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On the Cover: **Census Proceedings on the Campus Martius. Altar of Domitius Ahenobarbus. Decoration from the base of a statuary group. Rome. End of second century BCE.** Marble (78 cm × 559 cm). Louvre, Paris, France/Lauros/Giraudon/The Bridgeman Art Library Nationality/copyright status: out of copyright. **About the Cover p. 1974**

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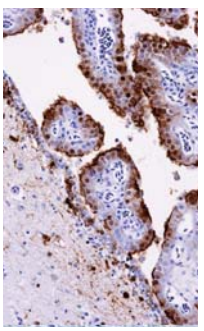

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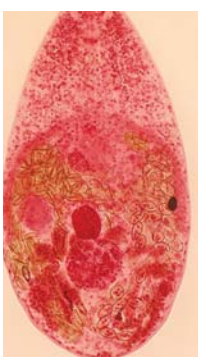

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Impact of Globalization and Animal Trade on Infectious Disease Ecology

Nina Marano,* Paul M. Arguin,* and Marguerite Pappaioanou†

The articles on rabies (*1*) and Marburg (*2*) virus featured in this month's Emerging Infectious Diseases (EID) zoonoses issue illustrate common themes. Both discuss zoonotic diseases with serious health implications for humans, and both have a common reservoir, the bat. These articles, and the excitement generated by this year's recognition of World Rabies Day on September 8, also described in this issue (3), remind us how globalization has had an impact on the worldwide animal trade. This worldwide movement of animals has increased the potential for the translocation of zoonotic diseases, which pose serious risks to human and animal health (*3*).

The magnitude of the global movement of animals is staggering. In terms of sheer numbers, 37,858,179 individually counted live amphibians, birds, mammals, and reptiles were legally imported to the United States from 163 countries in 2000–2004. These imports included Asian macaques, South American rodents, and African great cats (*4*).

Why do we have a global trade in animals? Animals are legally imported into the United States for many reasons. They are used for exhibitions at zoos; scientific education, research, and conservation programs; food and products; and in the case of companion animals, tourism and immigration. Increasingly, however, animals are being imported for a thriving commercial pet trade. In many cases the animals that are imported and traded are of species that are considered exotic (here defined as non-native species, animals not traditionally kept as pets, or both). This can be a risky business, as many shipments include a high volume of wild-caught versus captive raised. animals For most of these animals, there are no requirements for zoonotic disease screening either before or after arrival into the United States. There have been anecdotal reports of high rates of death among animals in these shipments.

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Animals imported for commercial trade represent a substantial risk to human health. In 2003, monkeypox was introduced into the United States when a shipment of African Gambian giant rats was sold to dealers, one of whom housed the rats with prairie dogs intended for the pet trade in a US distribution facility. The prairie dogs subsequently became ill and transmitted the infection to 71 humans, including prairie dog owners and veterinary staff caring for the ill animals (*5*). In addition to monkeypox, human tula-

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remia and salmonellosis outbreaks have been traced back to contact with prairie dogs and hedgehogs (*6*,*7*).

Exotic pet ownership brings unanticipated risks to agribusiness, wildlife conservation, and the ecosystem. For example, giant African land snails released into farmlands have become agricultural pests. They reproduce rapidly, consume large amounts of vegetation, and are hosts for parasites such as *Angiostrongylus cantonensis*. Pet pythons released into wetlands become unchecked predators, warping the balance of the existing food chain. Snakehead fish, imported as a delicacy for the live food markets, have turned up in ponds and waterways, where they quickly dominate the ecosystem at the expense of native species. And the illegal trade of exotic wildlife, with promises of considerable financial return in the underground markets, has disastrous implications for many endangered or threatened species.

How easy is it to get an exotic animal? Checked the Internet lately? It's now possible to obtain almost any type of exotic pet animal through the Internet, as opposed to purchasing them in pet stores, which are subject to licensure and inspection.

As a scientist, one might suggest solutions that employ familiar tools, such as postarrival screening of animals with reliable laboratory tests, empirical treatment for known diseases (if such tests and treatments already existed), or quarantine of the animals for an appropriate length of time. Many of these solutions are not feasible or practical to use on the large volume of animals that are being imported and cannot be employed to prevent new or emerging pathogens or infections. Ultimately, import restrictions may be the only means of preventing introduction of exotic infections.

Despite the societal costs of importing exotic animals, as well as the difficulties in regulating enforcement and coordination of efforts, there are also benefits and compelling reasons for importing certain species of wildlife. Many wildlife conservation and species survival programs depend on importing exotic animals, including endangered species kept by zoos. Much is learned from captive wild animals and the knowledge gained about how to manage disease problems. Zoologic societies' ability to use animals that are legally imported has enabled public education about endangered animals; were it not for legal animal importation and exhibition in zoological institutions, many species of animals, including bats, would be extinct in the wild.

Partnerships comprising experts and agencies involved with human, animal, and ecosystem health are critical to prevent and control imported zoonotic diseases. Such partnerships benefit public, animal, and ecosystem health. There are several unfortunate examples of the failure of partners across these areas to work together. They include governmental decisions in the People's Republic of China to slaughter dogs as a control measure for rabies and advocating extermination of storks as a control measure for avian influenza in Thailand (*8*,*9*) In the case of the dogs, an integrated team of animal and public health professionals might have implemented alternate control measures, such as leash laws and rabies vaccination of dogs. The storks were luckier: wildlife conservationists and other partners in the animal health sector eventually intervened to convince governmental authorities that slaughter of storks was not an appropriate control measure for avian influenza.

As in the past 3 EID zoonoses theme issues, we have called for renewed effort for the public health and animal sectors to work together, in this case to mitigate the impact on infectious disease ecology caused by unrestricted translocation of animals. Prevention efforts should include reducing both the supply of and the demand for exotic animals. However, navigating the myriad responsibilities of the different sectors for human, livestock, companion animal and wildlife health continues to be a challenge. Guidelines addressing the infectious disease risks associated with exotic animals that may help raise awareness of the risks and decrease the demand for exotic animals have been published (*10*). However, no single agency can solve this problem alone; it is only through partnership with other federal agencies, wildlife associations, veterinary medical associations and private industry that we will be able to better control the global movement of animals and reduce the risk of introducing emerging infectious diseases into new locations.

The "One Medicine Initiative" announced by Roger Mahr, the 2006 President of the American Veterinary Medical Association, has led to the 2007 formation in the United States of a "One Health Task Force" to bring wildlife, environmental, human, and domestic animal sectors together for a coordinated approach to improving and protecting human and animal health (*11*). This coordinated approach, actively supported by multiple stakeholders, takes into account the larger ecologic context of infectious diseases and improves our ability to prevent disease rather than simply reacting to new outbreaks as they emerge. We look forward to the work of the Task Force and other important cross-disciplinary initiatives, as well as the efforts of the informed readership of EID to make important contributions in stemming the magnitude of live animal trade that poses risks to human, animal, and ecosystem health.

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Effects of Local Anthropogenic Changes on Potential Malaria Vector *Anopheles hyrcanus* and West Nile Virus Vector *Culex modestus*, Camargue, France

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Using historical data, we highlight the consequences of anthropogenic ecosystem modifications on the abundance of mosquitoes implicated as the current most important potential malaria vector, *Anopheles hyrcanus,* and the most important West Nile virus (WNV) vector, *Culex modestus*, in the Camargue region, France. From World War II to 1971, populations of these species increased as rice cultivation expanded in the region in a political context that supported agriculture. They then fell, likely because of decreased cultivation and increased pesticide use to control a rice pest. The species increased again after 2000 with the advent of more targeted pest-management strategies, mainly the results of European regulations decisions. An intertwined influence of political context, environmental constraints, technical improvements, and social factors led to changes in mosquito abundance that had potential consequences on malaria and WNV transmission. These findings suggest that anthropogenic changes should not be underestimated in vectorborne disease recrudescence.

During the past 25 years, there has been a dramatic emergence and resurgence of epidemic vectorborne diseases affecting both humans and domestic animals (*1*). In most cases, sociodemographic changes, drug resistance, and anthropogenic environmental modifications appear to be the main factors responsible (*1–4*). The Camargue, the Rhone River Delta region in southeastern France, is an area relevant to the study of the influence of environmental changes on vector populations because 1) it has witnessed important anthropogenic ecosystem modifications in the past 60 years, 2) it contains a great abundance and diversity of mosquito-breeding sites and thus hosts large mosquito populations, and 3) it is a former zone of endemic malaria and a region of current and regular transmission of West Nile fever.

Until the beginning of the 20th century, malaria, mainly transmitted by *Anopheles (Anopheles) atroparvus* Van Thiel, was endemic in the Camargue and constituted a major health issue there (*5*,*6*). The last *Plasmodium vivax* malaria epidemic occurred in 1943, with ≈400 cases (*7*). Malaria disappeared from this area after World War II because of improved housing and living conditions and the extensive use of quinine. Among 8 anopheline species recorded in the Camargue, *An. atroparvus* was recently found to be rare and *An. (Anopheles) hyrcanus* (Pallas) very abundant with a high human-biting rate (*8*), findings that suggest that *An. hyrcanus* is currently the only *Culicidae* sp. likely to play a role in malaria transmission in the Camargue (*8*). Moreover, autochthonous transmission was recently suspected on the French Mediterranean Coast in 2006 (*9*), which also supports the idea that southern France remains suitable for malaria transmission.

The first description of West Nile virus (WNV) in France was in the 1960s, with human and equine outbreaks in the Camargue (*10*). After these episodes, the disease seemed to disappear from this region. However, WNV transmission apparently continued thereafter and

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was confirmed by serologic studies in the 1970s and 1980s (*11*,*12*). Since 2000, WNV-related disease has reappeared in southern France, causing equine outbreaks in 2000 (76 confirmed cases) and 2004 (32 confirmed cases) in the Camargue (hosting 7,000 equids) and sporadic human and/or equine cases in 2003 and 2006 elsewhere along the Mediterranean Coast (*13*–*16*). Among 7 *Culex* species recorded in the Camargue (*17*), *Culex (Barraudius) modestus* Ficalbi is considered the main WNV vector, based on abundance, feeding behavior, previous WNV isolations, and recent experimental transmission (*18*–*20*).

The aim of this article is to describe the history of the region and to examine the impact of the various anthropogenic environmental changes that have occurred in the Camargue over the past 60 years on 2 mosquito species, *An. hyrcanus* and *Cx. modestus.* Because rice fields are the quasi-exclusive breeding sites for *An. hyrcanus* and the most prolific sites for *Cx. modestus* (*5*), we focus on changes in rice cultivation, i.e., cultivated surfaces and agronomic practices, including insecticide spraying, related to socioeconomic and agronomic factors.

Context

The Study Area

The Camargue is the main wetland area in the southeast of France and covers the Rhone River Delta (Figure 1). This area has a Mediterranean climate characterized by warm, dry summers and mild, wet winters. Total annual rainfall is typically 500–700 mm and occurs mainly in autumn; the annual mean temperature is 14°C.

Landscapes in the Camargue are strongly affected by the duration of submersion and the salinity of the soils. The landscapes are organized roughly in a south-to-north gradient of salinity, with agricultural land and reed marshes in the north and natural salty ponds and salt marshes in the south. Most agricultural land belongs to a few large farms, which are able to rapidly change their production system (i.e., crop type), depending on the economic context (*21*,*22*). Rice is currently the main cultivated crop in the Camargue, which is almost the only French region that produces rice. Paddies are filled in April and May with ≈7 cm of water. From the end of June until the end of August, a depth of ≈20 cm of water is maintained in the paddies, and the rice plants cover their surfaces. The water is then drained and the rice harvested. Data on rice cultivation used in this article were provided by the French National Rice Center.

The Mosquito Species

A. hyrcanus is a Palearctic mosquito species belonging to the Hyrcanus group. It is distributed from Spain to People's Republic of China, covering the southern half of Europe, the Mediterranean area, and central Asia. Large populations are found in irrigated rice-growing areas in Turkey, Greece, and France (*23*,*24*), and this species was involved in malaria transmission in the north of Afghanistan (*25*).

Cx. modestus is also a Palearctic species, widely distributed from Europe to India, especially in delta areas, where its larvae can be found in semipermanent reed marshes, irrigation canals, and rice fields (*5*). The involvement of *Cx. modestus* in WNV transmission was established in the Camargue (*20*,*26*), the delta areas of the Caspian and Azov Seas (*27*,*28*), and the Volga region of Russia (*29*) and was suspected in the Danube Delta (*30*).

For our study, changes in *An. hyrcanus* and *Cx. modestus* abundance were assessed by using 1) literature data, 2) detailed annual activity reports that describe nuisance caused by mosquito pests and published from 1962 to 1996, and 3) data from regular human mosquito-landing collections conducted since 1969 in 12 sites in the western Camargue (for 15 minutes at sunrise, approximately once a week from June to October). These sites were sampled by using the same methods, thus allowing us to describe changes in *An. hyrcanus* and *Cx. modestus* abundance over a 38-year period. Collection sites were distributed in the western Camargue, which allowed a sampling of all ecosystems from the north to the south, reflecting mosquito abundance in the entire region (Figure 1). This human mosquito-landing survey did not focus specifically on *An. hyrcanus* and *Cx. modestus.* Thus, some sites were located some distance from the indicated area under cultivation (even if these areas have changed during the past 60 years) and were probably always negative for *An. hyrcanus* and *Cx. modestus*. Unfortunately, results of human mosquito-landing collections were not available per sample site. Thus, to avoid overrepresentation of uninformative and consistently negative sites, quantitative abundance of *An. hyrcanus* and *Cx. modestus* was assessed by the annual mean number of

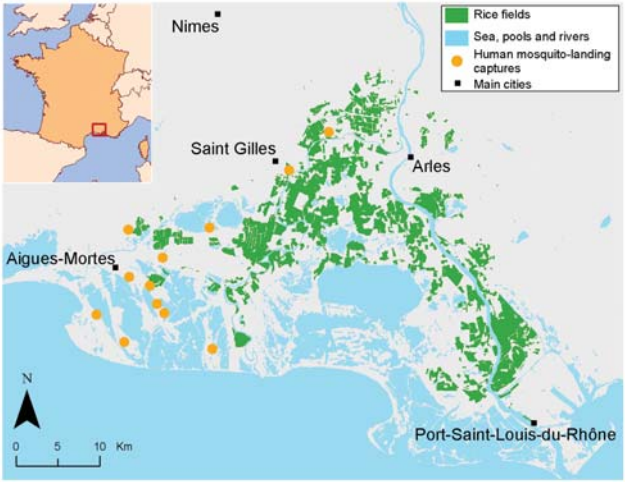

Figure 1. Map of the Camargue, France, indicating areas of rice cultivation as well as mosquito sampling sites, 2001.

mosquitoes caught by positive collection. Changes in rice cultivation and mosquito populations, shown in the online Appendix Figure (available from www.cdc.gov/EID/content/13/12/1810-appG.htm), were analyzed for 3 periods, described below.

Changes in Mosquito Populations and Rice Cultivation since the 1920s

1920s to 1960s: Proliferation of *An. hyrcanus* and *Cx. modestus*

In the Camargue, rice cultivation was rare before World War II, and both *An. hyrcanus* and *Cx. modestus* were only rarely reported after their first description in the 1920s until World War II (*5*,*31*–*36*). The development of rice cultivation started with the rice shortage caused by World War II and was supported by a guaranteed price and funds from the Marshall Plan in 1947 and by the agricultural equipment cooperative established in 1948. Rice cultivation was then mechanized and hugely increased to cover ≈30,000 ha during the 1960s (online Appendix Figure). In the 1950s and the 1960s, *An. hyrcanus* and *Cx. modestus* populations were described as widely distributed and very abundant in the entire Camargue, and these 2 species were included in the group of the 3 most abundant nuisance biters (*5*). *An. hyrcanus* was considered a major pest in the western Camargue in 1969 and 1970, and *Cx. modestus* attacks reached 300 bites per person per hour in reed marshes (*26*). From 1942 to the 1960s, the increase in *An. hyrcanus* and *Cx. modestus* populations seemed to follow changes in paddy surface area (online Appendix Figure), itself a product of the political consequences of World War II (e.g., agricultural support, mechanization).

1960s to 1999: Near Disappearance of Mosquito Populations

Decrease of Area under Rice Cultivation

In 1963, the enforcement of the Common Agricultural Policy of the European Community caused unfavorable conditions for French rice cultivation, which was confronted by the more competitive Italian rice cultivation. This situation depressed both prices and incomes for French producers, who abandoned rice cultivation and developed alternatives such as hard wheat. The area under rice cultivation started to decrease slowly after 1965 (online Appendix Figure).

Insecticide Implementation

In 1970, the striped rice borer, *Chilo suppressalis* (Walker), a pest insect that damages rice plants, was introduced into France on young rice plants imported from Spain (*37*). From 1972, rice producers implemented insecticide sprayings with fenitrothion, trichlorfon, and chlorphenamidine, which were conducted at the end of July each year, to control this pest (Table). The striped rice borer invasion reduced French rice competitiveness and consequently accelerated the decline in rice cultivation to 4,400 ha by 1981.

Human mosquito-landing collections showed a drastic drop in *An. hyrcanus* and a progressive decrease in *Cx. modestus* populations in 1972 and 1973, after the insecticide sprayings were initiated (online Appendix Figure). At the end of July, insecticide, also efficient against mosquito larvae, was sprayed by fixed-wing airplane that used low-volume applications (15 L/hectare); the insecticide reached the water even when rice plants covered the paddy surfaces. At this time of year, *An. hyrcanus* and *Cx. modestus* larvae usually massively colonize rice fields, which in summer are nearly the only available breeding sites for these species (N. Ponçon, unpub. data) (*26*). These sprayings likely reduced *An. hyrcanus* populations considerably, with the removal of water from paddies at the end of August limiting posttreatment population recovery. In September, flooding of reed marshes, which are natural breeding sites for *Cx. modestus*, allows only a limited maintenance of populations and probably explains the slower decrease of this species than of *An. hyrcanus*. Indeed, reed marshes cannot maintain important populations, as illustrated by the rareness of *Cx. modestus* before World War II.

Increase of Area under Rice Cultivation and of Insecticide Sprayings

In 1981 a French support plan was implemented that led to an increase in rice cultivation, which covered >20,000 ha by the early 1990s. In 1994 the General Agreement on Tariffs and Trade limited subsidies, and French rice cultivation, still fairly uncompetitive, experienced difficulties once again. These problems were accentuated by a new demand for perfumed rice varieties that are not produced in the Camargue. Some producers thus replaced rice with hard wheat, which explains the decrease in rice cultivation areas since 1994 (online Appendix Figure).

In 1988 a new rice variety, *Ariete*, was introduced into the Camargue and, from 1991 to 2000, it quickly became the most cultivated rice. This variety of rice is very susceptible to the striped rice borer. Consequently, producers sprayed large areas to avoid losses and to ensure high productivity. Sprayings were conducted by using the same methods as before except that alphamethrin, also efficient against mosquito larvae, replaced the former insecticides.

The intensive insecticide sprayings against the striped rice borer likely account for the low populations of *An. hyrcanus* and *Cx. modestus* over this period, despite the increase in the area of rice cultivation. Human mosquito-landing data showed a slight population peak in both

Table. Sprayed rice surfaces (hectares) to control striped rice borer in the Camargue

Years of insecticide spraying	Insecticides effective against mosquitoes		Lepidopteron-specific insecticides	
	Fenitrothion, trichlorfon, and chlorphenamidine	Alphamethrin	*Bacillus thuringiensis kurstaki*	Tebufenozide
1972	10,000	0	0	0
1973	6,500	0	0	0
1974	9,000	0	0	0
1975–1989	Very limited	0	0	0
1990–1996	0	Intensive, ≈2/3 of rice surfaces	0	0
1996–1999	0		Permitted but not often used	0
2000	0	11,500	500	0
2001	0	11,500	500	200
2002	0	10,000	2,000	300
2003	0	10,000	2,000	400
2004	0	4,500	1,000	500
2005	0	0	800	2,300
2006	0	0	Very limited	≈3,000

species in 1994, when rice cultivation covered a maximum of 24,500 ha (online Appendix Figure).

From 2000: Increase in Mosquito Populations

More recently still, rice producers have developed other cultivations in rotation with rice and have added new activities, such as hunting marshes and tourism, with the aim of diminishing their dependence on rice economics (*22*). Since 2000, the area under rice cultivation has remained stable at ≈18,000–20,000 ha.

Since 2000 the *Ariete* variety of rice has been progressively replaced by varieties less susceptible to the striped rice borer. Consequently, spraying was conducted over smaller percentages of the rice-cultivated areas: 61% in 2000 to 51% in 2003. Nevertheless, spraying was maintained to ensure the high productivity on which subsidies were based at that time. Since 2004, the terms of rice subsidies have changed yet again, leading to the disinterest in high productivity and to the high decrease in sprayed surfaces in 2004. Finally, controls on insecticide use were strengthened in 2005 (departmental order, Mar 5, 2004) to limit the impact on wild fauna; the use of alphamethrin by airplane was abandoned, and the emphasis now is on lepidopteron-specific insecticides (tebufenozide, *Bacillus thuringiensis kurstaki 3a*/*3b*). In parallel with the progressive abandon of insecticide, *An. hyrcanus* and *Cx. modestus* populations have increased continuously from 2000 to the present.

Untangling the Components of Anthropogenic Change

Before the 1970s, and in the absence of insecticide spraying, *An. hyrcanus* and *Cx. modestus* abundance followed the increase in the area under rice cultivation. After 1970, insecticide spraying, which was aimed at controlling the striped rice borer, likely influenced the size of the mosquito populations. From 1972 to 1974 and from 2000 to 2006 (surfaces sprayed were precisely known only during these years), the abundance of both mosquito species (human mosquito-landing data) was negatively correlated with the percentage of the rice areas sprayed with mosquito-efficient insecticides (Pearson coefficient $r = –0.84$, $p<0.001$ for *An. hyrcanus* and $r = –0.64$, $p<0.05$ for *Cx. modestus*).

This story highlights the intertwined importance of historical, political, environmental, technical, and social factors in explaining agricultural changes in the Camargue that could have directly contributed to variation in the abundance of both *An. hyrcanus* and *Cx. modestus* populations, with possible consequences for vectorborne diseases (Figure 2). *An. hyrcanus* is currently considered the main potential malaria vector in the Camargue, whereas the past periods of high *Cx. modestus* abundance, i.e., the 1960s and the 2000s, were associated with WNV outbreaks in the Camargue.

The amount of rice cultivation in this area was determined by national or European decisions, which were influenced by the global historical and political context. Favorable economic conditions for rice cultivation (Marshall Plan, guaranteed prices, and subsidies) were the results of the World War II and the developing Cold War; later economic globalization forced a decrease in this support. The close relationship between political decisions and variations in rice surface area in the Camargue is due to the organization of farming into large units, itself a product of past massive funding investments and environmental constraints; this system allowed a rapid response to the changing economic climate. Currently, rice producers in the Camargue are adding tertiary activities to their historical role as food providers, in response to new social demands concerning leisure such as hunting and nature tourism. The extent and amount of spray applied depends on the presence of rice pests, permissiveness of the rice variety; and the insecticide cost in regard to production costs, sale price, and subsidies (indexed or not on productivity). The choice of rice variety

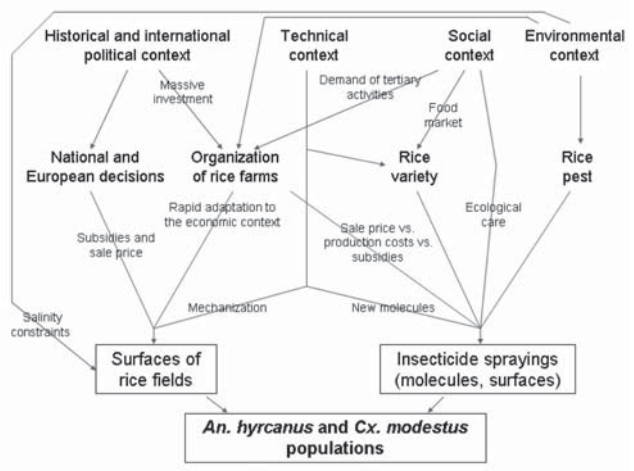

Figure 2. Impact of history, politics, technology, society, and environment on malaria and West Nile fever in the Camargue, France.

is determined by its adequacy within the food market and its agronomic performance in the production area.

What does the future hold for these mosquito populations in the Camargue? On the one hand, the Common Agricultural Policy will face another round of debates about subsidies in 2013. If subsidies are reduced, rice cultivation is expected to decrease; *An. hyrcanus* and *Cx. modestus* will therefore also likely decrease in abundance and may even become as rare as they were before World War II. Conversely, because rice cultivators are important actors in maintaining the ecosystems of the Camargue, the French government may decide to continue to support rice cultivation there. Without any other disturbance of this ecosystem, *An. hyrcanus* and *Cx. modestus* populations might then continue to increase.

Climate change is considered by some authors as being responsible for the vectorborne disease recrudescence (*38*,*39*). However, as our data indicate, environmental modifications and changes in the economic, social, and cultural environments can have strong and rapid effects on mosquito populations.

