and azide (100 mg/L). The conjugant T1 showed resistance to β-lactams, including all carbapenems, as well as decreased susceptibility to ciprofloxacin.

The KLZA strain and its transconjugant harbored other determinant of resistance, namely $bla_{CTX\text{-}M\text{-}15}$, $bla_{CMY\text{-}16}$, and *qnrA6*. Plasmid incompatibility groups, determined by a PCR-based replicon typing method, belonged to the incA/C replicon type.

This report of an NDM-1–producing *K. pneumoniae* in Croatia adds to those of other cases in patients from patients hospitalized in the Balkan area. The patient in this report had no apparent link to the Indian subcontinent.

In a survey conducted by the European Centre for Disease Prevention and Control to gather information about the spread of NDM-1–producing *Enterobacteriaceae* in Europe and reporting cases from 13 countries during 2008–2010, five of the 55 persons with known travel histories had traveled to the Balkan region during the month before diagnosis of their infection: 2 to Kosovo and 1 each to Serbia, Montenegro, and Bosnia and Herzegovina. All had received hospital care in Balkan countries because of an illness or accident that occurred during the journey (*7*). Two of the latter cases (*4*,*8*) and a case from Germany (*9*) were subsequently published. No patient had any apparent link to the Indian subcontinent.

Although the way NDM-1 isolates might have been imported to western Europe not only from the Indian subcontinent but also from Balkan countries (*10*) has been highlighted, awareness of western Europe as a possible area of endemicity remains limited. The aforementioned report from Germany, although recognizing that the patient had been repatriated after hospitalization in Serbia, declared "no evidence about contact with people from regions where NDM-enterobacteria are endemic" (*9*). This limited awareness shows the threat of neglecting to screen patients who are transferred from countries thought not to be at risk for NDM-1. Furthermore, it means that specimen are not sent to the local reference laboratories and recognized as positive for NDM-1, thus permitting wide dissemination of NDM-1–producing enterobacteria in the community (*4*). The accumulating evidence of NDM-1 from the Balkan area could suggest a possible multifocal spread of this enzyme, with the Balkans as a possible second area of endemicity, in addition to the Indian subcontinent, and prompts for widespread epidemiologic surveillance.

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Adherence to Oseltamivir Guidelines during Influenza Pandemic, the Netherlands

To the Editor: In the Netherlands, the outbreak of pandemic influenza A (H1N1) 2009 led to a 100-fold increase from 2008 in prescriptions for the antiviral neuraminidase inhibitor oseltamivir (*1*). The guidelines for prescribing oseltamivir during the 2009 pandemic were adapted throughout the year. After August 7, prescribers were advised to restrict prescriptions to patients with influenza symptoms plus 1 additional risk factor (*2*) (Table).

Community pharmacists dispensed oseltamivir as a 5-day course of sachets produced exclusively for the Dutch government program and documented all prescriptions. Our objective was to assess whether oseltamivir dispensed through community pharmacies was prescribed according to the national guideline for the pandemic virus and to investigate how patients used oseltamivir. The Institutional Review Board of the Division of Pharmacoepidemiology and Clinical Pharmacology of Utrecht University approved the study.

Pharmacists in 19 pharmacies belonging to the Utrecht Pharmacy Practice Network for Education and Research (UPPER) selected all patients who had filled a prescription for oseltamivir during May 1, 2009–February 8, 2010. These patients were contacted by phone and, after giving consent, completed a structured questionnaire. The questionnaire contained questions about potential risk factors, the reason for receiving the oseltamivir prescription (influenza symptoms or other reasons), and whether the oseltamivir course was started and completed.

Of the 630 patients eligible for contact, 361 (57.3%) completed the questionnaire. To assess whether the current guidelines were adhered to, because of the changes in policy throughout the year, we analyzed only the 300 respondents who had filled the oseltamivir prescription at the height of the pandemic, i.e., after August 7, 2009.

A total of 156 (52.0%) participants were female patients; most participants were 18–59 years of age. Of the 212 patients >18 years of age, education level was available for 195; of these, 55 (28.2%) had a low education level, 94 (48.2%) a middle education level, and 46 (23.6%) a high education level.

Of the 300 respondents, 111 (37.0%) received a prescription while they did not meet guideline criteria (Table). They had risk factors but did not experience influenza symptoms (67 [22.3%] of all respondents); had influenza symptoms but not risk factors (34 [11.3%]); or had neither influenza symptoms nor any risk factors (10 [3.3%]).

Compared with respondents who had a low education level, respondents >18 years of age who had a middle or high education level were 2× more likely to receive an oseltamivir prescription that was not in accordance with guideline criteria (odds ratio 2.20; 95% CI 1.12–4.32). Sex and age were not associated with the likelihood of receiving off-guideline oseltamivir.

Of the 189 respondents who received oseltamivir in accordance with guideline criteria, 184 (97.4%) started treatment and 167 (90.8%) completed the oseltamivir course. Of the 111 respondents who received a prescription for oseltamivir that was not in accordance with guideline criteria, 62 (55.9%) started treatment, and 56 (90.3%) completed the course.

We showed that during the pandemic the guideline criteria were not met by nearly one third of patients who received an oseltamivir prescription. Patients with a higher education level more often received a prescription, suggesting that they are more informed or empowered than patients with a lower education level to request a prescription. Another explanation for the inadequate adherence to guideline criteria is that prescribers themselves were not immediately aware of the current criteria, possibly because of changes throughout the year.

In addition, in nearly half of instances in which guideline criteria were not met but in which oseltamivir was prescribed, the patients did not start the oseltamivir course. These prescriptions could have been used for stockpiling, which

Table. Reported risk factors of patients with and without influenza symptoms who were dispensed a prescription for oseltamivir, the Netherlands, 2009–10

Risk factor	No. (%) patients*		
	Total, n = 300	Influenza symptoms, n = 223	No influenza symptoms, n = 77
Chronic condition	211 (70.3)	154 (69.1)	57 (74.0)
Chronic respiratory disease	127 (42.3)	98 (43.9)	29 (37.7)
Lower immune resistance caused by illness or medical treatment	76 (25.3)	50 (22.4)	26 (33.8)
Cardiovascular disease	52 (17.3)	34 (15.2)	18 (23.4)
Diabetes	44 (14.7)	28 (12.6)	16 (20.8)
Renal disease	10 (3.3)	5 (2.2)	5 (6.5)
Other	125 (41.7)	86 (38.6)	39 (50.6)
Age >60 y	66 (22.0)	35 (15.7)	31 (40.3)
Age <2 y	36 (12.0)	35 (15.7)	1 (1.3)
Regular patient contact by health care worker	22 (7.3)	15 (6.7)	7 (9.1)
Pregnancy	5 (1.7)	2 (0.9)	3 (3.9)
No. risk factors			
0	44 (14.7)	34 (15.2)	10 (13.0)
1	137 (45.7)	111 (49.8)	26 (33.8)
2	72 (24.0)	53 (23.8)	19 (24.7)
≥3	47 (15.7)	25 (11.2)	22 (28.6)

*Percentages may total >100% because of rounding.

also occurred during the influenza A (H5N1) outbreak in 2005 (*3*). In the Netherlands, stockpiling did not lead to drug shortages, but in countries where oseltamivir is not reimbursed by the government, stockpiling might lead to problems with availability for patients truly in need of antiviral therapy but without the necessary means to acquire it.

The limited effect of oseltamivir on reducing disease duration, usually only shortening the duration by 1 day in healthy persons (*4*), the possibility of serious side effects (*5*), the possibility of the virus developing resistance to neuraminidase inhibitors (*6*,*7*), and the cost to health care of unnecessary prescriptions are reasons to strive for better adherence to prescribing guidelines. Prescribers need to be properly informed about current guidelines to reduce overprescribing caused by lack of knowledge. Furthermore, improving communication between prescribers and patients might help relieve patients' concerns and increase awareness about the limited benefits of oseltamivir treatment in healthy persons.

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Serologic Evidence of Nipah Virus Infection in Bats, Vietnam

To the Editor: Bats are potential reservoir for highly pathogenic viruses, such as Nipah virus (NiV) and Hendra virus, which can cross species barriers (*1*). However, only limited surveillance has been conducted to assess risk for infection by these deadly emerging viruses. We conducted a study in Vietnam from 2007 to 2008 to assess the prevalence of these pathogens in bats.

Different species of live bats were obtained from hunters or were captured in caves, pepper fields, and residential areas by using mist nets or harp traps (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/pdfs/11-1121-Techapp.pdf). A total of 451 serum samples were collected and subjected to IgG ELISA by using an *Escherichia coli*–derived recombinant nucleocapsid (N) protein of NiV (NiV-N-ELISA) (*2*). Two Leschenault's rousette bats (*Rousettus leschenaulti*) were vaccinated with the same recombinant N protein to obtain positive serum specimens that contained antibodies against NiV-N. An optical density of 492 nm for negative control serum (1,000× dilution) was designated as 1:1,000 ELISA units. ELISA titers of sample serum specimens were obtained at a single dilution (1,000×) by using a standard curve of positive serum with high titers. A sample titer ≥3,000 was considered positive for IgG against NiV.