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Need for Improved Methods to Collect and Present Spatial Epidemiologic Data for Vectorborne Diseases

Lars Eisen* and Rebecca J. Eisen†

Improved methods for collection and presentation of spatial epidemiologic data are needed for vectorborne diseases in the United States. Lack of reliable data for probable pathogen exposure site has emerged as a major obstacle to the development of predictive spatial risk models. Although plague case investigations can serve as a model for how to ideally generate needed information, this comprehensive approach is cost-prohibitive for more common and less severe diseases. New methods are urgently needed to determine probable pathogen exposure sites that will yield reliable results while taking into account economic and time constraints of the public health system and attending physicians. Recent data demonstrate the need for a change from use of the county spatial unit for presentation of incidence of vectorborne diseases to more precise ZIP code or census tract scales. Such fine-scale spatial risk patterns can be communicated to the public and medical community through Web-mapping approaches.

Risk for human exposure to arthropod vectors and their associated pathogens (e.g., the tickborne Lyme disease spirochete *Borrelia burgdorferi*, fleaborne plague bacterium *Yersinia pestis*, and mosquitoborne West Nile virus [WNV]) is spatially highly heterogeneous in the United States (*1–16*). This concept can be exemplified by the spatial distributions of plague cases and areas with high projected plague risk in Arizona, New Mexico, Utah, and Colorado (Figure 1) and incidence of endemic Lyme disease in California (Figure 2) (*8*,*9*). Such heterogeneity in spatial risk patterns results in part from variability in environmental suitability for the vectors, especially with regard to climate factors and habitat type, and abundance of vertebrate hosts or pathogen reservoirs (*17–21*). Three examples can illustrate this point. First, exposure to *Ixodes pacificus* nymphs, which serve as primary vectors of *B. burgdorferi* in California, is largely restricted to dense woodlands with a ground cover dominated by leaf litter and lacking emergent vegetation (*22*,*23*). Moreover, density of nymphs and *B. burgdorferi*–infected nymphs differs between different woodland types; oak woodlands show a greater risk for exposure to the Lyme disease agent than redwood habitats (*6*,*7*). These differences represent crucial knowledge in assessment of probable pathogen exposure sites for Lyme disease cases in California.

Second, spatial patterns of distribution and abundance of the mosquito *Culex tarsalis*, which is considered the primary vector to humans of WNV in the western United States, are related to both climatic conditions and suitable mosquito larval habitats (*13*,*14*,*24–28*). In Colorado, which had a WNV disease outbreak with 2,947 reported human cases in 2003, the spatial pattern of abundance of *Cx. tarsalis* is highly heterogeneous. The mosquito occurs commonly only at lower elevations <1,800 m (*24*,*25*), and its presence in the semiarid plains landscape characteristic of eastern Colorado is strongly correlated with availability of water sources (natural or resulting from irrigation) for the immature aquatic mosquito life stages. Assessments of probable WNV exposure sites in Colorado are complicated by inadequate knowledge of the fine-scale spatial distribu-

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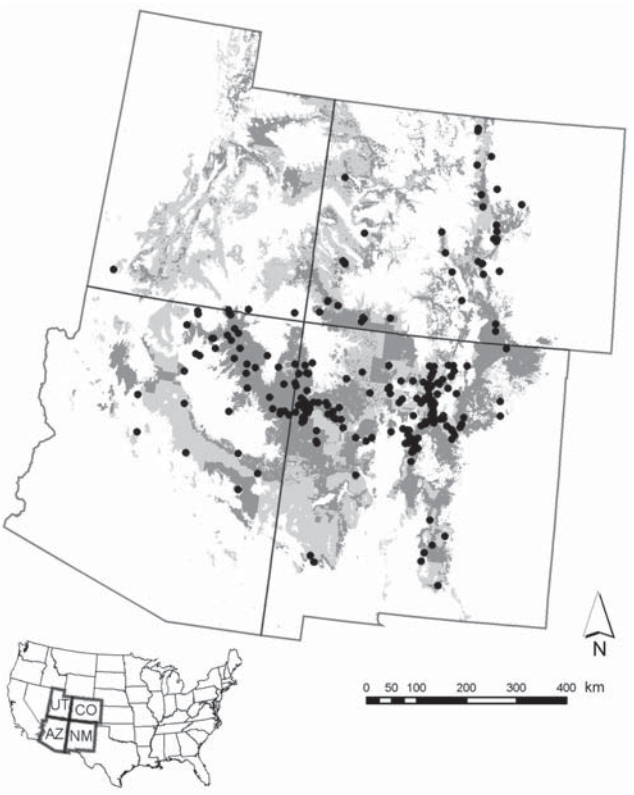

Figure 1. Areas predicted by a model based on peridomestically acquired plague cases from 1957 through 2004 to pose high risk to humans in the Four Corners Region (Arizona, Colorado, New Mexico, and Utah) are depicted in light gray. Those high-risk areas on privately or tribally owned land are shown in dark gray. Black circles represent locations of peridomestically acquired human plague cases. States comprising the Four Corners Region are shown within the United States in the inset. Reprinted with permission of the Journal of Medical Entomology from Eisen et al. (*9*).

tions of key *Culex* spp. WNV vectors (*Cx. pipiens*, *Cx. tarsalis*) and the fact that people commonly are bitten by other mosquitoes in areas where these vectors and WNV are absent (e.g., the high mountains in central Colorado).

Third, human plague cases in the southwestern United States are closely associated with ecotonal piñon-juniper habitat and elevation (*9*). The etiologic agent of plague is transmitted primarily by flea bite, and human cases are typically associated with epizootic activity, which most commonly occurs in clearly defined habitat types and under climatic conditions favoring build-up of dense rodent and flea populations (*17,18,29,30*). Exhaustive plague case investigations by state health agencies or the Centers for Disease Control and Prevention (CDC) ensure reliable assessments of probable exposure sites for *Y. pestis* in the United States.

Improving Data for Probable Pathogen Exposure Site

Over the past decade, advances in geographic information system technology have facilitated the development of predictive spatial models for risk for exposure to key vectors and pathogens in the United States (*1,3,5,7–12,16*). However, lack of reliable data for probable pathogen exposure sites has emerged as a major obstacle to the development of spatial epidemiologic and ecoepidemiologic models. In the United States, comprehensive case investigations by teams that include epidemiologists and vector ecologists and the determination of probable pathogen exposure sites are routinely conducted only for plague. Although the plague case investigation can serve as a model for how to ideally generate needed information for probable pathogen exposure sites, this exhaustive approach is cost-prohibitive for more common and less severe diseases such as Lyme disease and WNV disease. Unless the public health system is willing to invest funds needed to conduct comprehensive case investigations for a given vectorborne disease, determinations of probable pathogen exposure sites will remain the responsibility of the attending physician. Physicians may not be willing to spend the time required to obtain extensive patient travel histories to determine probable pathogen exposure site, and their lack of training in vector ecology impedes their ability to collect relevant information.

To solve this problem, new methods are needed to determine probable pathogen exposure site that yield reliable results while taking into account economic and time constraints of the public health system and attending physicians. These methods could, for example, include sets of standardized questions developed by CDC and tailored to a given vectorborne disease. A critical minimal need includes a basic assessment of whether pathogen exposure likely occurred in 1) the peridomestic environment, 2) outside the peridomestic environment but within the county of residence, or 3) outside the county of residence. The role of this issue for spatial epidemiologic modeling was demonstrated by our recent study of Lyme disease in California where reexamination of Lyme disease case files from 1993 through 2005 showed that 27% of the 1,325 case-patients had likely been exposed to the pathogen outside the county of residence (*8*). Other possible approaches include the point-radius method for georeferencing of probable pathogen exposure sites on the basis of patient activity space patterns (*31*). Research is needed to determine the value and feasibility of implementing these or other methods into routine public health activities.

Spatial Unit for Calculation and Presentation of Incidence of Vectorborne Disease

CDC and individual state health agencies routinely use county as the spatial unit for calculating and presenting

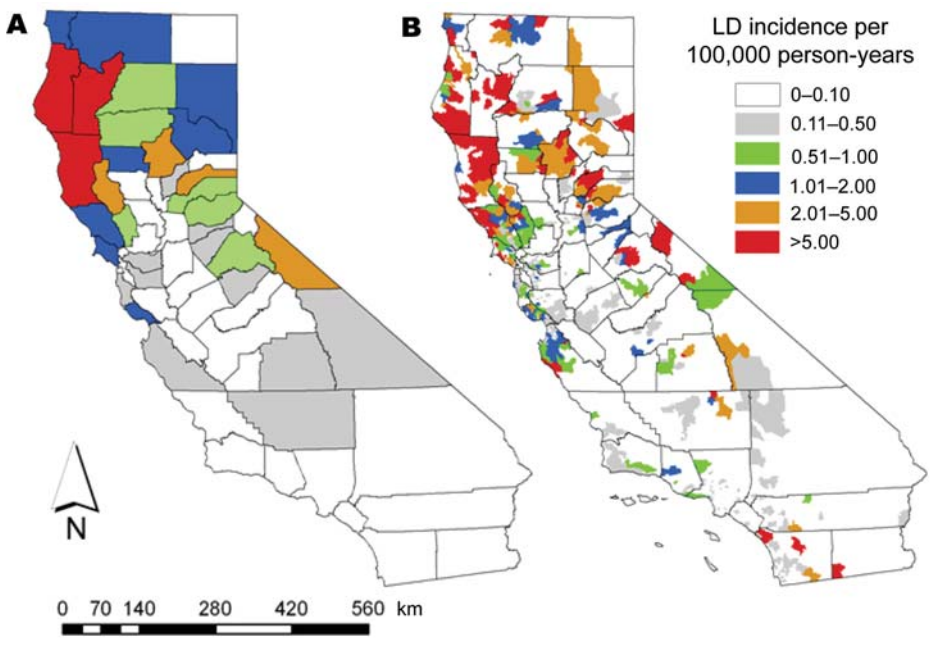

Figure 2. Comparison of spatial distributions of areas of California with different incidences of endemic Lyme disease (LD), 1993–2005, when calculated by A) the county spatial unit and B) the 5-digit ZIP code spatial unit. Adapted from a figure published in the American Journal of Tropical Medicine and Hygiene by Eisen et al. (*8*).

incidence of vectorborne disease. The main problem with using county-based incidences for vectorborne diseases is that incidences calculated at this relatively crude spatial scale obscure fine-scale risk patterns commonly occurring within a county. This is especially problematic in the western United States, where many counties cover extensive areas (Figure 3) and encompass considerable ecologic and climatic variability. It was therefore not surprising that Eisen et al. (*8*) found that calculation and presentation of incidence of endemic Lyme disease in California at the county spatial unit, relative to the 5-digit ZIP code spatial unit, served to obscure small, isolated high-risk areas in the southern part of the state and the spatial variability of risk within high-risk counties (Figure 2).

Knowledge of local areas and habitats representing risk for vector exposure can be a major component in a diagnosis of probable Lyme disease or plague because infected persons may be unaware of receiving a tick or flea bite (*32–34*). Such knowledge is crucial in areas of the United States where the disease in question occurs but is rare. For example, in the absence of a documented tick bite and without knowledge that there are local areas with risk for exposure to the Lyme disease agent, a physician may be unlikely to consider Lyme disease as a possible diagnosis unless the patient has visited some other area the physician perceives to pose risk for exposure to the Lyme disease agent. Recognizing heterogeneity in spatial risk patterns for plague is similarly critical because it will aid local public health workers in targeting education of healthcare providers and the public to areas with a high risk for exposure to the plague agent (*9*,*35*). Prevention and treatment guidelines are well established for plague, but outcome of infection is improved by early diagnosis followed by appropriate treatment with antimicrobial drugs (*36*).

The 2 primary options in a shift away from using the county spatial unit for vectorborne disease incidence calculations are 1) ZIP code/ZIP code tabulation area and 2) census tract. There are pros and cons for each option. The 5-digit ZIP code unit is convenient because information regarding ZIP code of home address is readily collected during a visit to a physician, and the public is well aware of their ZIP code of residence and therefore can make ready use of information in map formats for ZIP code–based risk patterns. Conversely, a recent publication (*37*) raised concerns regarding increasing use of ZIP codes/ZIP code tabulation areas in spatial analyses of epidemiologic data because of their lack of standardization and dynamic spatial structure.

The more permanent census tract spatial unit, which tends to be smaller than the 5-digit ZIP code unit in population centers but can be larger than the ZIP code unit in sparsely populated areas, is attractive because it has a more uniform population base (typically 1,500–8,000 persons) than the ZIP code unit and therefore is less prone to the problem of overestimation of disease incidence on the basis of a few cases among a low population base. As demonstrated for WNV disease in Colorado by the Colorado Department of Public Health and Environment, some state level agencies have already adopted the practice of using the census tract unit to present spatial patterns of vectorborne diseases (www.cdphe.state.co.us/dc/zoonosis/wnv). Research is needed to evaluate the relative benefits of using the ZIP code compared with the census tract unit for

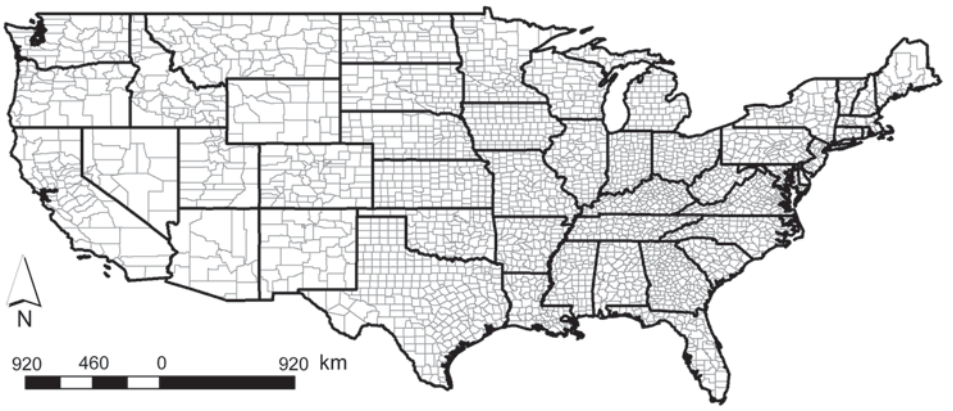

Figure 3. State and county boundaries within the contiguous United States. Note the increasing size of counties from east to west.

calculation and presentation of spatial patterns of different vectorborne diseases.

Finally, advances in geographic information system technology and the ever-increasing use of the Internet as a primary knowledge resource provide tremendous possibilities for disseminating information regarding spatially explicit risk for exposure to vectorborne pathogens. Using a Web-mapping approach, one could easily convert static maps for plague and Lyme disease (Figures 1, 2) into a Web-based information delivery system in which selecting a county of interest provides a closeup view of the county, showing risk patterns for labeled ZIP codes and the location of major roads, population centers, and heavily used recreation areas.

Conclusions

New methods of determining probable pathogen exposure site that yield reliable results while taking into account economical and time constraints of the public health system are urgently needed to improve capability for developing predictive spatial risk models for vectorborne diseases in the United States. Recent data also demonstrate the need for a change from use of the crude county spatial unit for presentation of incidence of vectorborne diseases to finer ZIP code or census tract scales. Communication of such fine-scale spatial risk patterns to the public and medical community can be achieved through Web-mapping approaches.

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Susceptibility of Canada Geese (*Branta canadensis*) to Highly Pathogenic Avian Influenza Virus (H5N1)

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Migratory birds have been implicated in the long-range spread of highly pathogenic avian influenza (HPAI) A virus (H5N1) from Asia to Europe and Africa. Although sampling of healthy wild birds representing a large number of species has not identified possible carriers of influenza virus (H5N1) into Europe, surveillance of dead and sick birds has demonstrated mute (*Cygnus olor*) and whooper (*C. cygnus)* swans as potential sentinels. Because of concerns that migratory birds could spread H5N1 subtype to the Western Hemisphere and lead to its establishment within free-living avian populations, experimental studies have addressed the susceptibility of several indigenous North American duck and gull species. We examined the susceptibility of Canada geese (*Branta canadensis*) to HPAI virus (H5N1). Large populations of this species can be found in periagricultural and periurban settings and thus may be of potential epidemiologic importance if H5N1 subtype were to establish itself in North American wild bird populations.

Wild aquatic birds belonging to the orders Anseriformes and Charadriiformes have long been recognized as the natural reservoirs for all influenza type A viruses (*1*). Spread from such wild birds to domestic poultry and various mammalian species occurs intermittently. Most viruses that initially infect domestic poultry will replicate only within respiratory or digestive tracts and cause no or very mild disease, referred to as low-pathogenic avian influenza (LPAI) (*2*). However, once introduced into domestic poultry, some viruses of the H5 and H7 hemagglutinin (HA) subtypes can mutate to a highly pathogenic form, producing a systemic infection referred to as highly pathogenic avian influenza (HPAI) (*2*). The hypothesis that HPAI H5 and H7 viruses emerge from low-pathogenic precursors only after the H5 and H7 LPAI precursors have been introduced into domestic poultry has been supported by work demonstrating that HPAI viruses do not appear to form separate phylogenetic lineages in waterfowl (*3*). Except for A/tern/South Africa/1961 (H5N3), no evidence existed before 2002 that an HPAI virus could cause deaths or be maintained within wild bird populations.

In late 2003, an HPAI (H5N1) outbreak of unprecedented magnitude began in Southeast Asia. Approximately 1 year before this, a high mortality rate attributed to HPAI virus (H5N1) was observed in waterfowl and other wild birds in Hong Kong (*4*). This led to speculation that wild birds may have contributed to the virus spread. In the spring of 2005, mass dieoffs of wild birds occurred at Qinghai Lake, People's Republic of China (*5*,*6*), an event heralded as the beginning of the long-range spread of HPAI (H5N1) from Asia into Europe and subsequently Africa, with migratory birds implicated as playing a role (*7*,*8*). Identifying which species of birds were involved in this spread is not only of academic interest but also of practical importance to surveillance activities because of concerns that migratory birds could also introduce H5N1 subtype into the Western Hemisphere. We examined the susceptibility of Canada

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geese (*Branta canadensis)* to infection with an HPAI virus (H5N1) and the effect that pre-exposure to an LPAI virus (H5N2) has on clinical disease, pathology, and virus shedding.

Materials and Methods

Viruses

The influenza viruses used in this study included A/chicken/Vietnam/14/2005 (H5N1) and A/mallard/British Columbia/373/2005 (H5N2). Vietnam/05 stocks were grown and titrated on Japanese quail fibrosarcoma (QT-35) cells. This isolate bears a PQRERRRKR/GLF HA_0 cleavage site (GenBank accession no. EF535027), has an intravenous pathogenicity index of 2.97, and produced a 100% mortality rate in oronasally inoculated leghorn chickens receiving 10^5, 10^4, and 10^3 PFU by 3, 4, and 6 days postinfection (dpi), respectively. British Columbia/05 stocks were grown and titrated in 9-day-old chicken embryos. Prior characterization of this isolate demonstrated that it has a PQRETR/GLF HA_0 cleavage site (GenBank accession no. DQ826532) typical for LPAI viruses.

Animals

Twenty-two Canada geese were captured with the permission of Environment Canada (Canadian Wildlife Service permit no. CWS06-M009) and were handled and cared for in accordance with Canadian Council on Animal Care guidelines and the animal use protocol approved by the Institutional Animal Care Committee. The geese consisted of 11 adult (6 male + 5 female) and 11 young-of-year (6 male + 5 female) birds. The latter were estimated to be ≈40 days of age at capture. Adult and juvenile birds were randomly assembled into 3 experimental groups, and each group subsequently housed in separate Biosafety Level-3 biocontainment cubicles: 1) a control group comprising 1 juvenile + 1 adult bird, 2) a pre-exposure group comprising 5 juvenile + 5 adult birds, and 3) a naive group comprising 5 juvenile + 5 adult birds.

After a 3-week acclimation period, the pre-exposure group was inoculated with 10^6 50% egg infectious dose (EID_{50}) of British Columbia/05 applied to the nares, oral cavity, and cloaca. Twenty-eight days later, pre-exposure and naïve groups were challenged with 1.7×10^5 PFU of Vietnam/05 applied to the nares, oral cavity, and eye. The control group received a sham inoculum of minimal essential medium. Timed necropsies involving 1 juvenile and 1 adult bird from pre-exposure and naïve groups were performed on days 3 and 6 postchallenge (dpc). All remaining birds were either humanely euthanized when moribund or allowed to survive until 20 or 21 days if they showed mild disease or remained clinically normal.

ELISA and Hemagglutination-Inhibition (HI) Assays

Group A specific nucleoprotein (NP) antibodies were detected with a competitive ELISA as described previously (*9*). H5-specific antibodies were detected by microtiter plate HI test that used 4 HA U of A/duck/British Columbia/26–6/2005 (H5N2) and chicken erythrocytes.

Virus Neutralization Assay

We incubated 200 EID_{50} of Vietnam/05 with an equal volume of 2-fold serially diluted test serum (1:4 to 1:512), incubated for 60 min at 37°C, and then used it to inoculate 9-day-old chicken embryos through the allantoic cavity. Egg deaths and HA titers were monitored and virus neutralization titers determined.

Real-Time Reverse Transcription–PCR (RT-PCR) Assays

Specimens were stored at –70°C before RNA was extracted. Total RNA was extracted from 0.5 mL of 10% (wt/vol) tissue emulsions or clarified swab specimens by using an RNeasy Mini Kit (QIAGEN, Mississauga, Ontario, Canada). A semiquantitative real-time RT-PCR (*10*) that targets the M1 gene of influenza A virus segment 7 was conducted. Full-length, in vitro transcribed segment 7 RNA, serially diluted in buffer, was run with each assay to give a semiquantitative estimate of the viral load in each tissue.

Immunohistochemistry

Formalin-fixed, deparaffinized, and rehydrated 5-μm tissue sections were quenched for 10 min in aqueous 3% H_2O_2, rinsed in MilliQ water, and placed into Tris-buffered saline plus Tween (TBST) buffer for 5 min. Sections were pretreated with proteolytic enzyme (DakoCytomation, Carpinteria, CA, USA) for 15 min, rinsed twice with TBST, and incubated for 1 h with a monoclonal antibody specific for influenza A nucleoprotein (Clone 1331, Biodesign, Sasco, ME, USA) at a dilution of 1:5,000. The sections were washed with TBST, then incubated for 30 min with the Envision + anti-mouse (horse radish peroxidase–labeled) polymer kit (DakoCytomation), followed by a TBST rinse. Diaminobenzidine was used as the substrate chromagen, and slides were counterstained with Gill's hematoxylin.

Results

A/mallard/British Columbia/373/2005 (H5N2) Pre-Exposure

Upon arrival, 12 of 12 juvenile geese tested negative and 10 of 12 adult geese tested positive for influenza A virus NP antibodies (Table 1). To determine the HA subtype specificity of the seropositive birds, HI assays were

Table 1. NP and H5 antibody levels in juvenile and adult Canada geese*

Animal ID	0 dpi cELISA (NP % inhibition)	0 dpi H5 HI assay†	14 dpi (H5N2) cELISA (NP % inhibition)	21 dpi (H5N2) H5 HI assay†	20–21 dpi (H5N1) cELISA (NP)	20–21 dpi (H5N1) H5 HI assay†
Juveniles						
852S/27R	Neg (20)	<8	Pos (66)	16	Euthanized or died‡	
853S/28R	Neg (13)	<8	Pos (64)	64	Pos (46% inhibition)	8
856S/31R	Neg (21)	<8	Pos (57)	256	Euthanized	
858S/33R	Neg (22)	<8	Pos (55)	128	Pos (48% inhibition)	64
859S/34R	Neg (19)	<8	Pos (49)	256	Euthanized	
851S/26R	Neg (24)	<8			Euthanized	
854S/29R	Neg (23)	<8			Euthanized	
855S/30R	Neg (22)	<8			Euthanized	
860S/35R	Neg (24)	<8			Euthanized	
861S/36R	Neg (20)	<8				
857S/32Y	Neg (18)	<8				
Adults						
842S/42Y	Pos (93)	<8	Pos (99)	512	Euthanized	
844S/44Y	Neg (23)	<8	Pos (99)	64	Pos (46% inhibition)	<8
845S/45Y	Pos (58)	<8	Pos (96)	8	Euthanized	
846S/46Y	Pos (76)	<8	Pos (96)	<8	Pos (63% inhibition)	<8
847S/47Y	Pos (74)	<8	Pos (99)	ND	Euthanized	
840S/40Y	Pos (45)	<8			Pos (99% inhibition)	>4,096
841S/41Y	Neg (22)	<8			Euthanized	
843S/43Y	Pos (78)	<8			Euthanized	
848S/48Y	Pos (39)	<8			Pos (98% inhibition)	64
849S/49Y	Pos (93)	<8			Pos (98% inhibition)	32
850S/50Y	Pos (85)	<8				

*NP, nucleoprotein; cELISA, competitive ELISA; dpi, days postinfection; Neg, negative (<30% inhibition); Pos, positive (≥30% inhibition); HI, hemagglutinin inhibition; ND, not determined.
†4 hemagglutinin units of A/duck/British Columbia/26–6/2005 (H5N2) used in assay.
‡Euthanized or died before day 20–21 postinoculation with virus (H5N1).

run with 4 HA U of the following antigens: H1N1 (A/Ck/BC/3/98); H2N9 (A/Pintail/AB/293/77); H4N6 (A/Dk/BC/14/99); H5N2 (A/mallard/BC/373/05); H6N1 (A/Tk/ON/844–2/04); and H7N3 (A/Ck/BC/514/04). All tests were negative, indicating that the birds did not appear to have pre-existing H5-specific antibodies. Real-time RT-PCR–negative cloacal swab specimens indicated that the birds were also not actively infected.

After inoculation with 10^6 EID_{50} of British Columbia/05, all birds remained clinically normal. The juvenile birds gained weight, but 3 of 5 adult birds had a 6%–10% loss of bodyweight after infection. Cloacal swabs from juvenile birds were real-time RT-PCR positive at 3 dpi; swabs from adult birds were negative (oropharynegeal swabs not tested). At 6 and 10 dpi, cloacal and oropharyngeal swabs from both juvenile and adult birds were real-time RT-PCR negative, indicating that viral shedding was brief. Although most of the British Columbia/05 infected birds developed H5-specific HI antibody titers (Table 1), these sera did not neutralize Vietnam/05 in a chicken embryo–based neutralization assay.

A/chicken/Vietnam/14/2005 (H5N1) Challenge

Twenty-eight days after pre-exposure to British Columbia/05, birds in the pre-exposure and naïve groups were challenged with Vietnam/05. Juvenile birds were estimated to be 13 weeks of age at this time. Adult birds in the British Columbia/05 pre-exposure group exhibited mild decreases in feed consumption and mild depression 5–7 dpc. Except for 1 bird with a positive oropharyngeal swab sample at 6 dpc, oropharyngeal and cloacal swab specimens for the adults tested real-time RT-PCR negative at 2, 3, and 6 dpc. Juvenile birds in the British Columbia/05 pre-exposure group exhibited clinical signs similar to those of the adults with the addition of transient nervous signs manifested as repetitive jerking head movements. Viral shedding, as determined by real-time RT-PCR and confirmed by isolation, was detected at 3 dpc in oropharyngeal swab samples in 3 of 5 birds and in a cloacal swab sample in 1 of 5 birds. Complete necropsies showed no gross lesions in juvenile or adult birds at 3, 6, 11, and 21 dpc. The cerebrum, brain stem, and spinal cord of juvenile birds exhibited low levels of viral nucleic acid at 11 and 21 dpc (online Appendix Table, available from www.cdc.gov/EID/content/13/12/1821-appT.htm). Other organs were weakly positive by real-time RT-PCR to varying degrees.

In contrast, juvenile birds in the naïve group showed 100% morbidity after Vietnam/05 challenge; clinical signs included severe depression, inappetence, bright yellow diarrhea, ruffled feathers, hunched posture, repetitive jerking

head movements, weakness, staggering gait, distressed vocalization, wing droop, and terminal coma. All birds died or were humanely euthanized by 5 dpc. Viral nucleic acid was detected in the oropharyngeal swab specimens collected at all time points before euthanasia or death; cloacal swab specimens were not as consistently positive. Adult birds also showed 100% morbidity but with clinical signs and viral shedding less pronounced than that observed in juveniles. Necropsies were performed on 2 adults on days 3 and 5; the remaining 3 birds survived until 20 dpc.

Gross pathologic lesions included congestion of the mucosal surface of the trachea, edema and multifocal pinpoint hemorrhages on the serosal surface of the pancreas, splenomegaly, hemorrhage within the ceca, conjunctivitis, congestion of the meninges and cerebral blood vessels, and hemorrhages on the surface of the brain. Virtually all tissues collected from juvenile birds in the naïve group were real-time RT-PCR positive; heaviest viral loads were found in cerebrum, brain stem, and spinal cord. Adult bird 841S/41Y, which required euthanasia at 5 dpi, also had levels of viral nucleic acid in the central nervous system (CNS) comparable to those found in naïve juveniles. This was one of the adult birds with no pre-existing NP antibodies at the beginning of the acclimation period (Table 1). Viral nucleic acid was found in the CNS of a second adult (840S/40Y), euthanized at 20 dpc, but at levels that were 5–7 logs lower than those found in juveniles or the adult bird euthanized at 5 dpc.

Specific influenza A virus immunolabeling was found in all tissues collected from naïve juvenile birds (Table 2). The most consistently affected tissues were the brain, spinal cord, parasympathetic ganglia of the gastrointestinal tract, heart, and pancreas (Figures 1, 2). Within the small intestine and cecum, the strongest and most consistent immunolabeling involved the parasympathetic ganglia of the submucosal and myenteric plexi (Figure 1, panel D) with only the occasional scattered smooth muscle and vascular endothelial cell within the gut mucosa positive for viral antigen. In the 3 birds in which the proventriculus was affected, viral antigen was detected in numerous cell types, including both surface columnar and glandular epithelium, smooth muscle cells of the muscularis mucosa, vascular smooth muscle, and the parasympathetic ganglia (Figure 2, panel C). In the lungs, antigen could be identified in a few capillary endothelial cells. Positive immunolabeling within trachea, liver, kidney, and breast muscle was minimal and observed in only a few birds. Immunohistochemical analysis of tissues collected from naive adult birds detected specific immunolabeling in only 1 bird (841S/41Y) euthanized at 5 dpc; tissues and cells affected were similar to those observed in naive juveniles.

Discussion

Deaths of mute *(Cygnus olor*) and whooper (*C. cygnus*) swans have signaled the arrival of HPAI virus (H5N1) in Europe (*11*,*12*). The affected swans had nervous signs

Table 2. Distribution of influenza virus antigen in tissues of naïve juvenile Canada geese tissues after challenge with influenza virus (H5N1)

Tissue	Animal 861S/36R dpi 3*	Animal 855S/30R dpi 4*	Animal 851S/26R dpi 5*	Animal 854S/29R dpi 5*	Animal 860S/35R dpi 5*	IHC-positive cell types
Trachea	+	+	–	–	–	Vascular endothelium
Lung	++	++	–	+	–	Vascular endothelium, mononuclear cells
Esophagus	++	++	+	+	–	Epithelium, Vascular smooth muscle, Smooth muscle of muscularis externa, Mucous glands
Proventriculus	+	+	–	++	–	Epithelium (columnar, glandular), muscularis mucosa, vascular smooth muscle, parasympathetic ganglia
Ventriculus	++	++	–	++	–	Epithelium
Gut	++	+++	–	++	+	Parasympathetic ganglia, mucosal smooth muscle, vascular endothelium
Cecal tonsil	++	++	+	++	–	Parasympathetic ganglia
Pancreas	++	+++	++	++	+	Exocrine acinar cells
Liver	–	–	–	+	–	Hepatocytes
Spleen	++	–	+	–	–	Vascular smooth muscle, mononuclear cells
Kidney	+	–	–	–	–	Tubular epithelium
Muscle	+	+	–	–	–	Vascular smooth muscle
Heart	++	++	++	+	+	Myocytes
Brain	++	+++	+++	+++	+++	Neurons, glial cells, ependymal cells, choroid plexus epithelium
Spinal cord	+	++	++	++	++	Ependymal cells, neurons, glial cells, leptomeninges
Sciatic nerve	+	–	–	–	–	Vascular endothelium
Brachial nerve	–	–	–	–	–	None

*Numbers of immunohistochemically positive cells: +, few; ++, moderate; +++, numerous; –, virus antigen negative; dpi, days postinfection.

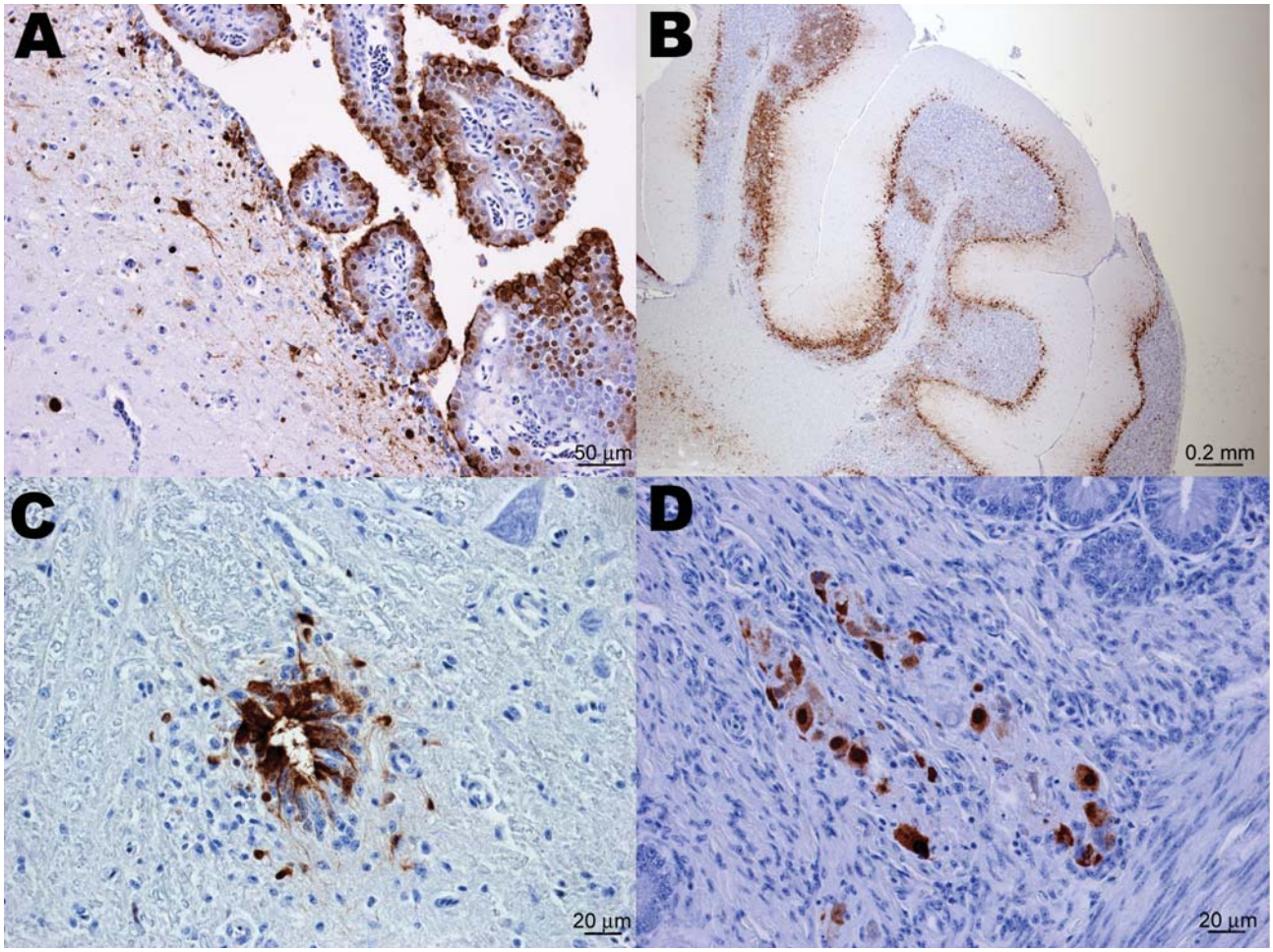

Figure 1. Immunohistochemical staining for influenza virus nucleoprotein in central and peripheral nervous system of naive juvenile Canada geese tissues after challenge with influenza virus (H5N1). A) Cerebrum. Positive immunolabeling of neurons, glial cells, ependymal and choroid plexus epithelial cells. B) Cerebellum. Extensive positive immunolabeling of Purkinje cells and neurons of the granular layer. C) Spinal cord. Positive immunolabeling of ependymal cells of the central canal and adjacent neurons and glial cells. D) Small intestine. Positive immunolabeling of neurons of the submucosal plexus.

that included somnolence, incoordination, and ataxia (*11*) and gross pathology that included multifocal hemorrhagic necrosis in the pancreas, pulmonary congestion and edema, and subepicardial hemorrhages (*13*). Recent studies addressing the susceptibility of North American waterfowl species to HPAI virus (H5N1) have shown wood ducks (*Aix sponsa*) and laughing gulls (*Larus atriculla*) to be highly susceptible, while mallards (*Anas platyrhnchos*), northern pintails (*A. acuta*), blue-wing teals (*A. crecca*) and redheads (*Aythya Americana*) to be refractory (*14*,*15*). Previous reports from Asia (*4*) and Europe (*13*) have indicated that HPAI virus (H5N1) can produce deaths in naturally infected Canada geese. Our study supports these observations and further demonstrates this susceptibility to be dependent on the age and immunologic status of the animal.

Adult birds were generally more resistant to Vietnam/05 than juveniles, regardless of which experimental group they belonged to. Although results of this study indicate that prior infection with a North American LPAI virus (H5N2) protects juvenile Canada geese against a lethal H5N1 subtype challenge, the mechanism responsible is unresolved. Although HI titers in poultry strongly correlate with protection against virulent challenge from viruses expressing the same HA subtype (*16*), the ability of British Columbia/05 H5-specific antibodies to neutralize Vietnam/05 in vitro was not demonstrated. British Columbia/05 and Vietnam/05 have 84% amino acid similarity in their HA_1 subunits. The receptor binding domain (*17*), which comprises an α-helix (190-helix, HA_1 188–190) and 2 loop structures (130-loop, HA_1 134 to 138, and 220-loop, HA_1 221 to 228) in addition to residues Tyr^{96}, Trp^{153}, and His^{183} is remarkably conserved for both viruses. Multiple amino acid differences that cluster around the receptor-binding domain (data not shown) may explain the inability of Brit-

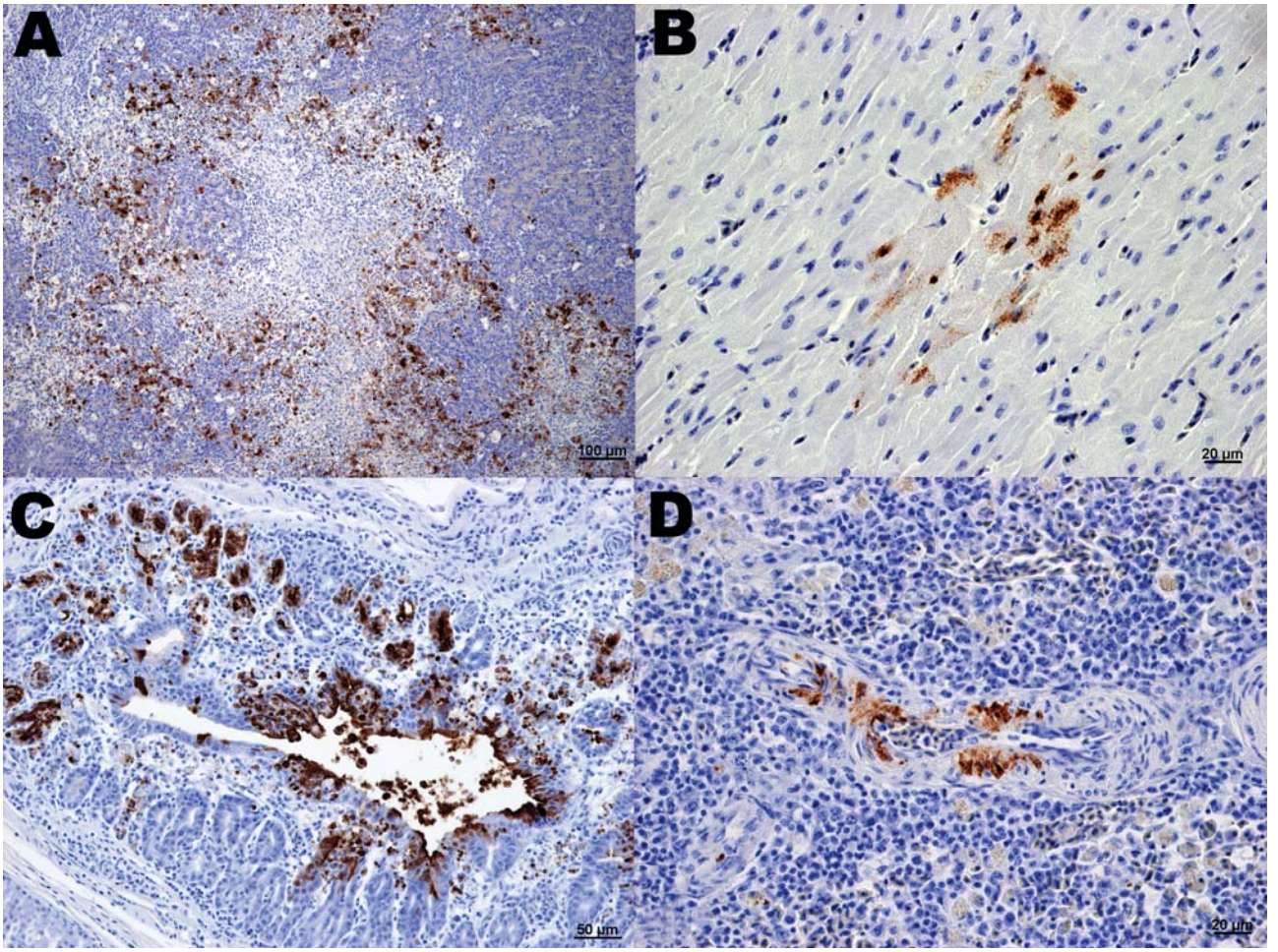

Figure 2. Immunohistochemical (IHC) staining for influenza virus nucleoprotein in tissues of naïve juvenile Canada geese after challenge with influenza virus (H5N1). A) Pancreas. Large areas of necrosis are surrounded by pancreatic acinar cells with strong positive intranuclear and intracytoplasmic immunolabeling. B) Heart. Positive intranuclear and intracytoplasmic immunolabeling of myocytes. C) Proventriculus. Strong positive immunolabeling of compound tubular gland epithelium. D) Splenic arteriole. Positive IHC staining of vascular smooth muscle cells.

ish Columbia/05 antisera to neutralize Vietnam/05 in vitro. Recent reports (*18*,*19*) have suggested that prior infection with viruses expressing heterologous HA subtypes can also protect chickens against a lethal (H5N1) challenge. Protection against HPAI virus (H5N1) in chickens that were previously infected with an H9N2 subtype correlated with the proportion of pulmonary $CD8^+$ T cells expressing gamma interferon (*19*). The hypothesis that cell-mediated immunity may have played a role in affording protection to the birds in this study is supported by the observation that even though NP antibody–positive naive adults did not appear to possess H5-specific antibodies, they were resistant to Vietnam/05 challenge.

The pronounced neurotropism that Vietnam/05 exhibited for Canada geese is similar to that reported for other susceptible wild bird species (*13*–*15*). A unique finding in our study was the widespread involvement of gastrointestinal parasympathetic ganglia. This has not been previously reported for wild birds, to our knowledge, although viral antigen within the parasympathetic ganglia of the small intestine of experimentally infected ducks has been documented (*14*). The mechanism by which avian influenza viruses invade the CNS has been most thoroughly investigated with mouse models (*20*–*22*). These studies have shown that after intranasal inoculation, neurotropic influenza A viruses can invade the CNS of mice by spreading along peripheral nerves; viral antigen is mainly detected in the vagal and trigeminal nuclei of the brainstem but not in the cerebral cortex. A compartmentalized mouse dorsal root ganglion neuron culture system (*22*) has further demonstrated that influenza A viruses could infect the distal parts of axons and reach the neuronal cell bodies by retrograde axonal transport in a microtubule-independent fashion. The involvement of the parasympathetic ganglia in our geese

suggests that CNS infection may occur by transmission of influenza virus via autonomic nerves to their centers in the brain stem. In contrast to the situation in mice, there is a more diffuse infection of cortical and midbrain neurons as well as choroid and ependymal epithelial cells. The latter may indicate that a hematogenous route involving penetration of the blood–brain barrier with infection propagated to glial cells and neurons (*23*) may also be involved.

Our work has demonstrated that Canada geese, and in particular immunologically naïve, young-of-year animals, may be suitable targets for dead bird surveillance activities. Based on our experiments, HPAI virus (H5N1) can be expected to produce pronounced neurologic signs and high deaths in this age group. CNS, pancreas, and heart specimens can be used in PCR or immunohistochemical diagnosis. However, prior exposure to North American lineage H5 viruses specifically, or avian influenza viruses of other HA subtypes more generally, may protect juvenile and adult geese against a virulent H5N1 subtype challenge, hence complicating detection. Determining the mechanism responsible for this apparent cross-protection will require further research.

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Fishborne Zoonotic Intestinal Trematodes, Vietnam

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Although fishborne zoonotic trematodes that infect the liver are well documented in Vietnam, intestinal fishborne zoonotic trematodes are unreported. Recent discoveries of the metacercarial stage of these flukes in wild and farmed fish prompted an assessment of their risk to a community that eats raw fish. A fecal survey of 615 persons showed a trematode egg prevalence of 64.9%. Infected persons were treated to expel liver and intestinal parasites for specific identification. The liver trematode *Clonorchis sinensis* was recovered from 51.5%, but ≥1 of 4 intestinal species of the family Heterophyidae was recovered from 100%. The most numerous were *Haplorchis* spp. (90.4% of all worms recovered). These results demonstrate that fishborne intestinal parasites are an unrecognized food safety risk in a country whose people have a strong tradition of eating raw fish.

Foodborne parasites are widespread and more common than generally recognized. Among these parasites, fishborne zoonotic trematodes (FZTs) are estimated to infect >18 million persons; worldwide the number at risk may be much greater (*1*–*3*). The FZTs include many species, especially representatives of the families Heterophyidae, Echinostomatidae, and Opisthorchiidae. Although their metacercarial cysts are easily inactivated by heating at 60°C or freezing to –20°C, they are highly prevalent in many regions, especially in Asia where food traditions include eating raw or improperly cooked fish dishes (Figure 1) (*4*). The fishborne liver flukes *Clonorchis sinensis*, *Opisthorchis viverrini*, and *O. felineus* cause cholangitis, pancreatitis, and cholangiocarcinoma in humans (*4*–*6*). During the past 10–20 years, a second large group of FZTs, the so-called minute intestinal flukes, has been increasingly recognized as widely distributed and a cause of illness (*1*–*8*).

The exponential increase in aquaculture is suggested to be the major cause of the emergence of FZTs in east and Southeast Asia (*2*,*3*). For example, in the People's Republic of China, the land devoted to aquaculture increased 75% (to 4.9 million hectares) since 1970, accompanied by a tripling of cases of infection with *C. sinensis* (*3*). The association of *O. viverrini* in Thailand and Lao People's Democratic Republic with fisheries has also been reported (*9*,*10*). However, wild fish are also frequently infected, but epidemiologic information to compare relative infection risks from eating wild fish and farmed fish in many FZT-endemic loci is insufficient (*1*,*4*).

A recent review of publications on FZTs in Vietnam indicated infections with only liver flukes (*C. sinensis* and *O. viverrini*) in humans (*11*). However, recent Vietnamese surveys for zoonotic parasites in cultured and wild fish in northern and southern Vietnam identified metacercarial stages of several zoonotic intestinal trematode species in fish (*12*,*13*). This finding is of concern because fish production has increased 9.3-fold (to 400,000 tons) over the past 40 years in Vietnam (*3*). Furthermore, human intestinal flukes are highly prevalent in neighboring countries such as Thailand (*14*), Lao People's Democratic Republic (*15*), and the People's Republic of China (*16*), which further raises the issue of whether human infections might be present in Vietnam but overlooked because of diagnostic difficulties in differentiating liver and intestinal fluke eggs in fecal examinations (*11*,*17*). A more reliable approach to detect and characterize human FZTs is to treat egg-positive patients and recover and identify the expelled adult worms (*7*,*15*).

We conducted a study in April 2005 in Nam Dinh Province, an area of Vietnam in which persons are known for eating raw fish, and where previous investigations have

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Figure 1. Typical dish of raw fish (slices of silver carp) sold in Vietnamese restaurants.

shown a high prevalence of liver flukes (*11*,*13*). Identification of worms expelled from egg-positive persons showed that intestinal FZTs are present in Vietnam and represent a major public health risk for a population with the habit of eating raw fish.

Materials and Methods

Site, Sampling, and Examination Procedure

A cross-sectional survey for fecal eggs was conducted in 2 communes in Nghia Hung District, Nam Dinh Province, Vietnam, southeast of the capital of Hanoi (Figure 2), a clonorchiasis-endemic area (*11*). The rural population in these communes is mostly farmers with fish ponds that are integrated into their farming systems, e.g., pig farming. In 2005, the Nghia Phu commune had a population of 9,608, including 2,214 families, and the Nghia Lac commune had a population of 9,147, including 2,160 families. Households in these 2 communes were randomly selected from a list provided by community authorities, and from each household 1 man or 1 woman who was head of household was selected. Trained personnel delivered labeled plastic containers to the selected persons and instructed them how to collect a fecal sample and store it until it was retrieved the next day. The label requested the person's name, age, address, and date of stool collection.

Permission to conduct this research was obtained from the National Institute of Malariology, Parasitology and Entomology (NIMPE), Hanoi, and the Faculty of Tropical Medicine, Mahidol University, Bangkok. Each study participant signed a consent form, which is on file at NIMPE.