Positive results were detected from only 2 fruit bat species, *R. leschenaulti* (31 bats [49.1%]) and *Cynopterus sphinx* (3 bats [2.8%]) (Table). Of the 34 samples positive by ELISA, only 22 (20 from the former and 2 from the latter bat species), which had enough volume left, were further analyzed by Western blot (WB) with *E. coli*–expressed recombinant N protein of NiV (Table; online Technical Appendix Figure 2). Twenty of the 22 specimens were confirmed as positive by WB. ELISA-positive samples with high titers were also positive by WB for both bat species. However, only 1 sample from an *R. leschenaulti* bat was positive by WB that used a baculovirus-expressed recombinant N protein. Because of the different protein expression systems, the reactivity of bat antibody against NiV protein in WBs showed different patterns (online Technical Appendix Figure 2). Neutralization tests (NTs) in which live NiVs (strain Ma-JMR-01–98) were used were performed at the Institute of Tropical Medicine, Nagasaki University. No specimens of *C. sphinx* bats were positive by NT; however, 2 specimens from *R. leschenaulti* bats, both positive with low titers, were confirmed to be positive by NT (50% cytopathic effect after NT, titers of 33.6 and 14.1). However, the latter specimen was negative by WB analysis.

Seroepidemiologic studies in other countries have indicated that *Pteropus* spp. bats (fruit-eating bats) are the main reservoirs for NiV (*3*–*6*). Pteropid bats are usually found only in southern Vietnam. We could not obtain these bats for our study. However, a relatively high prevalence (49.1%) of henipavirus antibody was found in *R. leschenaulti* specimens from Hoa Binh Province. *Rousettus* spp. bats are the only megabats that use echolocation. These bats hang together on cave ceilings in a tightly packed manner and in groups composed of bats of both sexes and of different ages. They roost in large colonies and fly vast distances to find fruit (*7*). This behavior might be related to their high rate of seropositivity for viral infections. In southern China, bats of the same species showed a high prevalence of henipavirus antibody (*8*). *R. leschenaulti* bats are distributed from central to northern Vietnam and southern China. *C. sphinx* bats are common all over Vietnam, and their

Table. Results of serologic tests for Nipah virus on bats captured in Vietnam, 2007–2008*

Bat species	No. samples	No. (%) ELISA+	No. WB+/no. ELISA+	No. micro-NT+/no. ELISA+†
Megachiroptera				
Cynopterus sphinx	109	3 (2.8)	2/2	0/3
Rousettus leschenaulti	74	31 (41.9)	18/20	2‡/31
Total	183	34 (18.6)	20/22	2/34
Microchiroptera				
Chaerephon plicata	130	0		
Hipposideros armiger	1	0		
Hipposideros cineraceus	3	0		
Hipposideros larvatus	3	0		
Hipposideros pomona	5	0		
Hypsugo cadornae	25	0		
Megaderma spasma	3	0		
Miniopterus magnater	1	0		
Scotophilus kuhi	45	0		
Unidentified	52	0		
Total	268	0		

*+, positive; WB, Western blot; NT, neutralization test. Blank cells indicate that test was not done.
†Micro-NT was done only on specimens positive for Nipah virus by ELISA.
‡One of the NT-positive samples was negative by WB analysis.

habitat overlaps with that of pteropid bats in southern Vietnam.

Previous studies showed that IgG ELISA results for NiV-positive flying foxes correlated well with NT results (*3*,*4*). However, in our study, discrepancies existed between NT results and NiV-N-ELISA and WB results. A reason for these differences could be that Nipah-like viruses are circulating among bats in Vietnam, producing antibodies that are cross-reactive by ELISA and WB, but poorly cross-reactive by NT. The cross-reactive antibodies were probably not directed against neutralizing epitopes. To date, no reports have been made of an increased number of febrile encephalitis cases among the residents in Hoa Binh and Dak Lak Provinces where seropositive bats were captured. The circulating viruses may lack the pathogenic potential of Hendra and Nipah viruses.

A survey by questionnaire was conducted among residents of Dak Nong and Dak Lak Provinces, where NiV-N-ELISA–positive *C. sphinx* bats were captured, to determine the frequency of contact between humans and bats. Risk factors for infection were observed in this study, such as bat hunting and cooking and drinking bat blood. In such situations, persons have direct contact with bat body fluids and feces and might be bitten during bat hunting. Thus, long-term systematic surveillance of bats is needed to determine the ecologic relationship between bats, humans, other animals, and the environment.

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Discordance in *Mycobacterium tuberculosis* Rifampin Susceptibility

To the Editor: Multidrug-resistant tuberculosis (MDR TB), i.e., TB resistant to at least the 2 most effective first-line antituberculous drugs (isoniazid [INH] and rifampin [RIF]), is increasing globally. World Health Organization estimations of 390,000–510,000 new MDR TB cases and 150,000 related deaths in 2008 highlight the need for timely drug susceptibility testing and improved therapies (*1*). Although novel rapid drug susceptibility testing tools are increasingly available, their clinical applicability is unsettled. We report a patient with pulmonary TB relapse with discordant genotypic and in vitro phenotypic drug susceptibility testing results associated with a mutation outside the RIF resistance determining region (RRDR) of the *rpoB* gene.