From each stool sample, 2 Kato-Katz smears were prepared and analyzed by using the standard kit provided to NIMPE by the World Health Organization and originally obtained from Vestegaard Frandsen Pvt. Ltd. (New Delhi, India). Fecal slides were examined by light microscopy (×400). Helminth eggs were identified and enumerated, and the number of eggs was multiplied by 23 to obtain the number of eggs per gram (epg) of feces.

Parasite Expulsion

Thirty-three persons who had ≥1,000 epg on fecal examinations were selected for worm expulsion. Selected patients were asked to eat a light liquid dinner the evening before treatment. The following morning they were given oral praziquantel, 25 mg/kg; 1 hour later, they were given a saturated solution of 30 g of $MgSO_4$ dissolved in water. Subsequently, 3–4 consecutive posttreatment stools were collected. Worms were recovered by a series of washing steps (*15*).

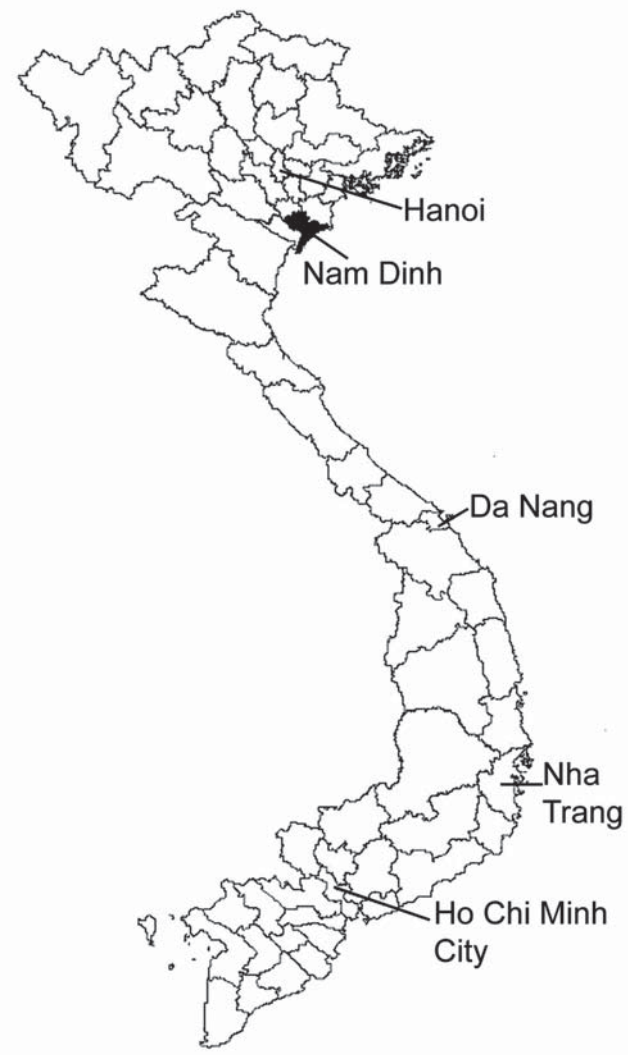

Figure 2. Map of Vietnam showing location of Nam Dinh Province, investigated for fishborne zoonotic trematode infections, April 2005.

All persons who were positive for eggs were provided free drug treatment. Patients with nematode infections were given 1 dose of albendazole (400 mg) or mebendazole (500 mg), and patients with trematode infections were given praziquantel (25 mg/kg, 3× a day for 1 day).

Identification of Adult Worms Recovered

Adult worms were identified by direct light microscopy while still alive; those that needed further examination were fixed in 10% formalin and stained with Semichon acetocarmine, mounted on a slide, and measured (*18*). Identifications were made by using published taxonomic references (*19*,*20*).

Data Analysis

Results of fecal examinations for helminth eggs were analyzed for prevalence and intensity of infection (epg), as measured by enumeration of eggs per gram of feces. Species infection rates (number of expelled worms) were descriptively analyzed by using SPSS version 11.0 software (SPSS Inc., Chicago, IL, USA) and χ^2 and Student *t* tests.

Results

Parasite Diversity, Prevalence, and Intensity

A total of 615 persons, 563 men (91.5%) and 52 women (8.5%), were selected and submitted stool for examination. Fecal egg examinations showed that 554 persons (90.1%) were positive for helminth parasites (Table 1). *Trichuris trichiura* (whipworm) nematode eggs were found in 58.2% of the stool samples. A total of 64.9% were infected with small trematode eggs (<50 μm long), and 39.5% were infected with *Ascaris lumbricoides* (roundworm). Hookworm eggs (3.1%) and large (≥50 μm long) trematode eggs (0.8%) were infrequently seen (Table 1). Multiparasitism was common in this community, with 65.1% of the persons expelling eggs having ≥2 species or types of eggs. Small trematode eggs, all <50 μm long, were presumed to be either those of *C. sinensis* or of intestinal trematodes of the family Heterophyidae. However, differentiation was not considered reliable by light microscopy, and selected persons were treated to expel their helminth parasites.

On the basis of egg count data, small trematode infection prevalence differed significantly between men (68.7%) and women (23.1%) (χ^2 43.56, p<0.05). The infection rate for small trematode infection in men also differed significantly between age groups; it was significantly higher for those ≥40 years of age (χ^2 7.95, p<0.05). In contrast, women did not show a significant difference in infection rates between age groups (χ^2 0.85, p>0.05).

Table 1. Helminth infections in persons living in Nghia Phu and Nghia Lac communes, Nam Dinh Province, Vietnam, April 2005

Helminth egg species or type	Fecal examination result, no. positive (%)
Small trematodes (<50 μm long)	399 (64.9)
Large trematodes (≥50 μm long)	5 (0.8)
Ascaris lumbricoides	243 (39.5)
Trichuris trichiura	358 (58.2)
Hookworm	19 (3.1)
Total positive	554/615 (90.1)

Most persons with small trematode eggs showed low infection intensity (epg); 344 (86.2%) of 399 shed <1,000 epg, and 55 (13.8%) of 399 shed 1,000–9,999 epg. Infection intensity differed significantly between those ≤40 years of age and those >40 years of age (χ^2 4.17, p<0.05) (Table 2).

The prevalence of *A. lumbricoides* was 39.3% in men and 42.3% in women. There was a significant increase in prevalence with age only in women (χ^2 6.4, p<0.05). Infection with *T. trichiura* infection did not differ significantly by sex or age (p>0.05).

FZT Species Identification

Trematodes responsible for releasing small eggs were identified by using morphologic characterization of adult stages expelled from patients. A total 15,185 adult worms were collected from 33 patients. The number and prevalence of individual species of expelled trematodes are shown in Table 3. *C. sinensis* and 4 species of intestinal fishborne zoonotic flukes were identified (Figure 3); *C. sinensis* was isolated from 51.5% of patients. Intestinal fluke species identified (mean body length × width measurements in μm) were *Haplorchis pumilio* (632 × 291), *H. taichui* (756 × 421), *H. yokogawai* (760 × 400), and *Stellantchasmus falcatus* (468 × 298). Prevalence of intestinal flukes was *H. pumilio*, 100%; *H. taichui*, 69.7%; *H. yokogawai*, 6.1%; and *S. falcatus*, 3.0%. *H. pumilio* was the most common trematode (90.4%) of all worms isolated; 13,734 adult worms were isolated from 33 persons (mean 416.2); 1 patient expelled 4,525 worms. The plantborne intestinal pig trematode *Fasciolopsis buski* was isolated from 1 patient.

Multiple infections with FZTs were common (Figure 4): 54.5% of patients were infected with 2 trematode spe-

Table 2. Intensity of small trematode infections in 2 age groups, Nam Dinh Province, Vietnam, April 2005*

Age group	No. positive	No. (%) with light infection†	No. (%) with moderate infection†
<40 y	111	102	9
≥40 y	288	242	46
Total	399	344 (86.2)	55 (13.8)

*epg, eggs per gram (of feces).
†Light infection = 1–999 epg; moderate infection = 1,000–9,999 epg.

Table 3. Species and no. trematode adult worms recovered, Vietnam, Nam Dinh Province, April 2005

Trematode sp.	No. positive persons (%)	Total worms recovered	Average no. worms recovered (range)
Liver fluke			
Clonorchis sinensis	17 (51.5)	72	4.2 (1–18)
Intestinal flukes			
Haplorchis pumilio	33 (100)	13,734	416.2 (1–4,525)
H. taichui	23 (69.7)	1,323	40.1 (1–307)
H. yokogawai	1 (3.0)	3	3
Stellantchasmus falcatus	2 (6.1)	52	26 (15–37)
Fasciolopsis buski	1 (3.0)	1	1

cies, 33.3% with 3 species. A total of 9% were infected with only 1 species. One person (3%) was infected with 4 FZT species and *F. buski*.

Discussion

Our results demonstrate that zoonotic fishborne intestinal trematodes are endemic in Vietnam. These trematodes represent, to our knowledge, a new and previously unrecognized public health problem. To our knowledge, in the many publications on human parasites originating in Vietnam since the 19th century colonial era, no reports on these intestinal fishborne parasites have appeared (*11*). Whether this zoonosis is newly emerging in Vietnam because of changes in agriculture/aquaculture, demographics, social, or environmental changes or if it has been overlooked because of diagnostic problems is not known. However, snail vectors (e.g., *Melanoides tuberculata*) and suitable vertebrate intermediate (fish) and reservoir hosts (fish-eating birds, dogs, cats, pigs) for FZTs are common in this country (*1*,*4*,*11*,*19*,*21*). Furthermore, *H. taichui*, *H. pumilio*, *H. yokogawai*, *and S. falcatus* are endemic in neighboring countries such as Thailand (*14*), Lao People's Democratic Republic (*10*,*15*), and the People's Republic of China (*16*).

It is puzzling why zoonotic heterophyids have only recently been isolated from fish in Vietnam (*12*,*13*,*22*) if they are endemic. These parasites may have been recently introduced into this country and then became a zoonotic risk. Intensification of aquaculture, use of human and animal manure for pond fertilization, and increased consumption of fish because of increasing affluence by a population with a tradition of eating raw fish may be contributing factors for infection. These issues need to be investigated if effective means for prevention of transmission are to be developed. Use of manure and waste water in aquaculture is a well-recognized risk factor for trematode infections in fish (*2*,*12*,*13*) and has been the focus of 2 hazard analysis, critical control point–based control projects (*4*). Major sources of infected fish responsible for trematode transmission to humans must be ascertained because FZT metacercariae have been found in both wild and farmed fish in Vietnam, as well as elsewhere in Asia (*4*,*10*,*12–16*).

Public health and agricultural/fishery agencies should consider intestinal and liver flukes as an FZT complex because they share most biologic features and are risk factors for human infection. Although intestinal flukes are less well characterized clinically than liver flukes, they are increasingly being recognized as a cause of intestine, heart, brain, and spinal cord abnormalities in humans (*1*,*4*,*8*,*23*).

The potential economic effect of FZTs on alleviation of poverty is also a concern. Aquaculture in Vietnam is a major economic activity in rural areas. During 2000–2006, Vietnam tripled the value of its export of fish, increasing its revenue to >3 billion US dollars. Domestic availability of farmed fish is also a way of increasing protein availability

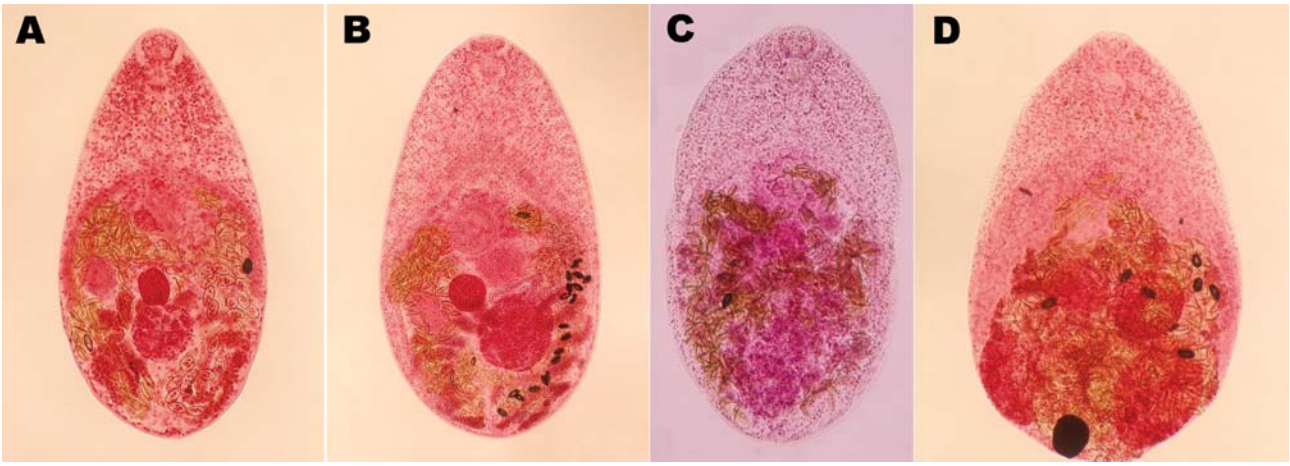

Figure 3. Adult trematodes isolated from Vietnamese persons. A) *Haplorchis pumilio*. B) *H. taichui*. C) *H. yokogawai*. D) *Stellantchasmus falcatus*. (Semichon acetocarmine stained, magnification ×120.)

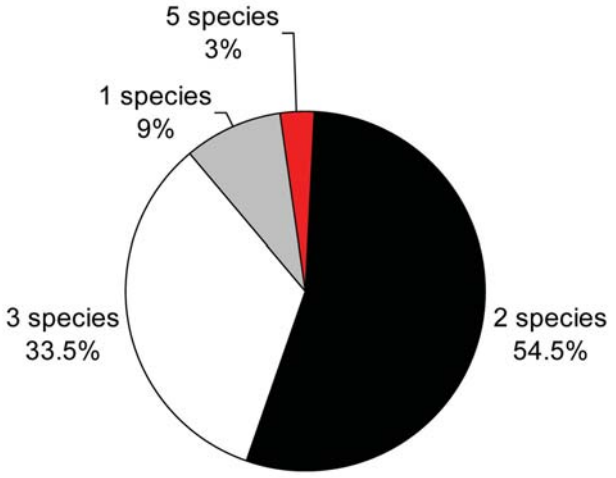

Figure 4. Multiple fishborne trematode infections in humans, Nam Dinh Province, Vietnam, April 2005.

to humans. Therefore, a newly recognized fish safety risk associated with aquaculture could have a serious constraint on market access (*24*), especially because consumer expectations and economic levels are increasing as predicted for Vietnam. These expectations can result in greater demand for safe fish by consumers, marketing agencies, and the tourist industry.

Our results showed a difference in infection rates of small trematodes between men (68.7%) and women (23.1%). Differences in liver infections with *C. sinensis* by sex of the patients are well known (*1*,*4*,*6*). Our results are similar to those of surveys conducted for this parasite in northern Vietnam (*25*,*26*). High infection rates for men in Vietnam are often associated with male-oriented social gatherings during which they consume raw or pickled fish, although this sex-related difference appears to be narrowing in some countries (*4*,*9*).

The relationship between prevalence and intensity of infection and age of the host is also a characteristic of FZT epidemiology (*1*,*4*). In our study, infection rates were higher for persons >40 years of age, a pattern believed to be caused by longer exposure and accumulation of parasites (*4*,*9*,*11*). However, the life span of intestinal flukes in humans is not well documented. Therefore, accumulation of worms as an explanation for age-related infection patterns is speculative. These behavioral factors in the epidemiology of FZT warrant greater collaboration between epidemiologists and anthropologists in designing approaches for mitigating risk in a population with great resistance to change in eating habits.

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Emergence of Methicillin-Resistant *Staphylococcus aureus* of Animal Origin in Humans

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In 2003 in the Netherlands, a new methicillin-resistant *Staphylococcus aureus (*MRSA) strain emerged that could not be typed with *Sma*1 pulsed-field gel electrophoresis (NT-MRSA). The association of NT-MRSA in humans with a reservoir in animals was investigated. The frequency of NT-MRSA increased from 0% in 2002 to >21% after intensified surveillance was implemented in July 2006. Geographically, NT-MRSA clustered with pig farming. A case–control study showed that carriers of NT-MRSA were more often pig or cattle farmers (pig farmers odds ratio [OR] 12.2, 95% confidence interval [CI] 3.1–48.6; cattle farmers OR 19.7, 95% CI 2.3–169.5). Molecular typing showed that the NT-MRSA strains belonged to a new clonal complex, ST 398. This study shows that MRSA from an animal reservoir has recently entered the human population and is now responsible for >20% of all MRSA in the Netherlands.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has traditionally been considered a nosocomial pathogen. However, for several years the number of reports of so-called community-onset MRSA (CO-MRSA) has been rapidly increasing (*1*). CO-MRSA has no relation to healthcare and is usually associated with the presence of Panton-Valentine leukocidin toxin (PVL) and SCC*mec* types IV and V (*2*,*3*). In 2004 and 2005, some unexpected cases of MRSA were found in patients who were associated with pig farms (*4*,*5*). Genotyping showed that these MRSA isolates were nontypable by pulsed-field gel electrophoresis (PFGE) and belonged to 1 *spa* type (t108). The aims of this study were to determine if nontypable MRSA (NT-MRSA) isolates are associated with pig farming and to compare the phenotypic, genotypic, and epidemiologic features of NT-MRSA with those of typable MRSA strains.

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Methods

National MRSA Database

The National Institute for Public Health and the Environment (RIVM) is the national reference center for MRSA in the Netherlands (www.rivm.nl/mrsa). According to national guidelines, all microbiology laboratories send the first isolate of newly identified carriers of MRSA to RIVM. Strains are confirmed to be MRSA by a Martineau PCR and by *mecA* PCR assay (*6*,*7*). Since 2002, all strains are typed by using PFGE (*8*), and the presence of PVL genes is determined (*9*).

Selection of Cases and Controls

Cases and controls were selected from the national MRSA database at RIVM. Case-patients were defined as persons carrying NT-MRSA who provided the first isolate from a cluster of 1 particular referring laboratory (index-patient) in the period January 2003 to September 2005. Cases were considered to be secondary to an index case when the strain was isolated within 3 months after the previous isolate with the same PFGE typing result. Controls were persons who carried MRSA that was typable with PFGE and who also fulfilled the index-patient definition. Controls were derived from the laboratories that provided cases and were selected at random. Twice as many controls as case-patients were selected.

[1]These authors contributed equally to this article.

Collection of Epidemiologic Background Information

Data were collected by questionnaires that were sent to the referring laboratories. The questionnaire contained items about patient characteristics (birth date, sex, postal code, presence or absence of infection, hospital admission dates, profession, profession of partner, profession of parents, and contact with animals, e.g., pigs, cows, horses, chickens, cats, or dogs) and microbiologic data (isolation date, source of culture, medical specialty). All data were collected and entered into the database without our knowing whether it concerned a case or control.

Initially, 41 cases and 82 controls from 26 different laboratories were selected from the national database. The response rate was 98% (40 cases and 81 controls). During workup, 5 cases and 5 controls were excluded for the following reasons: the confirmation test of the isolate indicated that it was not methicillin resistant (1 case), or the case did not fulfill the case definition because it was not the first case from a cluster (4 cases). Since 2 of these cases were from laboratories that had no other case in the study, the accompanying controls were excluded (n = 3). Two controls were identified outside the study period. Finally, 35 cases and 76 controls from 24 different laboratories were included in the analysis.

Molecular Typing and Susceptibility Testing

All MRSA isolates were typed by PFGE (*8*). All isolates from case-patients and 74 isolates from controls were typed by *spa* typing (*10*). Multilocus sequence typing (MLST) was performed on all case isolates, as well as on 1 strain of each *spa* type of the control isolates (n = 37) (*11*). PCR of the staphylococcal chromosome cassette (SCC*mec*) was performed according to Zhang et al. on all isolates from case-patients and 74 isolates from controls (*12*). The presence or absence of PVL genes (*lukS-PV*/*lukF-PV*) was determined in all case isolates and in 71 control isolates. The PVL genes were detected by PCR according to the method of Lina et al. (*13*). The susceptibility to antimicrobial agents was tested for 32 case isolates and 74 control isolates, according to CLSI guidelines that used Mueller-Hinton agar and multipoint inoculation (*14*).

Statistical Analysis

Data were entered into an Excel database (Microsoft Windows version 97 SR-2, Redmond, WA, USA) and further analyzed by using SAS (version 9.1) software package (SAS Institute Inc., Cary, NC, USA). Chi-square test and Fisher exact test for ordinal variables and Student *t* test for continuous variables were used for univariate analysis. Variables associated with both case-control status and the exposure (i.e., contact with pigs or cattle, respectively) with a p value <0.2 were included in the multivariate logistic regression model. If such variables changed the risk estimate for >10%, they were left in the model. All statistical tests were 2-sided, and a p value <0.05 was considered statistically significant.

Results

Epidemic Curve

The first NT-MRSA isolate was found in February 2003. In subsequent years, an increasing number of NT-MRSA isolates were found. The percentage of NT-MRSA relative to the total number of MRSA isolates in the Netherlands that were unique or first from a cluster rose from 0% in 2002 to 5.5% in the first half of 2006 and to >21% in the second half of 2006, after the introduction of intensified surveillance in July 2006.

Geographic Distribution

Figure 1 shows the geographic distribution of NT-MRSA and typable MRSA isolates, plotted over the density of the pig and human populations, respectively. The density of NT-MRSA isolates corresponds to the density of pig farming, whereas the density of typable strains corresponds to the density of the human population. The density of cattle farms is more or less identical to the density of pig farms.

Epidemiologic Data

Results of the univariate analysis are shown in Table 1. Comparable values were observed for the baseline characteristics of sex and age. Case-patients more often lived in rural areas and indicated more frequent contact with pigs or cattle than did controls. Controls were more often associated with healthcare facilities.

Among case-patients, MRSA was more frequently found in clinical samples (an unexpected finding) compared with controls, whose MRSA was found more often by targeted screening in nose, throat, and perineum. Among persons infected by MRSA, respiratory tract infections were more frequent in case-patients, whereas skin and soft tissue infections predominated in controls.

Multivariate analysis that used a model with the variables describing type of residence (rural vs. other) and contact with pigs, cattle, cats, and dogs (yes, no, or unknown) showed that contact with pigs and contact with cattle were independent statistically significant variables. The adjusted odds ratios (OR) for pigs and cattle were 9.4 (95% confidence interval [CI] 1.8–47.7) and 13.5 (95% CI 1.0–179.3), respectively.

Molecular Typing

Thirty-two of 35 case-patients had MLST sequence type (ST) 398; 1 had ST 9; and the remaining 2 had ST 752 and 753, closely related to 398 (Figure 2). Among case-

patients, the most frequent *spa* types were t108, t011, and t034 (Table 2). These MLST and *spa* types were not found among the controls. Twenty-two different STs and 37 different *spa* types were found in the controls (Table 2 and Figure 2).

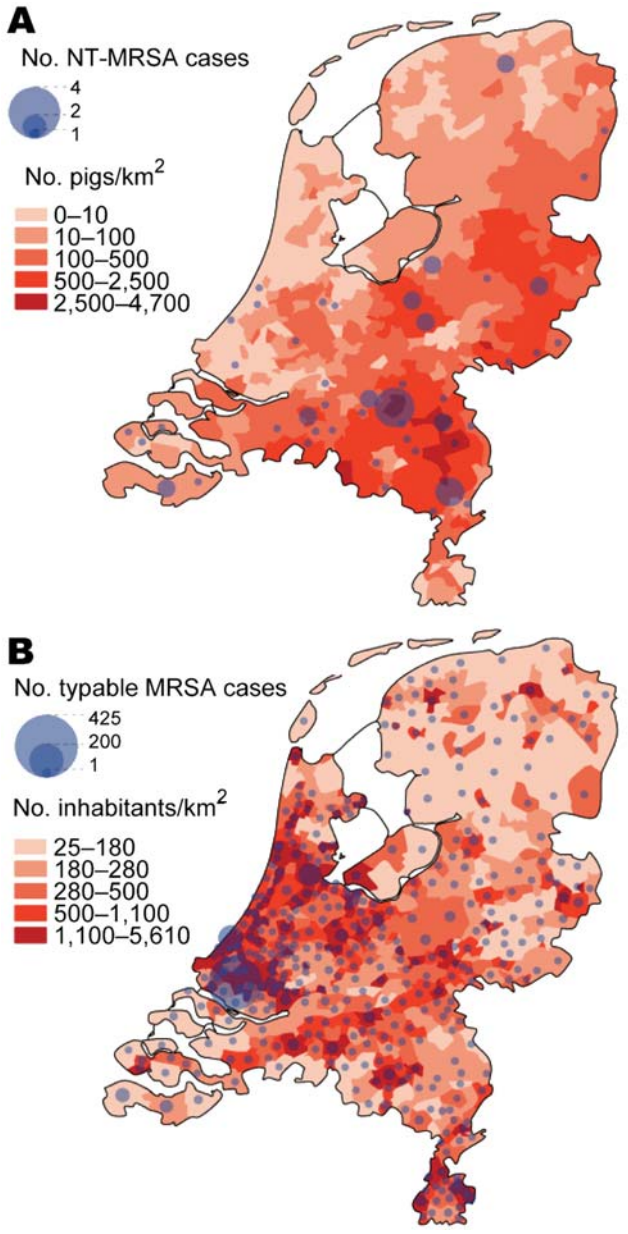

Figure 1. A) Number of nontypable methicillin-resistant *Staphylococcus aureus* (NT-MRSA) isolates per municipality received at the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands, January 2003–June 2005. The background color represents the density of pigs per km^2 in 2003. B) Number of typable MRSA per municipality received at the RIVM January 2003–June 2005. The background color represents the population density per km^2 (source: CBS Statline).

SCC*mec* typing showed that in isolates from cases SCC*mec* types III, IV, and V were found, whereas in isolates from controls all SCC*mec* types were found (Table 2). For 11 cases and 33 controls, the SCC*mec* type could not be determined. There was no difference in the presence of the PVL genes (Table 2).

Antimicrobial Agent Susceptibility

Table 3 shows the percentage of strains that were resistant to various antimicrobial agents. Isolates from case-patients were significantly more often resistant to doxycycline and clindamycin than were isolates from controls.

Discussion

A new type of MRSA recently emerged in the Netherlands. The first isolate was found in 2003, and since then it has been found with increasing frequency. The geographic origin of NT-MRSA correlates with the density of pig populations. This association was confirmed by the results from this case-control study, which show that NT-MRSA is significantly related to contact with pigs. In addition, a significant association was found with cattle. After multivariate analysis, contact with pigs and cattle were the only 2 significant independent variables. Screening of a representative sample of pigs in the Netherlands was recently performed and showed that nearly 40% of the pigs were colonized with a comparable strain of MRSA (MLST 398) and that ≈80% of the pig farms were affected (*15*). The association between NT-MRSA and cattle was not expected when this study was initiated and needs further evaluation.

On the basis of the above-mentioned findings, we conclude that this new MRSA strain is of animal origin (pigs and probably cows). Transmission of MRSA between animals and humans has previously been described, e.g., associated with colonized companion animals, horses, and persons who take care of them (*16–19*). However, the MRSA clones in these reports were known human clones, suggesting human-to-animal transmission in origin. Baptiste et al. found specific PFGE clones in horses that were never observed before (*20*). Until now, transmission of these clones to humans has not been reported.

We assume that this problem is not limited to the Netherlands. First, widespread dissemination in pigs in the Netherlands has been found. When one considers the intensive international transport of pigs, it is unlikely that this situation is limited to the Netherlands. Second, 3 of the case-patients came from abroad, 1 tourist and 2 adopted children from Asia. Also, MLST 398 was recently found in animals (pig, dog, and foal) and in humans in Germany (*21*). Finally, in Hong Kong Special Administrative Re-

Table 1. Results of univariate analysis of case-control study, the Netherlands, February 2007*

Variable	Cases		Controls		Odds ratio (95% CI)†	p value
	No.	No. (%) or mean ± SD with variable	No.	No. (%) or mean ± SD with variable		
Gender (male)	35	20 (57)	76	36 (47)	1.5 (0.7–3.3)	0.34
Age, y	35	42.7 ± 25.3	76	47.3 ± 24.7		0.37
Residence	35		75			
Rural area		14 (40)		6 (8)	7.7 (2.6–22.7)§	<0.01
Urban area		20 (57)		66 (85)		
Foreign country		1 (3)		3 (4)		
Contact with pigs	29	11 (38)	63	3 (5)	12.2 (3.1–48.6)	<0.01
Contact with cattle	29	7 (24)	63	1 (2)	19.7 (2.3–169.5)	<0.01
Unexpected MRSA	35	27 (77)	76	34 (45)	4.2 (1.7–10.4)	<0.01
Probable source	35		76			
Healthcare		5 (14)		39 (51)		0.01
Foreign country		3 (9)		5 (7)		
Other		12 (34)		10 (13)		
Unknown		15 (43)		22 (29)		
Active infection	35	19 (54)	76	29 (38)	1.9 (0.9–4.3)	0.11
Skin/soft tissue		10 (56)		24 (83)	0.3 (0.1–1.0)	0.05
Airways		3 (17)		0		
Other		6 (28)		5 (17)		
Hospital admission	35	17 (49)	76	24 (32)	2.0 (0.9–4.6)	0.08
Hospital stay, d	16	18.9 ± 20.2	22	23.5 ± 30.9		0.60

*SD, standard deviation; CI, confidence interval; No., number of cases or controls for whom data are available.
†Odds ratio was determined for rural area relative to urban area.

gion, People's Republic of China, MRSA with MLST 398 has been found in 2 patients with bacteremia (*22*).

The origin of the current NT-MRSA situation is difficult to elucidate. One earlier study can be found on carriage of *S. aureus* in pig farmers and pigs in France (*23*). It reported an increased carriage rate in pig farmers caused by transmission of *S. aureus* from pigs that also carried MLST ST 9 and 398. Further typing of the French ST 398 isolates at RIVM showed homology with the Dutch isolates. However, in the French study most of the MLST 398 strains were susceptible to β-lactam antimicrobial agents. The most likely explanation for the current findings is that MLST 398 is a commensal strain in pigs, which originally was methicillin susceptible. As most NT-MRSA isolates were resistant to doxycycline, the spread is facilitated by the abundant use of tetracyclines in pig and cattle farming (*15*).

What are the implications of these findings? Persons working or living in close contact with pigs or cows are at increased risk of becoming colonized and infected with MRSA. Infections can be severe, as is indicated by the hospital admission rate. Also, a case of endocarditis has been reported recently (*24*). At present, whether this strain is spreading further in the community is not clear. Before final recommendations for control can be made, the current size of the reservoir in farm animals and in humans has to be determined at an international level.

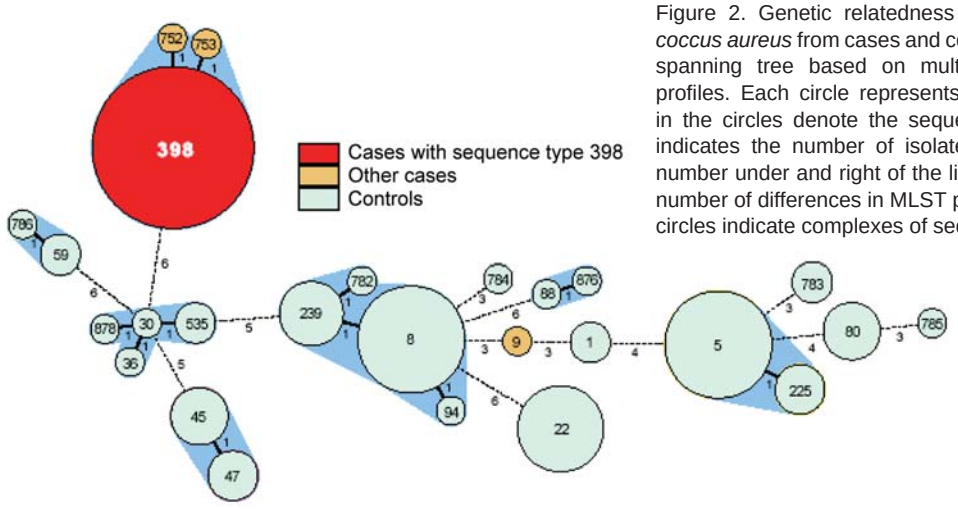

Figure 2. Genetic relatedness of methicillin-resistant *Staphylococcus aureus* from cases and controls, represented as a minimum spanning tree based on multilocus sequence typing (MLST) profiles. Each circle represents a sequence type, and numbers in the circles denote the sequence type. The size of the circle indicates the number of isolates with this sequence type. The number under and right of the lines connecting types denotes the number of differences in MLST profiles. The halos surrounding the circles indicate complexes of sequence types that differ by <3 loci.

Table 2. Typing results for cases and the number of controls with the same type, the Netherlands, February 2007

Type	Cases, no. (%)	Controls, no. (%)	p value
spa			
t108	14 (40)	0	<0.01
t011	8 (23)	0	
t034	6 (17)	0	
t571	3 (9)	0	
t567	2 (6)	0	
t337	1 (3)	0	
t898	1 (3)	0	
SCC*mec*			
I	0	4 (9)	<0.01
II	0	7 (16)	
III	4 (17)	6 (14)	
IV	2 (8)	21 (49)	
V	18 (75)	5 (12)	
Panton-Valentine leukocidin	3 (9)	10 (14)	0.21

Table 3. Number and percentage of resistant MRSA isolates for various antimicrobial agents, the Netherlands, February 2007*

Agent	Cases, no. (%)	Controls, no. (%)	p value
Doxycycline	25 (78)	10 (14)	<0.01
Ciprofloxacin	1 (3)	36 (49)	<0.01
Tobramycin	4 (13)	25 (34)	0.02
Gentamicin	2 (6)	12(16)	0.14
Clindamycin	12 (38)	15 (20)	0.05
Erythromycin	15 (46)	29 (39)	0.35
Cotrimoxazole	0	7 (10)	0.07
Rifampin	0	6 (8)	0.11
Mupirocin	0	5 (7)	0.15
Vancomycin	0	0	

*MRSA, methicillin-resistant *Staphylococcus aureus.*

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Dr van Loo is a medical microbiologist at the University Hospital Maastricht. The research described in this manuscript was performed as part of her training as a medical microbiologist.

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Hospitalizations and Deaths Caused by Methicillin-Resistant *Staphylococcus aureus*, United States, 1999–2005

Eili Klein,* David L. Smith,† and Ramanan Laxminarayan*

Hospital-acquired infections with *Staphylococcus aureus*, especially methicillin-resistant *S. aureus* (MRSA) infections, are a major cause of illness and death and impose serious economic costs on patients and hospitals. However, the recent magnitude and trend of these infections have not been reported. We used national hospitalization and resistance data to estimate the annual number of hospitalizations and deaths associated with *S. aureus* and MRSA from 1999 through 2005. During this period, the estimated number of *S. aureus*–related hospitalizations increased 62%, from 294,570 to 477,927, and the estimated number of MRSA-related hospitalizations more than doubled, from 127,036 to 278,203. Our findings suggest that *S. aureus* and MRSA should be considered a national priority for disease control.

Staphylococcus aureus is a leading cause of hospital-acquired infections. It is the primary cause of lower respiratory tract infections and surgical site infections (*1*,*2*) and the second leading cause of nosocomial bacteremia (*3*), pneumonia, and cardiovascular infections (*1*,*2*). Infections with *S. aureus* are especially difficult to treat because of evolved resistance to antimicrobial drugs. Resistance to penicillin and newer narrow-spectrum β-lactamase–resistant penicillin antimicrobial drugs (e.g., methicillin, oxacillin) appeared soon after they were introduced into clinical practice in the 1940s and 1960s, respectively (*4*). Penicillin resistance was initially confined to a small number of hospitalized patients, but resistance spread as use of penicillin increased, first to other hospitals and then into the community (*5*). By the late 1960s, >80% of community- and hospital-acquired *S. aureus* isolates were resistant to penicillin (*4*). Recent reports suggest that the evolution and spread of methicillin-resistant *S. aureus* (MRSA) seems to be following a similar wavelike emergence pattern (*5*).

MRSA is now endemic, and even epidemic, in many US hospitals, long-term care facilities (*6*), and communities (*7*,*8*). Contrary to the generally accepted view, community-associated MRSA strains may be spreading into the healthcare system rather than the other way around (*9*). Data from the National Nosocomial Infections Surveillance system suggest that in intensive care units the proportion of *S. aureus* isolates that are resistant to methicillin has increased to 59.5%–64.4% (*10*,*11*). Recent reports also suggest that community-associated MRSA infections have become the dominant cause of community-associated *S. aureus* skin and soft tissue infections (*9*,*12*). An understanding of the magnitude of the problem requires accurate national estimates of incidence. However, national studies examining the effect of *S. aureus* or MRSA on the healthcare system are >5 years old (*13*,*14*). For 2000–2001, Noskin et al. estimated that there were 290,000 *S. aureus*-related hospitalizations (*14*). Kuehnert et al. estimated a similar number of *S. aureus*–related hospitalizations for 1999–2000 and reported that 43.2% (125,969) were likely resistant to methicillin (*13*).

In this study, we estimated the magnitude of the effect and trend in the incidence and associated mortality rates of infections related to *S. aureus* and MRSA over a 7-year

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period, from 1999 through 2005, paying particular attention to the overall *S. aureus* infection level and the trend of typical community-associated infections. Evidence on the magnitude and trend of the problem on a national level informs rational, evidence-based decisions about how to allocate resources and adjust healthcare policy to address this issue. Infection trends are useful to clinicians, hospital administrators, insurers, and policymakers who make decisions regarding control measures, especially infection-control measures to contain the spread of nosocomial and community-associated pathogens.

Methods

Our analysis focused on the period 1999–2005 and followed an approach similar to that described by Kuehnert et al. (*13*). Estimated incidence of *S. aureus* was based on hospitalizations with *S. aureus*–related discharge diagnoses from the National Hospital Discharge Survey (NHDS). The NHDS covers ≈270,000 patients and 500 short-stay hospitals by using a stratified, multistage survey to create a nationally representative annual sample of discharge records. Children and general hospitals are included; federal, military, Veterans Affairs, or institutional hospitals are not included. Each discharge record contains ≤7 different International Classification of Diseases, Ninth Revision (ICD-9), Clinical Modification, discharge diagnosis codes and is population weighted on the basis of the probability of sample selection and adjusted for nonresponse. All acute-care hospitalizations, excluding those of infants born in the hospital, were considered.

S. aureus–related discharges were included if any of the 7 diagnosis codes contained specific *S. aureus* infection codes: 038.11 (*S. aureus* septicemias), 482.41 (*S. aureus* pneumonias), and 041.11 (other *S. aureus* infections). Records that contained multiple *S. aureus*–related discharge codes were only counted once, with septicemia preferentially included, followed by *S. aureus*–related pneumonia.

Because there is no MRSA-specific ICD-9 code, we indirectly estimated the proportion of *S. aureus*–related infections that were methicillin resistant by using antimicrobial drug testing data from The Surveillance Network (TSN) Database-USA (Focus Diagnostics, Herndon, VA, USA). TSN is an electronic repository of susceptibility test results collected from >300 microbiology laboratories in the United States; it has been used extensively to evaluate antimicrobial drug resistance patterns and trends (*15*). Participating laboratories are geographically dispersed and make up a nationally representative sample on the basis of hospital bed size and patient population. Patient isolates are tested for susceptibility to several different antimicrobial agents on site as part of routine diagnostic testing by using standards established by the National Committee for Clinical Laboratory Standards (NCCLS) and approved by the US Food and Drug Administration (*15*). Results are filtered to remove repeat isolates and identify microbiologically atypical results for confirmation or verification before being included.

We included *S. aureus* isolates from inpatient areas that were tested for susceptibility to oxacillin (which is used as a proxy for all β-lactam antimicrobial drugs, including methicillin) and classified as susceptible, intermediate, or resistant according to NCCLS breakpoint criteria. Data included >65,000 isolates annually, of which <0.01% had intermediate resistance and so were classified susceptible. To ensure comparability with NHDS data, isolates were stratified by the type of infection (i.e., isolates from the lungs were classified as pneumonias; those from the blood, as septicemias or bacteremias) and geographic region based on the US Census Bureau regions.

The annual estimated number of *S. aureus*–related hospitalizations was obtained from NHDS. The total number of MRSA-related hospitalizations was estimated by multiplying the number of *S. aureus*–related infections by the estimated percentage of *S. aureus* isolates that were resistant, stratified by infection type and region. Frequencies of primary and secondary diagnoses were also extracted for all hospitalizations that included *S. aureus*–related infections.

Relative standard errors for incidence of *S. aureus* were calculated by following guidelines for NHDS accuracy described by Dennison and Pokras (*16*). Standard errors and 95% confidence intervals (CIs) were calculated by multiplying the relative standard error by the estimated incidence. CIs for TSN data were calculated by using the Wilson score method and incorporating continuity correction as detailed by Newcombe (*17*). The variance of MRSA incidence was estimated by using the method described by Barnett (*18*) and Goodman (*19*).

NHDS reports whether or not hospitalization results in patient death but does not specify the cause of death. Because the primary diagnosis suggests that the disease played a role in patient death, we estimated the number of *S. aureus*–related deaths where the primary diagnosis code was an *S. aureus*–related code. We used the same procedure as described above to determine the estimated number of deaths for which MRSA was involved.

Results

From 1999 through 2005, annual hospital discharges in the United States increased ≈8%, from 32.1 million to 34.7 million. During this period, the estimated number of hospitalizations involving *S. aureus*–related infections increased 62%, from 294,570 (95% CI 257,304–331,836) to 477,927 (95% CI 421,665–534,189). *S. aureus*–related hospitalizations with diagnosis codes for septicemia and pneumonia increased 38% and 7%, respectively, and hospitalizations

involving other *S. aureus*–related infections in conditions classified elsewhere nearly doubled. Overall, the rate of *S. aureus*–related diagnoses per 1,000 hospitalizations increased 50%, from 9.17 to 13.79 (Table 1).

From 1999 through 2005, estimated MRSA-related hospitalizations more than doubled, from 127,036 (95% CI 112,356–141,716) to 278,203 (95% CI 252,788–303,619). MRSA-related hospitalizations with a diagnosis code for septicemia increased 81.2%, from 31,044 (95% CI 25,170–36,918) to 56,248 (95% CI 46,830–65,665), and MRSA-related hospitalizations with a diagnosis code for pneumonia increased 19.3%, from 30,632 (95% CI 24,597–36,666) to 36,540 (95% CI 29,527–43,554). The largest increase in MRSA-related hospitalizations involved infections outside the lungs or blood; these almost tripled from 65,361 (95% CI 55,801–74,920) to 185,415 (95% CI 162,102–208,728). Overall, the rate of MRSA-related discharges per 1,000 hospitalizations more than doubled, from 3.95 to 8.02 (Figure 1).

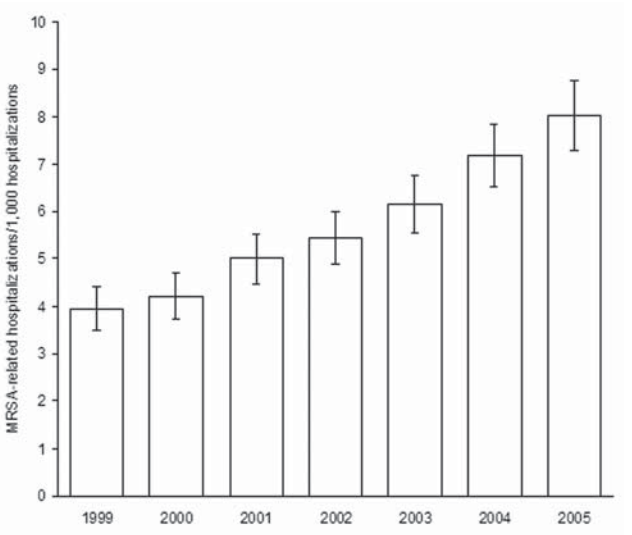

Figure 1. Estimated methicillin-resistant *Staphylococcus aureus* (MRSA)–related hospitalization rates, United States, 1999–2005. Rates are no. MRSA-related discharges/1,000 hospitalizations. Error bars represent 95% confidence intervals.

In hospitalizations for which *S. aureus*–related septicemia and pneumonia were listed as any 1 of the 7 discharge diagnoses, these diagnoses were coded as the primary diagnosis, on average, in 38% (standard deviation 6.4%) and 54% (3.7%) of records, respectively, over the 7-year period. The most frequent primary diagnosis associated with other *S. aureus*–related infections was other cellulitis and abscess (ICD-9 682), followed by postoperative infection (ICD-9 998.59), infections from an implanted device or graft (ICD-9 996), osteomyelitis (ICD-9 730), and diabetes mellitus (ICD-9 250). Cellulitis infections increased >25% per year from 22,451 (95% CI 17,007–27,895) to 87,500 (95% CI 75,485–99,515), which was nearly a 4-fold increase. No other primary diagnosis infection code increased over this time period (Figure 2).

Similar rates of discharge associated with *S. aureus*–related and, more specifically, MRSA-related infections per 1,000 hospitalizations were observed across all 4 US regions (Northeast, South, Midwest, and West; Table 2). Overall, the rate of *S. aureus*–related infections increased 5% per year in the Northeast, 7% in the Midwest and South, and 8% in the West. The rate of MRSA-related infections in the Northeast, Midwest, and South increased 9%, 11%, and 12% per year, respectively. In contrast, the West had the lowest incidence and frequency of MRSA-related infections, but the rate of MRSA-related infections increased 18% per year. Although increases were considerable, none of the rates in any region was significantly different in any year from the others at the 95% CI level.

In 2005, there were ≈11,406 *S. aureus*–related deaths (95% CI 7,609–15,203), of which 6,639 were MRSA-related (95% CI 4,429–8,850). Since 1999, no trend was seen in the number of deaths. We estimated that *S. aureus*–related deaths averaged ≈10,800 per year (range 7,440–13,676) and MRSA-related deaths averaged ≈5,500 per year

Table 1. *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA)–related hospital discharge diagnoses, by infection site and year, United States

Discharge diagnosis	1999	2000	2001	2002	2003	2004	2005
All discharges	32,131,876	31,705,672	32,652,588	33,726,612	34,738,412	34,864,168	34,667,316
S. aureus septicemias	75,125	73,206	77,998	82,813	92,247	92,785	103,300
% MRSA	41	45	48	49	52	54	54
MRSA septicemias	31,044	33,251	37,381	40,197	47,745	50,238	56,248
S. aureus pneumonias	58,833	53,692	63,759	64,294	58,511	71,275	63,185
% MRSA	52	54	56	58	58	59	58
MRSA pneumonias	30,632	29,210	35,893	37,120	33,965	41,988	36,540
Other *S. aureus* infections	160,612	161,614	189,715	211,310	245,971	272,873	311,442
% MRSA	41	44	48	50	54	58	60
Other MRSA infections	65,361	71,048	90,163	106,174	132,154	158,211	185,415
Total *S. aureus* infections	294,570	288,512	331,472	358,417	396,729	436,933	477,927
Overall % MRSA	43	46	49	51	54	57	58
Total MRSA infections	127,036	133,510	163,437	183,491	213,864	250,438	278,203

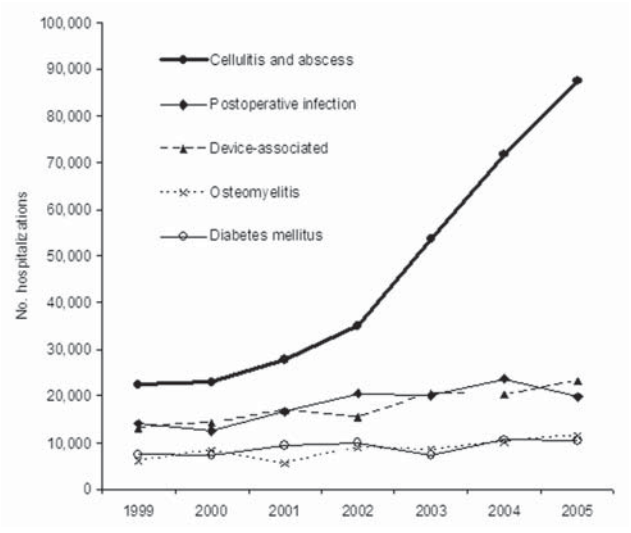

Figure 2. Primary diagnoses of *Staphylococcus aureus*–related hospitalizations. The most frequent primary diagnosis associated with other *S. aureus*–related infections was other cellulitis and abscess (International Classification of Diseases [ICD]-9 682), followed by postoperative infection (ICD-9 998.59), infections from an implanted device or graft (ICD-9 996), osteomyelitis (ICD-9 730), and diabetes mellitus (ICD-9 250). Cellulitis and abscess infections increased at a rate >25% per year from 1999 through 2005. No other primary diagnosis infection showed a major increase over this period.

(range 3,809–7,372) (Figure 3). However, the percentage of *S. aureus*–related and MRSA-related hospitalizations that resulted in death did show a trend, a decrease from ≈3.7% in 1999 to only 2.4% in 2005. We also calculated the number of deaths in which any diagnosis code was *S. aureus*–related. These calculations showed that deaths with an *S. aureus*–related discharge code increased 18% from 24,715 (95% CI 17,853–31,577) to 29,164 (95% CI 21,620–36,708) from 1999 through 2005. Deaths in which MRSA was likely present increased >50%, from 11,240 (95% CI 8,117–14,362) to 17,260 (95% CI 12,794–21,726) over the same period. However, despite the increases, the percentage of *S. aureus*–related hospitalizations that resulted in death decreased from 8.4% in 1999 to 6.1% in 2005, and the percentage of MRSA-related hospitalizations that resulted in death decreased from 8.8% to 6.2%.

S. aureus resistance to ampicillin/sulbactam, cephalothin, and erythromycin increased 21%, 35%, and 27%, respectively, during the study period. Resistance to gentamicin and trimethoprim-sulfamethoxazole decreased 76% and 64%, respectively. No instances of vancomycin-resistant (or intermediate-resistant) *S. aureus* in hospitalized patients were reported.