In August 2009, a 45-year-old homeless woman with AIDS ($CD4^+$ T-cell count 3 cells/mm^3) and a history of substance abuse sought care for fever, night sweats, weight loss, and cough (online Appendix Table, wwwnc.cdc.gov/EID/article/18/3/11-1357-TA1.htm). Pulmonary TB had been diagnosed in June 2008. At

that time, she received, by directly observed therapy, 6 weeks of INH, RIF, pyrazinamide (PZA), and ethambutol (EMB) through the local health department and was switched to RIF, PZA, and EMB on week 7 after the isolate was determined by liquid culture with BD BACTEC MGIT 960 Mycobacterial Testing System (BD Diagnostics, Sparks, MD, USA) to be INH resistant. During that period, she resided in American Lung Association–supported housing for TB patients and had 97% medication adherence by dose count. Her condition clinically improved, infiltrates completely resolved according to chest radiograph, 12 sputum inductions failed to yield sufficient material for analysis, and she began highly active antiretroviral therapy (HAART). In December 2008, however, because of crack cocaine use and belligerent behavior, she lost housing privileges. Caseworkers could not locate her to complete the 9-month planned directly observed therapy.

The woman was hospitalized in January and again in February 2009 with dyspnea and off medications. In both instances, chest radiographs showed no new changes, sputum specimens were negative for acid-fast bacilli (AFB) by microscopy and culture, and she was treated for presumptive *Pneumocystis* pneumonia and showed clinical improvement.

In August 2009, she was readmitted with cough and new cavitation on chest radiograph. Chest computed tomographic scan demonstrated right upper lobe infiltrates, bilateral lower lobe cavitation, and hilar and mediastinal lymphadenopathy. Sputum AFB smear was positive (graded 4+), and nucleic acid amplification (Amplified Mycobacterium Direct Test; Gen-Probe, San Diego, CA, USA) was positive for *Mycobacterium tuberculosis* complex. INH, RIF, PZA, and EMB, along with moxifloxacin (MXF) and amikacin (AMK), were initiated in accordance with 2003 national TB treatment guidelines for possible MDR TB (*2*). Shortly thereafter, a line probe assay (GenoType MTBDR*plus*; HAIN Lifescience, Nehren, Germany) performed by Southeastern National TB Center (Gainesville, FL, USA) on the culture of the sputum specimen obtained at admission indicated an *inhA* point mutation but no mutation in the RRDR region of the *rpoB* gene, which suggested that the isolate was INH resistant but RIF susceptible. AMK was discontinued, and the patient was discharged with RIF, PZA, EMB, and MXF.

One week later, drug susceptibility testing (BD BACTEC MGIT 960 System) results from the state mycobacteriology laboratory demonstrated that the *M. tuberculosis* isolate was resistant to INH and RIF. The patient was readmitted to resume injectable aminoglycoside therapy. After 5 weeks, sputum culture became negative, clinical and radiographic improvement was apparent, and HAART was reinitiated. She completed 2 months' INH/PZA/EMB/MXF/AMK inpatient therapy and was discharged to complete 6 additional months of PZA/EMB/MXF and streptomycin followed by 16 months of PZA/EMB/MFX. With HAART, her plasma HIV RNA viral load became undetectable, but her CD4 count remained low (9 cells/mm^3). She died from a motor vehicle accident 10 months after recurrent TB was diagnosed.

In this patient, RIF resistance was not predicted by line probe assay but was identified phenotypically by an automated system (BD BACTEC MGIT 960 System) that continuously monitors for growth and detection of mycobacteria. Through genotyping and DNA sequencing of the 2008 and 2009 *M. tuberculosis* isolates, the Mycobacteriology Laboratory Branch at the Centers for Disease Control and Prevention (Atlanta, GA, USA) established that the 2009 infection was a relapse, not re-infection, and confirmed the *inhA* mutation in both isolates. Using primers extending beyond the RRDR (the *rpoB* region surveyed by rapid molecular tests and responsible for >95% of RIF resistance mutations) the laboratory identified a novel *rpoB* gene mutation at codon 480 (ACC→AAC; Thr→Asn) and another previously described (*3*–*5*) mutation at codon 176 (GTC→TTC; Val→Phe) in the 2009 isolate, which has been implicated in RIF resistance. The role of the T480N mutation in RIF resistance is being investigated.

This case demonstrates the limitations of rapid molecular drug susceptibility testing (*6*). Rapid molecular diagnostics are valuable adjuncts to conventional phenotypic testing because they can quickly confirm clinically suspected MDR TB and have high agreement with other genotypic and phenotypic methods (*7*–*10*). However, they should not supplant phenotypic testing, and clinicians should understand their limitations. When rapid molecular tests are negative but suspicion for MDR TB is high, MDR TB treatment should be continued until phenotypic susceptibility results are available. DNA sequencing may be best suited for evaluating suspected drug-resistant *M. tuberculosis* isolates with discordant results for phenotypic susceptibility and rapid molecular testing.

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High Incidence of Group B Streptococcal Infection in Infants Born to HIV-Infected Mothers

To the Editor: In their cross-sectional study comparing group B streptococcus (GBS) carriage among HIV-infected and HIV-uninfected women in Malawi, Gray et al. found no differences in GBS carriage between both groups but found a higher carriage rate for HIV-infected women with high CD4 cell counts (*1*). They proposed that a GBS-specific immune defect might exist in HIV-infected pregnant women and suggested that this defect could be blurred by competitive exclusion of GBS as a consequence of changes in microbial flora at lower CD4 counts.

We recently reported an increased incidence of neonatal GBS sepsis in HIV-exposed uninfected (HEU) infants born in Belgium, compared with the general population (*2*). In our cohort, the risk for GBS infection was 20× higher in HEU infants than in infants born to HIV-uninfected mothers. Moreover, the episodes of GBS sepsis in HEU infants, compared with the general population, were more severe and mostly of late onset. We are currently looking prospectively at GBS carriage in HIV-infected and control uninfected pregnant women to learn whether our observation can be explained by a higher carriage rate in HIV-infected women or by increased susceptibility of HEU infants to this capsulated bacteria. The latter hypothesis would be in line with the higher susceptibility of HEU children to other types of severe infections, as has been described in several studies from sub-Saharan Africa and Latin America (*3*–*5*).

The incidence of GBS sepsis in HIV-exposed infants born in Africa is unknown. In addition to the need for further investigation of anti-GBS immunity in HIV-infected pregnant women, we believe that studies comparing the incidence of neonatal GBS sepsis in HEU and HIV-unexposed infants are warranted. If the increased risk for GBS sepsis is confirmed, prophylaxis should be implemented in the population concerned.

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Etymology of Cholera

To the Editor: I read with great interest the article by Männikkö (*1*) on the etymology of cholera. However, discovering the origin of the word with certainty is an intricate matter. The word cholera is undoubtedly Greek because Hippocrates was the first to mention it in his writings, although the exact disease he was referring to is unknown (*2*,*3*).

Apart from the rather probable derivation from *cholē* (the word for bile and a dominant term in the humoral theory, which is of Hippocratic and not Galenic [*1*] provenance), one more hypothesis has been suggested. The word *cholera*, sometimes *cholēdra*, originally meant a gutter (*4*). Following this connection, cholera came to mean a pestiferous disease during which fluids are forcefully expelled from the body, resembling a gutter (*4*). This etymology-derived definition could suggest that Hippocrates and Galen, the prolific medical writers of antiquity who each in his time referred to cholera, may have witnessed cases of this infectious disease, albeit not in the epidemic form it took in ancient India (*5*).

In addition, a missing clue on this issue is that cholera might derive from *cholās*, an Attic word meaning intestine, which has not survived in modern Greek (*4*). This new connection with the gastrointestinal tract further suggests possible knowledge of cholera in its present form, mainly diarrhea and vomiting. Hippocrates made such a reference, although loosely (*2*). Reaching a conclusion on the etymology of cholera remains intriguing.

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Paulus Potter (c. 1625–1654) *God Appearing to Abraham at Sichem* (1642) (detail) Oil on canvas (100.4 cm × 130.8 cm) From the collection of Dr. Gordon Gilbert, St. Petersburg, Florida, USA. Photo by Ray Bassett

The Shortest Follies Are the Best

—Pierre Charron, La Sagesse

Polyxeni Potter

"There is a kind of commerce betwixt beasts and us, a certain relation and mutual obligation, whereof there is no other reason, but that they belong to one and the same master, and are of the same family that we are," wrote French philosopher and theologian Pierre Charron. Not a new idea, this partnership between humans and animals dates back 9,000 years to the domestication of animals in the Stone Age. And as far back as the Paleolithic Age, art on cave walls suggested a close, if perilous, human association with wild and dangerous beasts—a short folly, not common today, except among hunters and adventurers.