Discussion

MRSA, a common cause of nosocomial infections, has emerged as an increasingly common cause of community-associated infections (*20*). Our analysis extends the work of Kuehnert et al. (*13*) and quantifies recent trends and the effect of *S. aureus* and MRSA on the US healthcare system.

This study focused on the effect and trends in the incidence of *S. aureus*–related infections generally and MRSA-related infections specifically. Although the number of hospitalizations associated with an *S. aureus* infection increased 62% or ≈8.4% per year, the number of *S. aureus* infections resistant to methicillin increased 119% or ≈14% per year. In addition, although steady growth was observed in the incidence of *S. aureus*– and MRSA-related septicemia, pneumonia, and device-associated infections that are typically nosocomial, dramatic increases were observed in the incidence of skin and soft tissue infections that are typically community associated. We also found no trend in the number of deaths caused by MRSA, and a decreasing trend in the percentage of *S. aureus*– and MRSA-related hospitalizations that resulted in death. These results suggest a change in the ecology of the disease; community-associated MRSA is spreading more rapidly and possibly making its way into hospitals.

The indication that community-associated MRSA is spreading rapidly into hospitals has implications for hospital and community infection control as well as empirical treatment. In hospitals, handwashing practices, which have been shown to be the leading intervention for limiting the spread of nosocomial infections, should be improved to meet recommended guidelines (*21*). Because of the increase in skin and soft tissue infections, standard precautions, including use of gloves, are likely warranted when dealing with all skin and soft tissue infections in outpatient clinics and acute-care facilities. Contact precautions, including use of gowns and gloves, should be implemented for all wound care in acute-care facilities, and institutional programs to enhance antimicrobial drug stewardship should be implemented. Programs to increase community

Table 2. Hospitalizations and rates of infections with *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) by region and year, United States*

Region	1999	2000	2001	2002	2003	2004	2005
Northeast	8.42 (3.58)	8.61 (3.9)	10.01 (4.9)	10.62 (5.22)	11.25 (5.65)	11.07 (5.84)	11.59 (6.12)
Midwest	8.53 (3.84)	9.59 (4.53)	9.8 (4.84)	9.33 (4.8)	9.65 (5.04)	11.29 (6.54)	12.47 (7.23)
South	9.71 (4.63)	9.44 (4.68)	10.14 (5.33)	11.17 (6.15)	12.5 (7.25)	13.46 (8.21)	14.77 (9.31)
West	9.75 (3.15)	8.33 (3.14)	10.85 (4.61)	11.05 (4.98)	11.57 (5.87)	13.75 (7.39)	15.84 (8.55)

*Rates are no. hospitalizations with *S. aureus* MRSA–related discharge diagnoses/1,000 discharges. Values in parentheses are rates for MRSA.

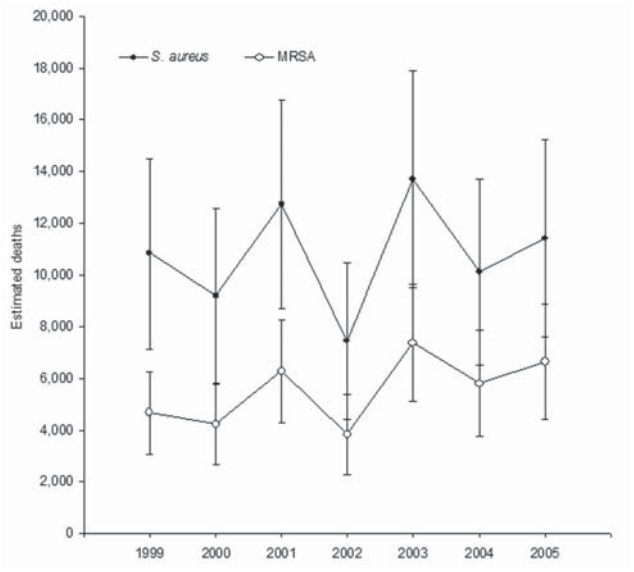

Figure 3. Estimated hospital deaths associated with *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA), United States, 1999–2005. Error bars represent 95% confidence intervals.

awareness to control spread of infections and initiatives to reduce inappropriate use of antimicrobial drugs should also be implemented, especially in institutions that are focal institutions such as daycare centers, schools, and prisons, as well as in high-risk groups such as immunodeficient persons, children, and elderly persons. Clinicians should be aware of the magnitude of the issue and consider MRSA a highly likely cause of skin and soft skin tissue infections, even in areas where the prevalence of MRSA is believed to be low.

Previous hospitalization has been associated with community MRSA carriage (*22*), and some recent studies have suggested that MRSA infection rates in the community are positively correlated with *S. aureus* infection rates in the hospital (*23*,*24*). Although a recent study suggests that community-associated MRSA is causing hospital-acquired MRSA (*25*), it is unclear from our study whether community-associated MRSA is responsible for increasing rates of nosocomial MRSA or the other way around. In all likelihood, MRSA is spreading in hospitals and communities and complicating efforts to prevent infections in hospitalized patients. Regardless, our findings demonstrate that recent reports of localized increases in community-acquired MRSA (*7*,*26*–*28*) are part of a larger trend of MRSA becoming rapidly endemic in communities all over the United states, emulating the wave-like pattern of emerging resistance to penicillin in the middle of the 20th century (*5*).

Hospital-acquired infections from all causes are estimated to cause ≥90,000 deaths per year in the United States and are the sixth leading cause of death nationally. Nosocomial infections increase patient illness and the length of hospital stays. The direct cost has been estimated to be >$6 billion (inflation adjusted) (*29*); costs of longer inpatient visits are shared by hospitals. The increasing trend in hospitalizations associated with *S. aureus* infections has considerable cost implications for the healthcare system, including costs when community-associated infections require hospitalization and the additional expenses from associated nosocomial infections.

Antimicrobial drug–resistant infections impose even greater costs than susceptible infections. Several studies have estimated that antimicrobial drug–resistant infections increase death, illness, and direct costs by 30%–100% (*30*). Estimates of the excess cost of an infection with MRSA compared with an infection with methicillin-sensitive *S. aureus* range from ≈$3,000 to $35,000 (*31*–*33*). This suggests that MRSA cost the healthcare system (patients and hospitals) an extra $830 million–$9.7 billion in 2005, even without taking into account indirect costs related to patient pain, illness, and time spent in the hospital.

Another important implication of our analysis is that the increasing incidence of MRSA in hospitalized patients, whether the infection was acquired in the hospital or the community, is likely to increase the demand for vancomycin. Despite several new (daptomycin, linezolid, tigecycline) and old (trimethoprim-sulfamethoxazole, clindamycin) antimicrobial drugs available for treatment of MRSA infections, vancomycin has remained the first-line drug for treating MRSA (*12*,*34*). This pattern has broad implications for the future control of MRSA as well as other pathogens. *S. aureus* infections resistant to vancomycin are already emerging (*35*), and vancomycin-resistant enterococci are already a major problem in hospitals. Vancomycin use should be restricted to methicillin-resistant *S. aureus* infections and used only for MRSA infections in situations where other drugs are not appropriate.

Our analysis has some limitations. First, it was restricted to the incidence of disease associated with acute-care management within the hospital setting. Recent reports suggest that MRSA has been increasing in outpatients (*36*,*37*). Thus, our results represent only a part of the problem, although hospitalizations outweigh outpatient visits by ≈4 to 1.

Second, NHDS data enables the coding of only 7 diagnosis codes; hospital information systems typically include 15–20 diagnosis codes for each admission (*38*). Thus, additional diagnoses in which *S. aureus* played a role may have been excluded. Errors in ICD coding when transcribing from doctors' discharge summaries are another potential source of bias, as is the possibility that multiorgan failure, an end stage of sepsis, was coded as septicemia. One study concluded that the positive predictive value of the 038 code on NHDS records to predict sepsis was 88.9%–97.7%, de-

pending on the criteria, and the negative predictive value was 80.0% (*39*). The authors of another study that examined whether sepsis was coded correctly on hospital bills concluded that strict reliance on administrative data may be prone to bias because only 75.4% of sepsis cases were accurately coded (*38*). Thus, our results may be an underestimate of the true effect, although trends are likely robust to coding errors.

Third, TSN data provide information concerning only the site of isolate collection and not the infection. Thus, some isolates from blood or the lung area may not be associated with septicemia or pneumonia, respectively. In addition, the code for *S. aureus* septicemia was given priority over the other more site-specific codes; this could have affected the estimates of MRSA infections. However only a limited number of records had overlapping codes.

Fourth, although the 2 data sources from TSN and NHDS used in this article are nationally representative, they may not represent a stratified random sample of hospitals by type and region. However, the trends are likely robust enough to avoid bias. In addition, the percentage of *S. aureus* isolates resistant to methicillin reported in the TSN database has increased similar to that reported by other national studies (Figure 4). Finally, our estimates of the number of hospitalizations and deaths are associated with, but cannot be directly attributed to, *S. aureus* and MRSA because NHDS does not report the immediate cause of death, and older, sicker patients are more likely to contract a nosocomial infection (*40*).

Our findings suggest that *S. aureus* and MRSA should become a national priority for disease control. Possible responses include expanding national surveillance or reporting requirements for *S. aureus* and MRSA infections, more research to quantify the relative importance and interaction between community- and healthcare-associated colonization and infection, improved investments in hospital-infection control, and greater public investment to support research and development of an *S. aureus* vaccine.

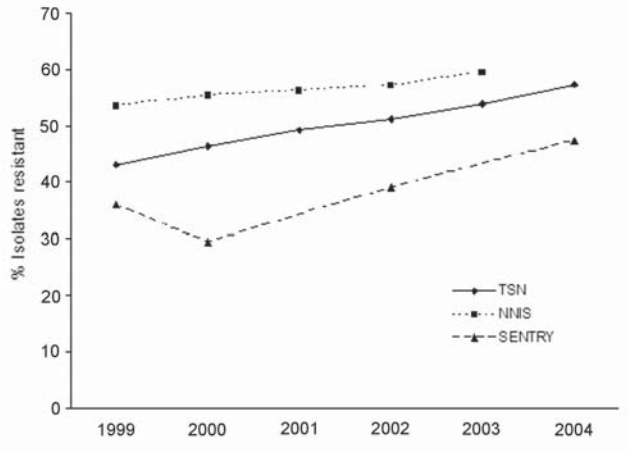

Figure 4. Percentage of *Staphylococcus aureus* isolates resistant to methicillin in national surveys, United States, 1999–2004. TSN, The Surveillance Network (data include hospital infections); NNIS, National Nosocomial Infections Surveillance System (data include only intensive care units); SENTRY, includes only skin and soft tissue infections.

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Studies of Reservoir Hosts for Marburg Virus

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To determine reservoir hosts for Marburg virus (MARV), we examined the fauna of a mine in northeastern Democratic Republic of the Congo. The mine was associated with a protracted outbreak of Marburg hemorrhagic fever during 1998–2000. We found MARV nucleic acid in 12 bats, comprising 3.0%–3.6% of 2 species of insectivorous bat and 1 species of fruit bat. We found antibody to the virus in the serum of 9.7% of 1 of the insectivorous species and in 20.5% of the fruit bat species, but attempts to isolate virus were unsuccessful.

Marburg virus (MARV) and Ebola virus, members of the family *Filoviridae,* cause outbreaks of severe hemorrhagic fever in Africa. Although humans have on occasion acquired infection from contact with tissues of diseased nonhuman primates and other mammals, the reservoir hosts of the viruses in nature remain unknown.

An outbreak of Marburg hemorrhagic fever ran a protracted course in the gold-mining village of Durba, northeastern Democratic Republic of the Congo, from October 1998 through September 2000. The outbreak involved 154 patients (48 confirmed and 106 suspected cases); the case-fatality ratio was 83% (*1*). Primary cases occurred in young male miners and spread as secondary cases to family members and, less frequently, to healthcare workers and others in the community. Most cases occurred in Durba, but a few secondary cases occurred elsewhere, including nosocomial infections in nearby Watsa village, where severely ill patients sought care. The occurrence of sporadic cases and short chains of human-to-human transmission suggested that infection had been repeatedly introduced into the human population; this suggestion was substantiated by the detection of at least 9 genetically distinct viruses circulating during the outbreak. Identical sequences of MARV were found in patients within but not across clusters of epidemiologically linked cases, although viruses with the same sequences reappeared at irregular intervals during the outbreak. Most (94%) affected miners worked underground in Goroumbwa Mine, rather than in the 7 opencast mines in the village. Cessation of the outbreak coincided with the flooding of Goroumbwa Mine. Interviews with long-term residents and healthcare workers and review of hospital records showed that a *syndrome hémorragique de Durba* [hemorrhagic syndrome of Durba] had been associated with the mine since at least 1987, and a survivor of a 1994 outbreak was found to have antibodies against MARV. The fauna of Goroumbwa Mine included bats, rodents, shrews, frogs, snakes, cockroaches, crickets, spiders, wasps, and moth flies (*1*). We present the results of virus reservoir host studies conducted during the outbreak.

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Methods

In parallel with human epidemiologic studies, visits were made to Durba in May and October 1999 to collect specimens for virus ecostudies. The ecostudies were approved by the International Scientific and Technical Committee for Marburg Hemorrhagic Fever Control, which was coordinated by the World Health Organization on behalf of the government of the Democratic Republic of the Congo. In view of the epidemiologic findings during the outbreak, emphasis was placed on the fauna of Goroumbwa Mine. Bats were caught with mist nets at mine entrances; rodents and shrews were caught live with Sherman traps within and close to the mine; and arthropods (cockroaches, crickets, spiders, wasps, and moth flies, plus streblid, nycteribiid, and mite parasites of bats) were collected by hand or with sweepnets. Vertebrates were euthanized and dissected on site. Blood samples were collected; and samples of liver, lung, spleen, kidney, testes, brain, salivary glands, and fetuses of pregnant females were preserved along with the arthropods in liquid nitrogen dry-shipping containers for transport to the National Institute for Communicable Diseases in South Africa. Extra liver samples were collected for phylogenetic studies on bats and rodents, and formalin-fixed tissue samples were kept for possible histopathologic and immunohistochemical examination. Carcasses were fixed in formalin for α-taxonomy purposes.

Vertebrate tissue and arthropod suspensions were processed and tested for filovirus nucleic acids by reverse transcription–PCR (RT-PCR) and nested PCR by using filovirus-specific large (L) protein gene primers and nested MARV-specific viral protein 35 (VP35) primers as described for samples from human patients during the outbreak (*1*). Nucleotide sequencing of amplicons and sequence data analysis were also performed as described previously (*2*), except that MEGA version 3.1 software was used (*3*). Initial RT-PCR and nested PCR were performed with pooled tissue samples of individual vertebrates; when possible, for specimens that produced positive results, all tissues were retested separately. In attempts to isolate virus as detected by indirect immunofluorescence, suspensions (≈10%) of vertebrate tissues pooled for individual animals and arthropods pooled by species were subjected to 3 serial passages in Vero 76 cell cultures. Serum samples from bats and rodents were tested for antibody to MARV by ELISA by using a modification of the technique described previously for human serum (*1*). ELISA antigen consisted of lysate of Vero cell cultures infected with the Musoke strain of MARV. Bat antibody was detected with antibat immunoglobulin–horseradish peroxidase conjugate (Bethyl, Montgomery, AL, USA) and rodent antibody with antimouse immunoglobulin conjugate (Zymed Laboratories, San Francisco, CA, USA). Net ELISA optical density values were expressed as percent positivity (PP) of a human serum sample confirmed positive for MARV and used as an internal control. Cutoff values for recording positive results were deliberately selected to be stringent at 3 × (mean + 3SD) PP values determined for stored bat (n = 188) and rodent (n = 360) serum samples that had been collected for unrelated purposes in Kruger National Park, South Africa, from 1984 through 1994, and tested at a dilution of 1:100. The Kruger bat samples were collected from 3 species of fruit bats (*Megachiroptera*) and 12 species of insectivorous bats (*Microchiroptera*), including samples from 56 *Chaerephon pumila*, 32 *Rousettus aegyptiacus*, 27 *Mops condylurus*, 16 *Hipposideros caffer,* plus 57 samples from 11 other species.

Results and Discussion

The numbers of specimens collected, plus the results of RT-PCR, nested PCR, attempts to isolate virus in cell culture, and ELISA antibody determinations, are summarized in the Table. With the exception of a *Nycteris hispida* bat, which was caught near a house in Durba, all specimens were collected within Goroumbwa Mine or its immediate surroundings. An estimated minimum of 10,000 Egyptian fruit bats (*R. aegyptiacus*) roosted in the mine, clustered within the upper galleries. Although the numbers of insectivorous bats were difficult to estimate because these bats roosted mainly in the deeper recesses of the mine, the catch rates indicated substantial numbers of the eloquent horseshoe bat (*Rhinolophus eloquens*) and the greater long-fingered bat (*Miniopterus inflatus*). Few microchiropterans were caught in May, but catch rates improved in October after adjustment of trapping hours and the gauge of mist nets used. Pregnancy was recorded in 12 (24%) of 50 *R. aegyptiacus* females in May and in 2 (4.2%) of 47 females in October; descended testes were found in 2 (6%) of 33 males in May and 19 (25%) of 76 in October. The only indication of breeding activity observed in microchiropterans was that 1/7 *Rh. eloquens* females was pregnant in May.

The L primer RT-PCR, which was applied to all specimens, produced no positive result. In contrast, the nested MARV VP35 PCR, which was applied only to specimens collected in October 1999, produced positive results on specimens from 12 bats: 1 (3.0%) of 33 *M. inflatus*, 7 (3.6%) of 197 *Rh. eloquens,* and 4 (3.1%) of 127 *R. aegyptiacus*. Nested VP35 PCR on individual tissues of the positive bats produced positive results for liver, spleen, kidney, lung, salivary gland (3/5 bats), and heart (2/5 bats). Attempts to isolate virus in cell cultures from pooled organs were uniformly negative. Applying an ELISA cutoff value of 16.4 PP, determined as 3 × (mean + 3 SD) of values recorded for 188 bat serum samples from Kruger National Park, antibody activity to MARV was detected by ELISA in 20 (9.7%) of 206 *Rh. eloquens* and in 32 (20.5%) of 156 *R. aegyptiacus* serum specimens from Durba (Table;

Table. Results from Marburg virus testing of specimens collected in Durba, northeastern Democratic Republic of the Congo, May and October 1999

Species	Total no. sampled	Marburg ELISA antibody, no. positive/no. tested (%)	Filovirus L RT-PCR and virus isolation	Marburg nested VP35 RT-PCR, no. positive/no. tested (%)
Chiroptera: Microchiroptera				
Hipposideros caffer	13	0/10	0/13	0/7
H. commersoni	17	0/16	0/17	0/13
Miniopterus inflatus	38	0/34	0/38	1/33 (3.0)
Nycteris hispida	1	0/1	0/1	0/1
Rhinolophus eloquens	222	20/206 (9.7)	0/222	7/197 (3.6)
Rh. landeri	1		0/1	
Chiroptera: Megachiroptera				
Lissonycteris angolensis	3	0/3	0/3	0/3
Rousettus aegyptiacus	230	32/156 (20.5)	0/230	4/127 (3.1)
Rodentia				
Lemniscomys striatus	10	0/10	0/10	
Lophuromys sikapusi	2	0/2	0/2	
Mastomys natalensis	4	0/4	0/4	0/1
Mus (Nannomys) minutoides	11	0/11	0/11	0/2
Praomys delectorum	14	0/14	0/14	0/4
Taterillus emini	1	0/1	0/1	0/1
Rattus norvegicus	5	0/5	0/5	0/1
Insectivora: Sorcidae (*Crocidura* spp.)	3		0/3	0/3
Amphibia: Anura (unidentified frog)	1		0/1	0/1
Arthropoda: Crustacea (unidentified crab)	4		0/4	0/4
Arthropoda: Hexapoda, Arachnida*	≈2,000		0/22†	

*Cockroaches, crickets, spiders, wasps, moth flies, streblids, nycteribiids, mites.
†Pooled specimens.

Figure 1). Prevalence of nucleic acid or antibody did not differ significantly between male and female bats or adults and juveniles (determined on the basis of body mass) or between bats collected in May and October. The only RT-PCR–positive bat that had antibody was a *Rh. eloquens* male collected in October. All other investigations produced negative results.

Phylogenetic analysis of the sequences determined for the twelve 302-nt MARV VP35 gene fragments amplified from bat specimens (GenBank accession nos. EU11794–EU118805) showed that 6 corresponded to sequences previously determined for virus isolates from humans during the epidemic (*1*), 1 corresponded to a 1975 human isolate from Zimbabwe, and the remaining 5 represented novel sequences; these last 6 variants from bats, combined with the 9 variants from humans, make a total of 15 distinct MARV sequences found to have been in circulation during the Durba epidemic (Figure 2). Although the differences observed between MARV sequences during the 1999 Durba outbreak were minor, the sequences were consistent in sequential isolates from individual patients and within groups of epidemiologically linked patients (e.g., intrafamilial transmission). In addition, phylogenetic analysis on L gene fragment sequences showed that the 33 virus isolates from patients resolved into exactly the same 9 groups as did the VP35 gene fragments of the same isolates (*1*). Nucleotide sequence divergences of up to 21% observed among the VP35 gene fragments detected in the Durba patients and bats are representative of the diversity of the complete MARV genome and encompass the entire genetic spectrum of isolates obtained over the past 40 years (*1*,*4*). This fact indicates that the virus evolves slowly and that any possible relationship with bats in the Goroumbwa Mine must have extended over a long period. The diversity of MARV sequences detected suggests compartmentalized circulation of

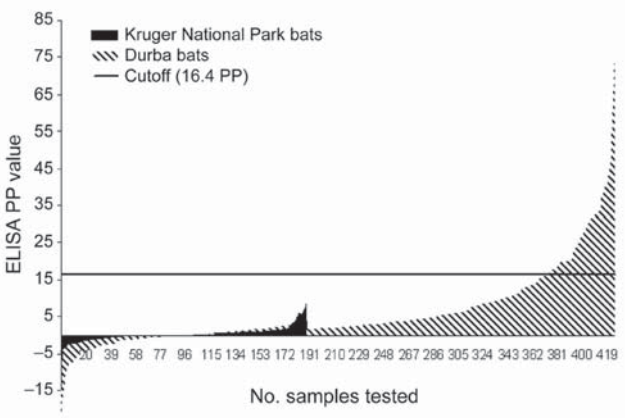

Figure 1. Marburg virus ELISA percent positivity (PP) values recorded on bat serum samples collected in 1999 in Durba, Democratic Republic of the Congo (n = 426), and from 1984 through 1994 in Kruger National Park, South Africa (n = 188). The cutoff PP value of 16.4 was fixed as 3 × (mean + 3 SD) of values observed in the Kruger National Park samples.

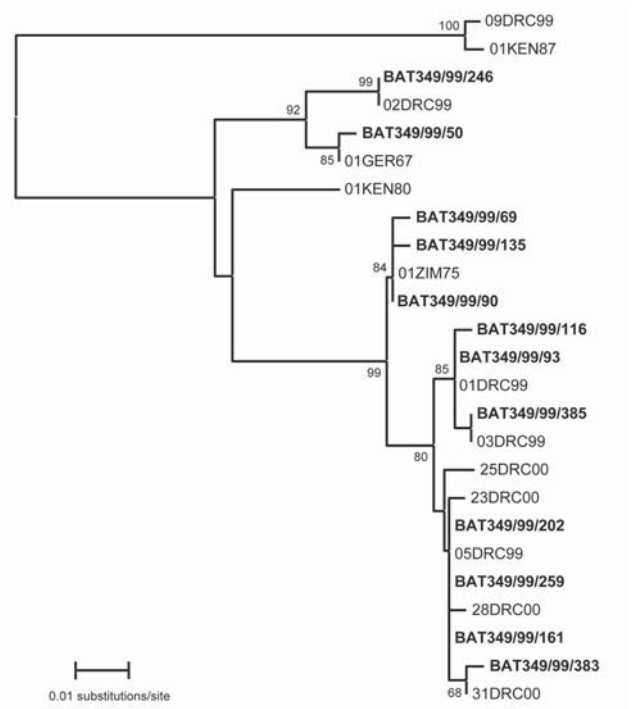

Figure 2. Phylogenetic analysis created by using a neighbor-joining algorithm (MEGA version 3.1, [*3*]) that related sequences of 302-nt fragments of Marburg viral protein 35 gene detected in 12 bats in Durba Mine (**boldface**) to sequences determined for isolates from human patients in the Durba plus previous outbreaks of the disease. Six bat-derived sequences were identical to sequences from human isolates during the outbreak; 1 corresponded to a 1975 human isolate from Zimbabwe, and the remaining 5 represented novel sequences, making a total of 15 distinct MARV sequences found to be in circulation during the Durba epidemic. Bootstrap values were determined by 500 replicates. DRC, Democratic Republic of the Congo; GER, Germany; KEN, Kenya; ZIM, Zimbabwe.

virus in bat colonies, as would occur if the species involved existed as metapopulations, spatially discrete subgroups of the same species, as opposed to panmictic populations in which there are no mating restrictions (*5*). Alternatively, bats could be intermediate hosts of the virus.

The history of filovirus outbreaks shows several instances from which it can be inferred that bats may have served as the source of infection. Anecdotal evidence indicates that during shipment from Uganda, the monkeys associated with the first outbreak of Marburg hemorrhagic fever in Europe in 1967 were kept in a holding facility on a Lake Victoria island that had large numbers of fruit bats. In the second filovirus outbreak in 1975, Marburg hemorrhagic fever developed in 2 tourists who had slept in rooms with insectivorous bats at 2 locations in Zimbabwe (*6*). In the first recognized outbreak of Ebola hemorrhagic fever in 1976, the first 6 patients had worked in a cotton factory in Sudan in which insectivorous bats were present (*7*). In 2 separate incidents in 1980 and 1987, infection with MARV was putatively linked with entry into Kitum Cave on the slopes of Mount Elgon in Kenya, where fruit and insectivorous bats are present (*8*,*9*). In 1994, a clan of chimpanzees in a forest reserve in Côte d'Ivoire had been observed feeding in a wild fig tree with fruit bats for 2 weeks before an outbreak of fatal disease, caused by a new strain of Ebola virus, occurred (*10*). The Reston strain of Ebola virus, which is apparently nonpathogenic for humans, was imported into the United States and Europe in infected monkeys from the Philippines; on each occasion, the animals came from a holding facility where they were potentially exposed to the excretions of large numbers of fruit bats (*11*).

The circumstantial evidence in the Marburg hemorrhagic fever outbreak in Durba strongly implicates Goroumbwa Mine as the source of human infection. At least 9 genetic variants of MARV circulated in humans during the outbreak. And because laboratory testing was limited to a few patients, additional variants could have been undetected, as substantiated by our evidence of 6 more variants in bats. The evolution and perpetuation of multiple genetic variants of virus in a fixed location would require a suitably large reservoir host population with constant recruitment through reproduction or migration of susceptible individuals, as generally occurs in small vertebrate and invertebrate populations such as the bat population of Goroumbwa Mine. Failure to isolate live virus may be because it was present in very low concentrations, either early or late in the course of infection. This was the first detection of filovirus nucleic acid and antibody in bats, a phenomenon which was subsequently demonstrated with Ebola virus and MARV nucleic acids and antibodies in fruit bats collected in 2002 and 2005 in Gabon, where it again proved impossible to isolate live virus (*12*,*13*).

The nature of filovirus infection in bats may vary with age and reproductive status. A seasonal pattern in the occurrence of human disease was noted over the 2 years of the epidemic in Durba; transmission began in October–November and peaked in January–February (*1*). In the caves of Mount Elgon in Kenya, Egyptian fruit bats breed in March and September; at other sites in Kenya, the timing varies markedly; no data are available for the Durba area (*14*). The remaining species of bats found in Goroumbwa Mine breed annually, but details for this location are unknown. Thus, although the reproductive status of bats differed in May and October, evidence is insufficient to establish a clear link between breeding patterns of bats in Goroumbwa Mine and the occurrence of Marburg hemorrhagic fever. Nevertheless, many examples in human and veterinary medicine indicate that the outcome of virus infection, development of carrier status, and shedding of virus are influenced by age and reproductive status, including stage of gestation at which infection occurs and the conferral to and duration of

maternal immunity in progeny (*15*). Likewise, whether insectivorous bats, fruit bats, or both, are likely to serve as the primary source of infection and whether particular species are involved with secondary transmission of infection to other species is unclear. The evolutionary distinction may exist between cave-roosting bats as hosts of MARV and forest bats as hosts of Ebola virus. Moreover, the ultimate source of infection could prove to be external, such as bat parasites or seasonally active insects in the bats' diet. Experimental infections in colonized bats could answer some of these questions (*16*).

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Invasive Group A Streptococcal Infection in Older Adults in Long-term Care Facilities and the Community, United States, 1998–2003[1]

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Limited information exists on the incidence and characteristics of invasive group A streptococcal (GAS) infections among residents of long-term care facilities (LTCFs). We reviewed cases of invasive GAS infections occurring among persons ≥65 years of age identified through active, population-based surveillance from 1998 through 2003. We identified 1,762 invasive GAS cases among persons ≥65 years, including 1,662 with known residence type (LTCF or community). Incidence of invasive GAS infection among LTCF residents compared to community-based elderly was 41.0 versus 6.9 cases per 100,000 population. LTCF case-patients were 1.5 times as likely to die from the infection as community-based case-patients (33% vs. 21%, p<0.01) but were less often hospitalized (90% vs. 95%, p<0.01). In multivariate logistic regression modeling, LTCF residence remained an independent predictor of death. Additional prevention strategies against GAS infection in this high-risk population are urgently needed.

Although group A *Streptococcus* (GAS) most commonly causes pharyngitis and soft tissue infections (*1*), it also produces severe invasive disease including bacteremia, pneumonia, necrotizing fasciitis (NF), and streptococcal toxic shock syndrome (STSS), especially at the extremes of age (*2*,*3*). In the United States, 9,000–11,000 cases and 1,100–1,800 deaths from invasive GAS infection occur each year (*3*). Those ≥65 years of age have the highest incidence and case-fatality rate: nearly a third of all cases and half of all deaths occur in this age group (*3*). In addition to advanced age, cardiac and vascular disease, diabetes, skin breakdown, corticosteroid use, and malignancy are associated with increased risk for invasive GAS infection among adults (*4*–*8*). Because underlying conditions are common among long-term care facility (LTCF) residents, this population may be especially vulnerable to invasive GAS infection. Although outbreaks of invasive GAS infections have been well described among LTCF residents (*9*–*16*), the extent and characteristics of sporadic invasive GAS infections in this population have not been well defined.

Since 1998, the Active Bacterial Core surveillance (ABCs) of the Emerging Infections Program Network (EIP)—a collaboration between the Centers for Disease Control and Prevention (CDC), state health departments, and academic centers—has collected information on residence (LTCF vs. community) of invasive GAS case-patients. We used ABCs data to compare incidence, characteristics, and factors contributing to death from invasive GAS infections of elderly LTCF residents and similar-aged persons residing in the community.

Methods

Surveillance

ABCs conducts active laboratory- and population-

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based surveillance for invasive infections due to GAS and other bacterial pathogens of public health importance. We reviewed ABCs reports of invasive GAS cases among persons ≥65 years of age occurring from January 1, 1998, through December 31, 2003, in the following sites: San Francisco, California (3 counties); Baltimore, Maryland (6 counties); Albany and Rochester, New York (15 counties); Portland, Oregon (3 counties); Chattanooga, Knoxville, Memphis, and Nashville, Tennessee (11 counties); and the entire states of Connecticut, Georgia, and Minnesota. Five counties in the Denver, Colorado, metropolitan area were added in 2000. The total surveillance area encompassed a 2000 Census population of 3,446,404 persons ≥65 years of age (10% of the total US population in this age group).

ABCs methodology has been published previously (*2*,*17*). Briefly, ABCs sites maintain active contact with clinical laboratories to identify all cases and perform audits of laboratory records at least every 6 months to ensure complete reporting. Surveillance officers review case-patient medical records to obtain information on demographic characteristics, clinical syndrome, underlying disease, and illness outcome. Case-patients with GAS-positive blood cultures but without an identifiable clinical syndrome are categorized as having bacteremia without focus. Otherwise, multiple clinical syndromes—including cases of pneumonia, cellulitis, osteomyelitis, non-skin abscesses, and other syndromes (*18*) when accompanied by a sterile site isolate—may be reported for each case. Underlying illness information (*18*) was not consistently collected in Georgia from 1998–1999, Maryland from 1998–2000, or Tennessee in 1998. Information on smoking status was collected beginning in 2000 and history of cerebrovascular accident (CVA) in 2001.

Case Definitions

ABCs defines a case of invasive GAS infection as isolation of GAS from a normally sterile site (e.g., blood, cerebrospinal fluid) or from a wound when accompanied by STSS or NF in a resident of an ABCs surveillance area. ABCs defines an LTCF as a skilled nursing facility, nursing home, rehabilitation hospital, or other chronic-care facility in which the patient has been living for at least 30 days before GAS infection. The definition did not include facilities in which the patient receives daily outpatient therapy or prisons, group homes, and assisted living facilities.

To determine whether outbreaks contributed significantly to GAS disease among LTCF residents, we looked for clusters within LTCFs. We defined a GAS LTCF cluster as ≥2 invasive infections with the same *emm* type occurring within 12 months (duration of some previously reported GAS outbreaks [*10*]) among residents ≥65 years of age living at the same facility. Surveillance staff confirmed the residence of case-patients within each cluster.

Descriptive Epidemiology

To describe incidence trends for persons ≥65 years of age (regardless of residence type) from 1998 to 2003, we analyzed GAS cases and deaths reported from 54 ABCs counties that conducted GAS surveillance during the entire 6-year period (1998 population: 1,981,662 persons ≥65 years of age). For annual rate calculations, we used national census and postcensus population estimates for these counties as the annual population denominators.

To calculate incidence of invasive GAS infection among persons ≥65 years of age stratified by residence type, we included ABCs GAS case-patients during the year 2000 and imputed cases with missing residence information on the basis of distribution of cases with known residence. For the denominator we used residence type–specific population estimates from the US Census 2000 Summary File 1 for ABCs counties (*19*); census data on residence type were only available for the year 2000. To calculate national estimates of disease, we applied age- and race-specific GAS rates from the ABCs surveillance area to the age and racial distribution of the US population in 2000; we redistributed those of unknown race on the basis of the reported distribution for known cases.

For residence-specific analyses, we excluded cases of invasive GAS infection if residence was missing or unknown. To calculate case-fatality ratios (CFRs) we included only case-patients with known outcomes.

Microbiologic Testing

ABCs sites forwarded all available GAS isolates to CDC's Streptococcal Genetics Laboratory. GAS isolates underwent T typing and amplicon restriction profiling of the *emm* gene as described at www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm (*20*). Using a reference database containing ≈180 group A streptococcal *emm* sequence types, we categorized an isolate as a given *emm* type if it had ≥92% identity over the first 30 codons encoding the processed M protein with one of the reference *emm* types (*21*).

Antimicrobial drug susceptibility testing of available GAS isolates in 1999, 2001, and 2003 was performed at CDC by using broth microdilution. To report antimicrobial susceptibility, we used established Clinical and Laboratory Standards Institute breakpoints for MICs and defined isolates with intermediate or high-level resistance as nonsusceptible (*22*).

Statistical Analysis

We used SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) for all analyses. To analyze incidence trends, we used Cochran-Armitage calculations for linearity and trend. In univariate analysis, we used Cochran-Mantel-Haenszel statistics to compare case-patient and GAS iso-

late characteristics stratified by case-patient residence; we also analyzed factors associated with death among LTCF residents and community-based case-patients separately.

We used logistic regression to characterize factors associated with death, checking for 2-way interactions and collinearity. We included in our model all variables associated with death on univariate analysis ($p<0.15$) controlling for age group, race, and sex. We stratified *emm* type into each of the 10 most common *emm* types and an 11th category including all remaining *emm* types ("other"). We classified case-patients with multiple clinical syndromes in the category with the highest CFR. The model was restricted to cases for which information on all variables was available. We considered p values <0.05 statistically significant.

Results

Disease Incidence and Estimated Disease Impact in the Elderly

From 1998 to 2003, a total of 5,889 cases of invasive GAS infection of all ages were reported, including 1,762 (30%) among persons ≥65 years of age. Incidence of invasive GAS infection in this elderly age group increased from 10.0 cases per 100,000 population in 1998 to 10.9 cases per 100,000 population in 2003 (Table 1). Type of residence was available for 1,662 elderly case-patients (94%). Of these, 383 case-patients resided in LTCFs, accounting for 23% of cases in those ≥65 years of age. In 2000 (the only year with reliable US Census population estimates for residence type), the incidence of invasive GAS among LTCF residents was almost 6 times higher than among community-based residents (41.0 vs. 6.9 cases per 100,000 persons, $p<0.01$). Projecting to the US population, we estimate that 650 cases among LTCF-residents and 2,250 cases among community-based residents ≥65 years of age occurred nationwide in 2000. Among both LTCF- and community-based residents, GAS incidence was highest among black men (78.9 and 13.8 cases per 100,000 persons, respectively) and lowest among white women (35.1 and 4.9 cases per 100,000 persons, respectively).

Demographic and Clinical Characteristics

In comparison to community-based case-patients, LTCF case-patients were older (median 83 years vs. 75 years for community case-patients, $p<0.01$) and more frequently female (Table 2). Underlying illness information was available for 1,538 (93%) case-patients. Congestive heart failure (CHF), diabetes mellitus, chronic obstructive pulmonary disease, and atherosclerotic cardiovascular disease were common in both groups. However, LTCF case-patients more frequently had CHF and a history of cerebrovascular accident but less commonly had diabetes mellitus or were current smokers than community-based case-patients. In addition, LTCF residents were less likely to have penetrating trauma preceding the infection (0.8% vs. 2.7%, $p<0.05$). Compared to community-based case-patients, LTCF case-patients more commonly had bacteremia without focus and pneumonia but less frequently had cutaneous or soft tissue infections as the possible source of the invasive GAS isolate identified (Table 3).

Isolate Characteristics

GAS was identified from blood cultures in 1,491 (90%) of the 1,662 elderly case-patients with known residence. Of the remaining 171 nonbacteremic patients, GAS was most commonly isolated from joint fluid (n = 57) and surgical specimens (n = 51). GAS was identified from multiple body sites in 125 (8%) case-patients.

GAS isolates were available in 1,414 (85%) of the 1,662 case-patients. From a total of 63 *emm* types identified, 5 (*emm1*, *emm3*, *emm12*, *emm28*, and *emm89*) accounted for most infections (57% among LTCF residents; 62% among community-based residents) (Table 4). Antimicrobial susceptibility testing was performed on 781 GAS isolates including 187 isolates from LTCF case-patients. Fourteen (7%) isolates from LTCF case-patients

Table 1. Invasive group A streptococcal infection cases and deaths among persons age ≥65 y, by site, ABCs areas, 1998–2003*

	1998	1999	2000	2001	2002	2003
No. cases/100,000 population						
CA	8.4	10.3	11.1	9.8	7.6	9.5
CT	8.7	9.4	11.3	9.8	10.2	11.5
GA	10.5	7.3	9.7	12.5	6.4	9.5
MD	13.7	9.0	9.3	15.4	11.4	15.3
MN	11.4	10.5	10.6	13.1	10.3	9.8
NY	7.7	12.6	10.3	10.2	12.9	10.2
OR	9.2	6.5	4.0	4.6	6.6	9.0
All sites	10.0	9.3	10.0	11.1	9.2	10.9
No. deaths/100,000 population						
All sites	2.2	1.9	2.3	2.2	2.2	2.6

*ABCs (Active Bacterial Core surveillance) areas: San Francisco, California (3 counties), Connecticut (entire state), Atlanta, Georgia, metropolitan area (20 counties), Baltimore, Maryland (6 counties), Minneapolis/St. Paul, Minnesota (7 counties), Rochester, New York (7 counties), and Portland, Oregon (3 counties).

Table 2. Characteristics of persons age ≥65 y with invasive group A streptococcal infection by known residence, ABCs areas, 1998–2003*

Characteristic	No. LTCF case-patients (%), n = 383	No. community-based case-patients (%), n = 1,279	p value
Age, y			<0.01
65–74	72 (18.8)	584 (45.7)	
75–84	149 (38.9)	465 (36.3)	
≥85	162 (42.3)	230 (18.0)	
Female sex	238 (62.1)	626 (48.9)	<0.01
Race†			0.16
White	282 (82.5)	914 (78.9)	
Black	50 (14.6)	182 (15.7)	
Other	10 (2.9)	63 (5.4)	
Case-fatality†	124 (32.6)	268 (21.1)	<0.01
Hospitalization†	346 (90.3)	1211 (94.8)	<0.01
Presence of underlying illnesses†			
Congestive heart failure	104 (29.3)	237 (20.5)	<0.01
Cerebrovascular accident	39 (16.8)	71 (9.4)	<0.01
Diabetes mellitus	86 (24.2)	346 (30.0)	<0.05
Current smoker	6 (2.1)	61 (6.5)	<0.01
Chronic obstructive pulmonary disease	62 (17.5)	172 (14.9)	0.24
Atherosclerotic cardiovascular disease	95 (26.7)	351 (30.4)	0.19
Renal failure/dialysis	30 (8.5)	103 (8.9)	0.78
Alcohol abuse	19 (5.4)	48 (4.2)	0.34
Immunosuppressive therapy‡	19 (5.4)	87 (7.5)	0.16

*ABCs, Active Bacterial Core surveillance;LTCF, long-term care facility. Case-patients with missing responses for residence type or individual characteristics were excluded from analysis.
†Data were not available for all case-patients. Denominators by residence varied for the following: race (LTCF 342, community 1,159), outcome (LTCF 380, community 1,270), hospitalization (LTCF 383, community 1,278), underlying illnesses (LTCF 355, community 1,154) except for cerebrovascular accident (LTCF 232, community 758) and current smoker (LTCF 285, community 936).
‡Includes steroids, chemotherapy, and radiation therapy.

and 34 (6%) from community-based case-patients were not susceptible to erythromycin (p = 0.38). Three isolates from LTCF case-patients and 5 from community case-patients were not susceptible to levofloxacin; 2 from community case-patients were not susceptible to clindamycin. No isolates were resistant to penicillin, ampicillin, cefazolin, vancomycin, or cefotaxime.

Predictors of Death

The CFR among case-patients ≥65 years of age was 24%. CFR increased with age among both LTCF- and community-based case-patients. However, when compared to the CFR for the 65- to 74-year-old group, the CFR among 75- to 84-year-old persons and those ≥85 years of age was significantly greater only among community-based case-patients (Figure). LTCF case-patients were 1.5 times as likely to die from the infection as community-based GAS case-patients (33% vs. 21%, p<0.01); however, this group was less often hospitalized (90% vs. 95%, p<0.01). CFRs among hospitalized and nonhospitalized case-patients were comparable in both LTCF (33% vs. 33%, p = 0.92) and community case-patients (21% vs. 25%, p = 0.44).

Univariate analysis of LTCF case-patients showed that those with CHF had significantly higher CFR (42%

Table 3. Clinical syndromes among persons ≥65 y with invasive group A streptococcal infection, by residence and overall CFR, ABCs areas, 1998–2003*

Clinical syndrome	No. LTCF case-patients (%), N = 383	No. community-based case-patients (%), N = 1,279	p value	Overall CFR, %
Bacteremia without focus	145 (37.9)	406 (31.7)	<0.05	25.1
Pneumonia†	97 (25.3)	225 (17.6)	<0.01	34.0
Cellulitis†	121 (31.6)	498 (38.9)	<0.01	16.3
Septic arthritis†	20 (5.2)	90 (7.0)	0.21	11.8
Osteomyelitis†	7 (1.8)	26 (2.0)	0.80	6.1
STSS	15 (3.9)	82 (6.4)	0.07	55.7
Necrotizing fasciitis	15 (3.9)	80 (6.3)	0.08	36.6
Abscess†‡	8 (2.3)	47 (3.9)	0.15	14.5

*CFR, case-fatality ratio; ABCs, Active Bacterial Core surveillance; LTCF, long-term care facility; STSS, streptococcal toxic shock syndrome. Case-patients with missing responses for residence type, outcome, or clinical syndrome were excluded from analysis. Data for case-patients could be categorized under ≥1 syndrome except for case-patients identified as having bacteremia without a focus.
†Occurring in conjunction with isolation of group A streptococcal infection from a sterile site (e.g., blood culture).
‡Data not available for all years. Denominators: LTCF 349; community 1,205.

Table 4. Most common *emm* types identified in persons ≥65 y with invasive group A streptococcal infection, by residence, ABCs areas, 1998–2003*

emm type	No. LTCF case-patients (%), N = 324	No. community-based case-patients (%), N = 1,090
1	55 (17.0)	233 (21.4)
3	44 (13.6)	141 (12.9)
28	39 (12.0)	122 (11.2)
12	21 (6.5)	116 (10.6)
89	27 (8.3)	61 (5.6)
77	9 (2.8)	39 (3.6)
6	12 (3.7)	22 (2.0)
18	6 (1.9)	28 (2.6)
11	10 (3.1)	23 (2.1)
4	11 (3.4)	21 (1.9)

*ABCs, Active Bacterial Core surveillance; LTCF, long-term care facility. Case-patients with missing responses for residence type and *emm* type were excluded from analysis. Table stratified by overall frequency.

with CHF died vs. 27% without CHF, p<0.01) as did those with infections caused by *emm1* (51% vs. 28%, p<0.01) or *emm3* (45% vs. 30%, p<0.05) when compared to other *emm* types. We also observed higher CFR among LTCF case-patients with STSS (73% vs. 31%, p<0.01), NF (64% vs. 31%, p<0.05), or pneumonia (42% vs. 30%, p<0.05) than those with other syndromes. Sex, race, and hospitalization of LTCF case-patients were not significantly associated with death. These same variables were associated with significantly higher case-fatality rates among community-based case-patients.

In the final multivariate logistic regression model, independent predictors of death included LTCF residence; lack of hospitalization; infection due to *emm1*, *emm3*, or *emm12*; disease manifesting as STSS, NF, pneumonia, or bacteremia without focus; and interaction between female sex and presence of congestive heart failure (Table 5). Age was not a significant risk factor associated with death.

Clustering of Cases

We identified 18 GAS clusters comprising a total of 40 cases (10% of LTCF cases). Fourteen clusters consisted of only 2 cases; the other 4 clusters had 3 cases each. The median interval between the first and second cases was 2.5 months (range 0.2–9.2 months). The most common *emm* types identified were *emm28* and *emm89*, which caused 4 and 3 clusters, respectively. Case-patients in clusters were of similar age (median 85.5 years), sex (68% female), and race (75% white) to overall LTCF GAS case-patients ≥65 years of age. The most common syndromes of clustered patients were cellulitis (40%) and bacteremia without focus (38%). Fifteen case-patients died (CFR 38%).

Discussion

Although the elderly have the highest rates of disease and death due to invasive GAS infection (*2*–*4*), we demonstrated that a subset of persons ≥65 years of age has an even greater risk. Invasive GAS infection was almost 6 times as likely to develop in elderly LTCF residents. Moreover, such case-patients were 1.5 times more likely to die from this infection than elderly persons living in the community. LTCF case-patients with invasive GAS infection were more likely to be older, female, have a history of CHF or CVA, and have pneumonia or bacteremia without focus compared to community-based case-patients. We found no significant differences in *emm* type distributions and antimicrobial resistance patterns among GAS isolates that caused infections in LTCF- or community-based case-patients.

The increased risk for death among elderly case-patients living in LTCFs compared to case-patients in the community remained significant on multivariate analysis and is likely attributable, in part, to the fact that LTCF residence is a proxy measure of individual frailty. While this surveillance system collects information such as age and underlying conditions, measurements of functional status such as the Karnofsky score or activities of daily living are not obtained. The common use of advanced directives among LTCF residents may also contribute to the higher CFR. Because some directives preclude aggressive clinical management, this may also explain the lower frequency of hospitalization among LTCF case-patients.

Other factors associated with higher CFR included specific *emm* types and several clinical syndromes. These findings are consistent with past studies in which disease due to *emm* types 1 and 3 as well as the clinical syndromes pneumonia or STSS were independent predictors of death among all age groups (*2*). Although advancing age has been found previously to contribute to overall case-fatality rates

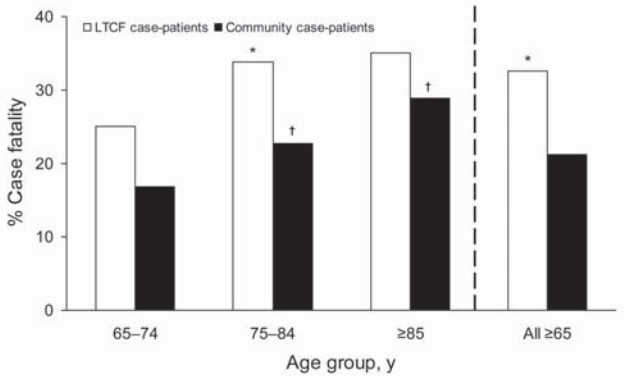

Figure. Comparison of case-fatality ratio from invasive group A streptococcal infections among persons by age group and residence, Active Bacterial Core surveillance areas, 1998–2003. Blank square, long-term care facility case-patient; black square, community-based case-patient. Case-patients with missing responses for residence type and outcomes were excluded from analysis. *p<0.05 for long-term care facility case-patients versus community-based case-patients. †p<0.05 indicates significance between the following groups: 75–84-year age group versus 65–74-year age group, or ≥85-year age group versus 65–74-year age group.