Animals feature prominently in art of all ages, and in the 17th century, no longer as part of human scenes but as the primary subject matter. This shift signaled a change in the balance of power between owner and subservient beast, between humans and work animals. Dutch painter Paulus Potter understood this and expressed it by moving away from the human figure, traditionally the artist's "noblest calling." Some of his paintings contained no humans at all, bringing instead to center stage the horse or milk cow, the donkey or the mule, which until the advent of machines, were primary providers of agricultural, industrial, and other services.

Potter received early instruction from his father, a noteworthy landscape and figure painter, and from Nicolaes Moeyaert and others, who painted biblical and mythologic scenes. But soon he took off in his own direction, precocious, prolific, and popular, learning from nature and painting furiously, as if anticipating his demise before the age of 30 from tuberculosis. He joined the guild of St. Luke in Delft, married well, and was soon noticed by Dutch surgeon and mayor of Amsterdam Nicolaes Tulp, who had also earlier commissioned young Rembrandt van Rijn to paint the famed *Anatomy Lesson of Dr. Nicolaes Tulp*. At the mayor's invitation, Potter moved to Amsterdam, where, like Rembrandt before him, he became Tulp's protégé. The portrait of Tulp's equestrian son Dirk was Potter's last work. His painting *The Young Bull* (1649) was to become as famous as Rembrandt's *Night Watch.*

During his brief artistic career, Potter became the most famous animalier of his day and a pioneer in featuring farm beasts outside the biblical or mythologic context. His approach influenced the presence and appearance of animals in European art. He painted them with sensitivity

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and exquisite naturalism in all their activities: standing, eating, drinking, sleeping, at work or at rest; integrated them in local valleys, trees, and waterfalls; and showed them in perfect harmony with nature, as humans rarely are.

The appearance and behavior of the diverse species of animals painted by Potter illustrate their interaction with humans during the 17th century, a period of land reclamation and sweeping socioeconomic and religious changes in Holland. All over Europe, agricultural production was increasing as a result of new technologies, ushering in unprecedented prosperity. An expanding art market brought in new buyers, whose perceptions and interpretations of the role and image of animals in society influenced artistic styles. At this time in Holland, the well-groomed milk cow was a symbol of stability and peace.

God Appearing to Abraham at Sichem is a bucolic scene with a biblical bent. A posthumous portrait of Potter by Bartholomeus van der Helst closely resembles the face of Lot in this painting—a self-portrait of Potter at age 16. Abraham may be depicted as the artist's father; Sara as his mother. The patriarchal family is anchored against a tree in the corner of the canvas. In the center, two prominent milk cows gaze directly at the viewer. The carefully constructed landscape is traversed by a convoy of animals and their attendants as far as the eye can see. This is a fine spot with a sprawling mountain view. Everything is balanced and peaceful. The children are touching the animals. A building with a tower stands nearby amidst abundant water and vegetation. The ample sky above is pregnant with anticipated divine promise.

As naturalistic as this scene might have been intended, it seems idyllic to us. Not that the milk cows, goats, and other animals would seem out of place in today's herds or that divine promise is no longer needed. What is missing, unknown to the painter, is the zoonotic touch. Much has happened since the painter was revolutionizing the presentation of food-producing animals.

We have learned a few things, and not only about what cut short the painter's life. Bovine tuberculosis, a long-time source of human infection, has declined in many parts of the world through control of the disease in cattle, affirming the application of "one medicine" for animal and human health. But progress in some is offset by new developments in other animal diseases, such as in the identification of a new orthobunyavirus in cattle in Europe and the newly defined European pathogen sheep in a devastating disease of New World bighorn sheep. In addition, chronic wasting disease continues to spread geographically among farmed and wild deer and elk in the United States and Canada, fanning concerns about its unknown zoonotic potential.

Many other infections continue to plague animals harvested as food and the humans who rely on them. For example, neurocysticercosis, a well-known neurologic disease in developing countries, is increasingly found in immigrants in the United States, where the infection is not endemic. Recent outbreaks in Europe and the United States suggest an expanding role of bovine non-O157 *E. coli* strains in human disease. Unpasteurized dairy products continue to be associated with disease outbreaks in the United States. And for reservoirs and interspecies transmission of highly pathogenic viruses like Nipah virus and Hendra virus, the relationships between bats, humans, other animals, and the environment remain difficult to understand and control.

Despite increasing knowledge of the closeness of human and animal destiny, the "mutual obligation" so clearly defined by Charron is slow to advance. And, despite public health efforts, long follies persist for lack of adequate study or application of existing epidemiologic and biomedical tools.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

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http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

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Washington State Convention Center
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http://www.retroconference.org

March 11–14, 2012
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Hyatt Regency
Atlanta, GA, USA
www.iceid.org

April 13–16, 2012
SHEA Spring 2012 Conference
Jacksonville, FL, USA
www.shea2012.org

May 6–9, 2012
8th International Symposium on Shiga Toxin (Verocytotoxin) Producing *Escherichia Coli* Infections
Amsterdam, Netherlands
www.vtec2012.org

May 9–13, 2012
8th International Congress on Autoimmunity 2012
Granada, Spain

July 22–27 2012
XIX International AIDS Conference (AIDS 2012)
Washington, DC, USA
www.aids2012.org/Default.aspx

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Article Title

Nonpasteurized Dairy Products, Disease Outbreaks, and State Laws—United States, 1993–2006

CME Questions

1. Your patient is a 12-year-old girl who presents for 3 days of gastroenteritis symptoms. Her father reports that most of the family has been consuming unpasteurized dairy products for the past year, and he is concerned that his children are at risk for foodborne illness.

On the basis of the current study by Langer and colleagues, what should you consider regarding the epidemiology of foodborne illness related to dairy products?

A. Fewer than 20 cases were reported to the CDC between 1993 and 2006
B. Unpasteurized dairy products accounted for most outbreaks
C. Yogurt was the dairy product implicated in most cases
D. Unpasteurized cheese was more likely to promote infection compared with unpasteurized milk

2. In your management of this patient, what should you consider from data reported in the current study regarding the clinical picture of infection associated with contaminated dairy products?

A. The rate of hospitalization was similar whether the infection was due to pasteurized or unpasteurized products
B. The overall hospitalization rate was 25%
C. The case-mortality rate approached 50% for infections due to unpasteurized products
D. Patients with infection due to unpasteurized products were younger compared with those with infection due to pasteurized products

3. What was the most common organism isolated from patients with infection due to consumption of unpasteurized dairy products in the current study?

A. Norovirus
B. *Salmonella* spp.
C. *Escherichia coli*
D. *Campylobacter* spp.

4. The patient's mother drinks pasteurized milk, and she, too, is not feeling well. What can you tell her was the most common source of contamination of pasteurized dairy products in the current study?

A. Infected food handlers
B. Temperature abuse
C. Consumption of dairy products beyond their expiration date
D. Poor pasteurization practices

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Community-associated *Clostridium difficile* Infections, Monroe County, New York, USA

CME Questions

1. On the basis of the study by Dumyati and colleagues, which of the following statements about the relative burden of and potential risk factors for community-associated (CA) *Clostridium difficile* infection (CDI) disease is most likely correct?

A. CA-CDI accounted for about half of CDI cases in Monroe County, New York, in 2008
B. The proportion of CA-CDI in this study was much higher than that reported in other recent surveillance studies
C. About three quarters of patients with CA-CDI had taken antibiotics in the 12 weeks before diagnosis
D. About half of patients with CA-CDI reported proton pump inhibitor use

2. On the basis of the study by Dumyati and colleagues, which of the following statements about clinical characteristics of patients with CA disease is most likely correct?

A. About one fifth of the hospitalized CA patients died or had serious complications
B. In addition to diarrhea, 86% reported abdominal pain, 48% nausea, 40% fever, and 17% bloody stool
C. CA-CDI patients were older than HA-CDI patients
D. Molecular testing showed significantly different distribution of strains between HA-CDI and CA-CDI

3. You are a public health official asked to consult on strategies for CA-CDI prevention and surveillance. On the basis of the review by Dumyati and colleagues, which of the following statements would most likely appear in your report?

A. Reducing exposure to specific foods and animals is a viable strategy
B. Outpatient clinicians should be educated about the risk for CA-CDI following oral antibiotic use and about the need for judicious antibiotic use
C. Risk factors leading to CDI in patients not exposed to antibiotics are well defined
D. *C. difficile* recovery rate from refrigerated stool swabs is poor

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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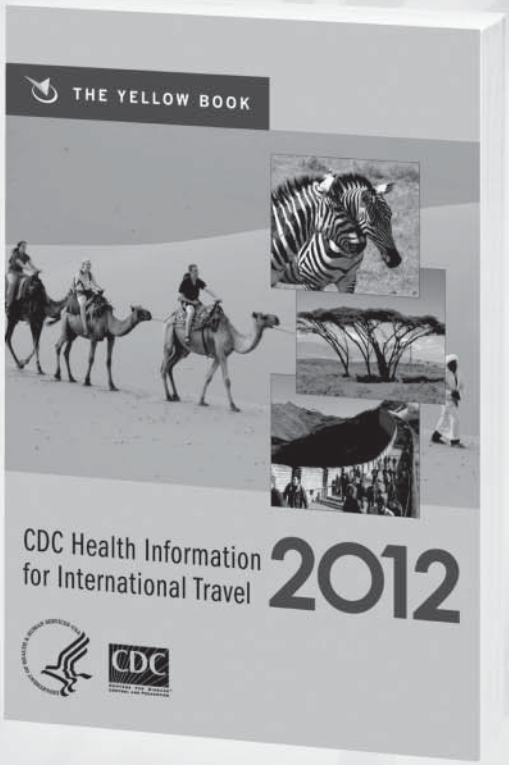

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JOURNAL BACKGROUND AND GOALS

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The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES®

February 2012

Infections New and Old

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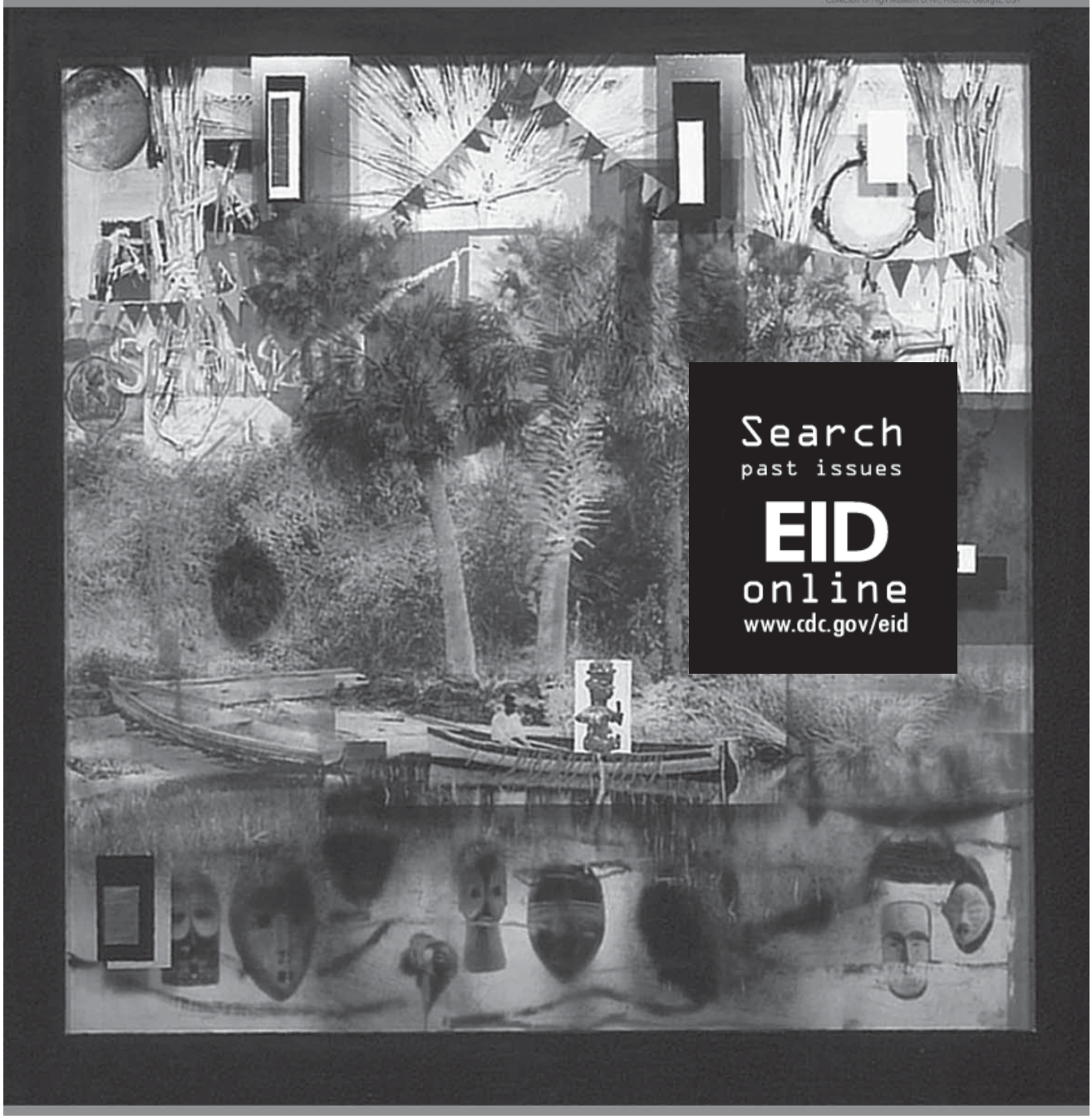

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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

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Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures as separate files, in the native format when possible (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpg or .tif files. Other file formats may be acceptable; contact fue7@cdc.gov for guidance. Figures should not be embedded in the manuscript file. Use color only as needed. Use Arial font for figure lettering. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced to print size. Large figures may be made available online only. Place figure keys within the figure; figure legends should be provided at the end of the manuscript file.

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.