Table 5. Results of multivariate logistic regression analysis of factors associated with death from invasive group A streptococcal infection among case-patients ≥65 y of age, ABCs areas, 1998–2003*

Characteristic	Adjusted odds ratio (95% CI)
Age group, y	
≥85	1.4 (0.9–2.1)
75–84	1.2 (0.8–1.8)
65–74	Reference
Race	
Black	0.8 (0.5–1.2)
Other than black	Reference
Residence	
Long-term care facility	**1.6 (1.1–2.2)**
Community	Reference
Hospitalized	
Hospitalized	**0.5 (0.3–0.9)**
Not hospitalized	Reference
Syndrome	
Bacteremia without focus	**2.6 (1.7–3.8)**
Pneumonia	**3.7 (2.4–5.8)**
Necrotizing fasciitis	**3.6 (1.7–7.4)**
STSS	**11.1 (6.4–19.3)**
Other syndrome	Reference
emm type	
emm1	**2.3 (1.4–3.6)**
emm3	**1.9 (1.1–3.1)**
emm4	1.7 (0.6–4.5)
emm6	0.6 (0.2–2.1)
emm11	0.4 (0.1–2.0)
emm12	**1.9 (1.1–3.4)**
emm18	1.3 (0.5–3.9)
emm28	0.9 (0.5–1.7)
emm77	1.3 (0.5–3.4)
emm89	1.5 (0.8–3.0)
Other *emm* types	Reference
Sex and history of CHF†	
Females with CHF	**2.4 (1.5–3.8)**
Females without CHF	0.9 (0.7–1.4)
Males with CHF	1.2 (0.7–2.0)
Males without CHF	Reference

*ABCs, Active Bacterial Core Surveillance; CI, confidence interval; STSS, streptococcal toxic shock syndrome; CHF, congestive heart failure. A total of 1,140 case-patients with complete data were included in the final model. Significant results are shown in **boldface**.
†Interaction between sex and history of CHF.

(*2*,*23*), our analysis showed advancing age (e.g., age 75–84 years or ≥85 years) was no longer significant once presence of CHF, residence type, and *emm* type were included in the statistical model.

The true extent of severe GAS infections in the LTCF population is likely greater than our study estimates. First, ABCs identifies only culture-confirmed invasive GAS infections, limiting recognition of GAS syndromes such as cellulitis, for which cultures are not commonly obtained. Furthermore, current guidelines developed through expert opinion do not recommend obtaining blood cultures in residents of LTCFs, largely because of the low yield of blood cultures in this setting (*24*). Consequently, many LTCF practitioners do not routinely obtain blood cultures in residents with fever; residents are either treated empirically or transferred to an acute-care facility (*25*,*26*). In our analysis of hospitalized LTCF case-patients, only 8% of positive GAS cultures were obtained before the day of hospitalization. Second, ABCs surveillance personnel have noted that residence-type is not always recorded in medical records, potentially leading to misclassification of LTCF residents as community residents. However, this misclassification would also underestimate the extent of severe GAS illness in the LTCF population.

We used available data to estimate the frequency of clusters of invasive GAS infection occurring in LTCFs. Although other studies suggest that many cases of invasive GAS may represent secondary transmission (*4*,*23*,*27*), we found that only 10% of cases among LTCF residents occurred within documented clusters. This finding likely represents underreporting for several reasons: use of empiric antimicrobial agents in LTCFs for mild and moderate infections; presence of disease manifestations for which cultures are not routinely obtained (e.g., cellulitis); and absence of GAS isolates (15%) for *emm* typing, a criterion we used to define a cluster.

Nonetheless, this study augments findings from other studies that note greater frequency of invasive bacterial infections among the elderly (*27–29*). Prior analyses of invasive group B streptococcal (GBS) and *S. pneumoniae* infections found that these infections were ≈4 times more common in LTCF residents than in community-dwelling elderly (*28*,*29*), likely due to the advanced age, multiple underlying conditions, and immobility in this population (*30*). Crowded living quarters may also play a role, as clusters of invasive GAS among healthy persons living in close proximity have been reported previously (*31*,*32*). Although less prevalent within nursing homes than illnesses such as urinary tract infection, invasive GAS, GBS, and pneumococcal diseases remain substantial causes for concern given the associated illness and higher deaths with these infections, the risk for outbreaks, and emerging antimicrobial resistance.

In addition to improved LTCF infection control practices, invasive GAS infections could be prevented with the use of an effective GAS vaccine. In the past, development of a GAS vaccine targeting the M protein, a major virulence determinant, has been halted over concerns of possible induction of antibodies that cross-react with brain, joint, and cardiac tissues (*33*,*34*). However, current vaccine candidates avoid the risks for cross-reactivity (*35*,*36*). Our analysis shows that 82% and 85% of strains causing invasive disease in both LTCF and community elderly, respectively, would be covered by the 26-valent M protein-based vaccine recently tested in phase II trials. If this vaccine also induces a protective response among older adults, it could substantially benefit LTCF residents.

In conclusion, our analysis noted that all older adults, but particularly those living in LTCFs, have significantly higher rates of disease and death from invasive GAS infection. This institutionalized population represents a unique opportunity for prevention through enhanced surveillance to improve case detection and secondary disease prevention, stringent infection control measures, and annual immunization against influenza, a disease for which GAS is a known secondary infection (*14*,*16*,*23*). Finally, vaccination of this population with an effective GAS vaccine may be highly beneficial.

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Dr Thigpen works for the Division of HIV/AIDS Prevention at CDC. He developed this project to analyze national data from CDC's ABCs system to determine the incidence and case-characteristics of invasive GAS among the elderly.

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Pig Herds Free from Human Pathogenic *Yersinia enterocolitica*[1]

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Pork products are a substantial source of human yersiniosis, a foodborne disease caused by *Yersinia enterocolitica*. Thus, the ability to eliminate this agent from pig herds would be an important step in producing human pathogen–free pork. Pig herds free from *Y. enterocolitica* O:3/biovar 4 have been established and maintained. According to serologic and cultural testing results, 15 of 16 specific pathogen–free herds were free from *Y. enterocolitica* O:3/biovar 4; this closed breeding pyramid has remained free from this organism since 1996. Pig herds free from human pathogenic *Y. enterocolitica* suggest that human pathogen–free herds could be attained to provide pork free from zoonotic agents.

Yersinia enterocolitica is a major cause of foodborne disease in the industrialized world (*1*,*2*). The emergence of *Y. enterocolitica* O:3 and O:9 in Europe and Japan in the 1970s and in North America by the end of the 1980s has been characterized as an example of a global pandemic (*3*). Outbreaks of *Y. enterocolitica* O:3 have occurred among black US infants due to cross-contamination during household preparation of raw pork intestines (chitterlings) (*4*,*5*), and the main reservoir for *Y. enterocolitica* O:3 in Europe is the domestic pig population (*6*). A case–control study conducted by the US Centers for Disease Control and Prevention (CDC) and the Norwegian Institute of Public Health (NIPH) indicated pork products as a major source of yersiniosis in humans in Norway (*7*). As a result of this and other epidemiologic studies (*6*,*8*–*10*), improved slaughtering and dressing procedures of pigs (*11*,*12*) were implemented in Norwegian abattoirs in 1994. The decline in the incidence of human yersiniosis (*13*), which started in 1995, is most likely the result of these preventive measures. Among the Nordic countries, Denmark, Norway, and Sweden started to improve slaughter hygiene by implementing the plastic bag technique during 1990–1995; however, Finland did not implement this technique, which may have contributed to the higher level of human yersiniosis in this country than in the other Nordic countries (*14*).

During an outbreak in January and February 2006, 11 human cases of *Y. enterocolitica* O:9/biovar 2 infection were identified in Norway; 2 patients died and reactive arthritis developed in 1 (*15*). A case–control study and microbiologic findings indicated a processed pork product (julesylte; Christmas brawn) as the probable source. Another, smaller, family outbreak of yersiniosis occurred, caused by *Y. enterocolitica* O:3/biovar 4 in brawn and was registered in the outbreak database at NIPH in 2006 (*16*).

Most Norwegian pig production is organized in a closed breeding system in which primary nucleus-herd farms sell breeding animals to secondary multiplying-herd farms. These multiplying-herd farms sell breeding animals to conventional-herd farms (farrowing to finishing herds or young pig production). In turn, animals from young pig–production farms are sold to fattening-herd farms. These breeding pyramids are kept free from animal diseases such as sarcoptic mange, swine dysentery, and enzootic pneumonia. If successful elimination of human pathogenic *Y. enterocolitica* could be accomplished on the top levels of the breeding pyramids, prevalence of human pathogenic *Y. enterocolitica* might be lowered in the general pig population. Previously, Skjerve et al. (*17*) indicated that intervention at herd level is a possible strategy for maintenance of *Y. enterocolitica* O:3/biovar 4–free pig herds in Norway.

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[1]Findings from this investigation were presented in part at the International Pig Veterinary Science Congress, July 16–19, 2006, Copenhagen, Denmark; and 9th International Symposium on *Yersinia*, July 10–14, 2006, Lexington, Kentucky, USA.

Serologic analysis showed 182 (63.4%) of 287 herds to be positive for *Y. enterocolitica* O:3. Among the seropositive herds in this study, significantly fewer were mixed herds of piglets and fatteners (53.1%) than fattening herds (86%). Mixed herds represent a significant protective factor against infection with *Y. enterocolitica* O:3/biovar 4 because the herd is not supplemented by animals brought in from outside sources. Thus, reducing the herd prevalence of *Y. enterocolitica* O:3/biovar 4 may be possible by minimizing contact between infected and noninfected herds.

The ability to create pig herds free of human pathogenic *Y. enterocolitica* has been evaluated. We report that a specific pathogen–free (SPF) breeding pyramid with focus on animal disease can be established and maintained free from *Y. enterocolitica* O:3/biovar 4.

Material and Methods

Herds

In 1996, the first SPF nucleus herd (herd 1; 100 breeding sows) was established by hysterectomy, and the piglets were reared without contact with other pigs. In 1999, a second nucleus SPF herd (herd 2; 65 breeding sows) was established with gilts from herd 1. These 2 herds have been totally isolated from other herds, except for artificial insemination. Since 1997, 14 new SPF herds have been established with gilts from 1 or both of the above-mentioned SPF nucleus herds; each has been maintained as a closed herd (or supplemented with replacement gilts from 1 of the 2 SPF nucleus herds). Each of these 14 new SPF herds had an average of 60 animals (range 20–150). All SPF herds are housed, the water supply is potable, and pest control systems are established. Pets and wild animals cannot enter the pig house. The owner, herdsmen, veterinarians, and technicians must shower and change clothes before entering the pig housing. Many pig herds organized in the general closed breeding system have also implemented many of these preventive measures.

Testing of Pigs

Previously, Nesbakken et al. (*18*) have shown that *Y. enterocolitica* O:3/biovar 4 can be detected in different age groups of pigs by 1) serologic testing of pigs at all ages from ≈100 days, including at slaughter when the pigs are 150–180 days old; and 2) bacteriologic examination of feces from pigs of all ages from 85 days until ≈135 days. In most instances, the testing of pigs in our study has been in accordance with the conclusions of Nesbakken et al (*18*).

Collection of Blood Samples

After the original 54 samples were tested in 1996, blood samples from 30–60 pigs in herd 1 were tested for antibodies against *Y. enterocolitica* O:3 every year from 1998 through 2007, and samples from 30 pigs in herd 2 were tested each year from 2001 through 2006. Periodically, from 2002 through 2007, blood samples from 19–60 pigs from the 14 secondary SPF herds were tested (Table). Most blood samples were collected from 4- to 6-month-old fatteners or gilts. Through 2001, some samples from pigs in the 2 nucleus herds were from sows. In total, blood samples from 1,083 pigs from 16 different herds were tested for antibodies against *Y. enterocolitica* O:3.

Collection of Fecal Samples

Each herd was sampled once. In total, 286 samples were collected from 18–24 animals from each of 4 herds in 2005 and 10 herds in 2006 (Table). Fecal samples were not collected from herds 5 (the owner did not give permission) and 9 (no longer registered as an SPF herd since 2006). Fecal samples weighed 0.1–36.8 g. The average amounts per herd tested varied from an average of 0.8 g (range 0.1–3.3 g) to an average of 23 g (range 8–31 g). The fecal samples were aseptically collected from the rectum of the pigs (86–150 days of age) by use of a clean plastic glove.

Serologic Methods

Serum samples were analyzed for antibodies against *Y. enterocolitica* O:3 by using an indirect pig immunoglobulin lipopolysaccharide ELISA (*19*) at the Danish Veterinary Institute, Technical University of Denmark, Copenhagen. A basic cut-off of optical density (OD) 20% was used to maximize the specificity of the ELISA.

Isolation and Characterization of *Y. enterocolitica*

Y. enterocolitica were cultured and isolated according to the International Organization for Standardization (*20*) with modifications (*21*,*22*). Colonies characteristic for *Yersinia* were confirmed biochemically, first by selecting only lactose-negative, urease-positive colonies and later with Vitek (BioMerieux Limited, Marcy l'Etoile, France) by using the revised biogrouping scheme for *Y. enterocolitica* (*23*) as a key, and serologically for O:3 and O:9 reactivity (63501 and 63502; Sanofi Diagnostics-Pasteur, Marnes la Coquette, France).

Results and Discussion

The serologic and the bacteriologic results showed a low rate of exposure to *Y. enterocolitica* O:3/biovar 4 in the pigs from the closed SPF herds (Table). During the first 5 years, 10 of 174 blood samples from pigs in herd 1 had low levels of antibodies against *Y. enterocolitica* O:3; however, because some of these pigs were old sows, the low titers (OD >20% but <31%) are consistent with past exposure to the organism or nonspecific cross-reaction rather than active infection. Bowman et al. (*24*) report that gestating sows had the second highest prevalence of human patho-

Table. Antibodies against *Yersinia enterocolitica* O:3 in blood samples and culture of feces from pigs in a closed system of 16 SPF herds in Norway*

Herd no. (year established)	Serologic testing (1996–2007), no. pos/no. tested	Culture (2005–2006), no. pos./no. tested
1 (1996)	10/397†	0/20
2 (1999)	0/150	0/20
3 (1997)	1/61	0/21
4 (1997)	0/19	0/20
5 (1998)	0/30	NA‡
6 (1999)	0/34	0/20
7 (1999)	0/20	0/20
8 (2000)	0/60	0/20
9 (2001)	0/30	NA‡
10 (2002)	1/61	0/20
11 (2002)	0/20	0/20
12 (2003)	0/30	0/22
13 (2003)	0/51	0/18
14 (2004)	15/30	11/24§
15 (2004)	0/50	0/23
16 (2004)	0/30	0/20

*SPF, specific pathogen–free. Herds 1 and 2 are nucleus herds. Herds 3–16 were established with gilts from 1 or both of the nucleus herds. A basic cut-off of optical density of 20% was used to maximize the specificity of the ELISA.
†During the first 5 years, 10 of 174 blood samples from pigs in herd 1 had a low level of antibodies against *Y. enterocolitica* O:3 (OD >20 but <31). None of the 223 blood samples taken from pigs in this herd from 2002 through 2007 was positive. The low-positive reactions from pigs in herd 1 might have been the result of nonspecific reactions because a few of these samples were from old sows, which might have more serologic interference.
‡NA, not applicable; no culture of feces.
§Positive for *Y. enterocolitica* O:3/biovar 4.

genic *Y. enterocolitica* among the different age categories at herd level; *Y. enterocolitica* was never detected in the farrowing sows. Gürtler et al. (*25*) did not detect human pathogenic *Y. enterocolitica* among sows. However, according to these 2 reports, the sows were investigated by culture and not by serologic testing (*24*,*25*). In the past 5 years (2002–2007), none of the 223 blood samples taken from pigs in this herd has been positive for *Y. enterocolitica*. Although some of the blood samples from the 2 nucleus herds were from old sows, most were from fattening pigs at slaughter. If nucleus herd 1 had been truly positive, pigs purchased from this herd would probably have infected the other herds because this herd was at the top of the breeding pyramid. In herds 3 and 10, 1 of 61 animals was positive. When a herd has a history of infection with *Y. enterocolitica* O:3/biovar 4, antibodies are widely distributed among the animals (*17*,*18*). Accordingly, it is not likely that herds 1, 3, and 10 were infected by *Y. enterocolitica* O:3/biovar 4. The specificity of the serologic ELISA used is not fully known; false positives might appear. Only 1 of the 16 herds examined (herd 14) was classified as serologically positive for antibodies against *Y. enterocolitica* O:3. Among the 30 animals tested, 15 were positive (OD average 39%; range 0%–109%). This herd was also the only one that was positive for *Y. enterocolitica* O:3/biovar 4 according to culture result. The isolation method used in our study has proven to be sensitive for isolation of *Y. enterocolitica* O:3/biovar 4 even when the fecal samples are small (*18*,*21*). On the basis of intestinal tract content samples (n = 120), there was no statistical difference between the isolation method used in our study and the BUGS'n BEADS (Genpoint, Oslo, Norway) detection method (PCR) for virulent *Y. enterocolitica* (*21*).

According to serologic testing results, 15 of the 16 SPF herds examined were free from *Y. enterocolitica* O:3/biovar 4. The first basic nucleus herd at the top of this breeding pyramid has remained free from this pathogenic variant since the herd's establishment in 1996. A total of 13 herds were confirmed negative for *Y. enterocolitica* O:3/biovar 4 by culture of feces. Broadly, these findings show that clusters of pig herds free from *Y. enterocolitica* O:3/biovar 4 can be established and kept free from this human pathogenic variant for many years. Christensen (*26*) also documented a low level of human pathogenic *Y. enterocolitica* in 4 SPF herds examined by tonsil swabs in Denmark during 1978–1979. From 99 pigs he found only 1 isolate of *Y. enterocolitica* serovar O:3/biovar 4.

The low prevalence of human pathogenic *Y. enterocolitica* observed in the herds' immediate environment (e.g., water, rodents, flies) by Pilon et al. (*27*) suggests that the environment does not represent the main source of contamination of pigs by human pathogenic *Y. enterocolitica*. Rather, transmission is more likely from other infected pigs. Thus, mixed herds in closed health and breeding pyramids represent an important barrier against infection with *Y. enterocolitica* O:3/biovar 4. Reduction in prevalence of human pathogenic *Y. enterocolitica* at the top levels of the health and breeding pyramids may also reduce the prevalence of *Y. enterocolitica* O:3/biovar 4 in the general pig population. The meat industry could then categorize herds by serologic or bacteriologic methods and use these results in its strategy to reduce the risks for consumers. Serologic

testing is preferable to bacteriologic methods on the basis of practicality, time-saving aspects, and costs. If human pathogenic *Y. enterocolitica*–free segments of the pig population could be established, preharvest risk management might be possible by using serologic methods to categorize herds. If this experience is used in the general health and breeding pyramids of pig herds, the Norwegian meat industry could provide pork from pigs raised in herds free from human pathogenic *Y. enterocolitica*, which might be the starting point for providing human pathogen–free (HPF) pork on the market. The following facts should be considered in discussions of the possibility of establishing HPF herds: 1) <0.1% of the pigs in Norway harbor *Salmonella* (*28*); 2) the most recent case of *Trichinella* infection in pigs was in 1994 (*28*); 3) 2.6% of 1,605 pigs from 321 herds had antibodies against *Toxoplasma gondii* (*29*), and only 1.3% of the mixed herds had antibodies against *T. gondii* according to the data on which this article is based; and 4) ≈100% of the pigs harbor *Campylobacter* spp. (*21*).

Closed SPF pig herds are probably nearly free from *Salmonella, Trichinella, T. gondii,* and, according to our findings, even human pathogenic *Y. enterocolitica*. Freedom from *Campylobacter* spp. in pigs is probably impossible. However, blast chilling after the slaughtering process seems to reduce the number of *Campylobacter* spp. ≈100% (*30*; Nesbakken et al., unpub. data). Thus, in the future, pork from Norwegian SPF pig herds and even mixed herds in closed breeding pyramids might be marketed as HPF.

Another aspect to consider is the environment. Usually manure from pig farms is spread in fields and may contaminate wild animals, lakes, and rivers. Drinking water may thereby be contaminated with pathogenic *Y. enterocolitica.* This contamination has a human health aspect because one of the risk factors for human yersiniosis might be drinking water that has not been disinfected (*7*). Thus, in addition to their public health benefits, human pathogenic *Y. enterocolitica*–free herds might have a positive environmental effect.

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Swine Influenza (H3N2) Infection in a Child and Possible Community Transmission, Canada

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An influenza A virus (H3N2) of probable swine origin, designated A/Canada/1158/2006, was isolated from a 7-month-old hospitalized child who lived on a communal farm in Canada. The child recovered uneventfully. A serosurvey that used a hemagglutination-inhibition assay for A/Canada/1158/2006 was conducted on 54 of the 90 members of the farm. Seropositivity was demonstrated in the index patient, 4 of 7 household members, and 4 of 46 nonhousehold members; none had a history of hospital admission for respiratory illness in the preceding year. Serologic evidence for this strain of swine influenza was also found in 1 of 10 pigs (12 weeks–6 months of age) on the farm. Human infection with swine influenza virus is underrecognized in Canada, and because viral strains could adapt or reassort into a form that results in efficient human-to-human transmission, routine surveillance of swine workers should be considered as part of pandemic influenza preparedness.

Influenza A is endemic in a broad range of species, with avian and swine strains having the greatest potential for transmission to humans. Pandemics of influenza A occur when a major change occurs in the proteins of circulating strains of the virus. During the pandemics of the past century, this antigenic shift resulted from reassortment of human and avian strains or adaptation of avian viruses to facilitate person-to-person transmission (*1*). Avian influenza preferentially binds to sialic acid–galactose receptors with an α-2,3 linkage that is abundant on duck intestinal epithelium; human influenza preferentially binds to sialic acid–galactose receptors with an α-2,6 linkage that is abundant on human respiratory epithelium. The respiratory epithelium of swine contains both types of receptors and can potentially be simultaneously infected with avian and human influenza (*2*). Human infection with avian influenza subtype H5N1 is of great concern, with 194 deaths of 321 cases reported worldwide through August 16, 2007 (*3*). Swine infected with avian subtype H5N1 have been identified in Vietnam (*4*), raising the possibility that swine could act as the "mixing vessel" that allows avian influenza (H5N1) to reassort with a human influenza strain, resulting in a virus with high pathogenicity and a high potential for person-to-person spread.

Another theoretical mechanism for the origin of an influenza pandemic would be the adaptation of a swine strain that results in efficient person-to-person transmission, although cross-protection by antibodies to recently circulating human strains may prevent this from occurring with swine influenza virus (SIV) H1 and H3 strains. Infection of humans with SIV was first recognized in 1974 with an H1N1 strain (*5*); the solitary outbreak occurred in military recruits at Fort Dix, New Jersey, USA, in 1976 (*6*). Human infection with SIV subtype H3N2 was first described in Europe in 1993 (*7*). The first reported case of probable infection of a person in North America with a non-H1N1 subtype of SIV occurred in Ontario, Canada, in 2005 with an H3N2 strain detected in the respiratory tract of an adult with no serologic evidence of infection (*8*). We describe a case of SIV (H3N2) infection in a Canadian infant, confirmed by viral isolation and serologic testing.

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Case Report

A 7-month-old boy was admitted to the hospital on September 10, 2006, with a 3-day history of fever, rhinitis, and cough. He had had no previous contact with ill persons. The child was born at term and was hospitalized for 21 days at 5 weeks of age when he received ventilation for 6 days for pneumonia due to respiratory syncytial virus. He lived on a communal farm (90 occupants) with horses, cows, swine, sheep, dogs, cats, turkeys, geese, ducks, and chickens but had no direct contact with the animals. The swine were contained in barns and did not mix with the other animals. His household contacts did not work directly with animals, but his father occasionally spent time in the barns, and his uncle, who lived next door, worked in the swine barns.

On admission, the child was afebrile with a heart rate of 120 beats/min, respiratory rate 56/min, and oxygen saturation of 85% on room air. Diffuse wheeze was noted. Chest radiograph results were unremarkable. Direct fluorescent antibody testing on a nasopharyngeal aspirate was positive for influenza A, and the virus was isolated in rhesus monkey cell culture. The isolate was sent to the National Microbiology Laboratory for influenza subtyping as a requirement of the Canadian influenza surveillance program, where it was subsequently designated A/Canada/1158/2006. The child stayed in the hospital for 2 days and then made an uneventful recovery at home. A cough and rhinitis developed in his 19-month-old brother on the day the index patient was admitted to the hospital, but the brother was not assessed by a physician.

Methods

Antigenic Analysis

For the antigenic characterization of A/Canada/1158/2006, hemagglutination-inhibition (HI) assay was performed by using 4 hemagglutination units of virus, 0.7% v/v guinea pig erythrocytes, and postinfection fowl serum specimens for the currently circulating human strains (A/New Caledonia/20/99 [H1N1], A/Wisconsin/67/2005 [H3N2]), past circulating human strains (A/Panama/2007/99 and A/Nanchang/933/95), and swine serum for A/Swine/Texas/4199–2/98 (H3N2) treated with receptor-destroying enzyme (*9*).

Molecular Characterization

All 8 RNA segments of A/Canada/1158/2006 were amplified by reverse transcriptase–PCR (RT-PCR) and sequenced. A universal primer set for the full-length amplification of all influenza A viruses was used for the RT-PCR (*10*). Viral RNA was extracted from 100 μL of tissue culture fluid with the RNeasy Mini Kit (QIAGEN, Mississauga, Ontario, Canada). Viral RNA was amplified in a OneStep RT-PCR reaction (QIAGEN) following the manufacturer's recommendations. Briefly, 5 μL RNA was added to the RT-PCR mixture containing 2 μL QIAGEN OneStep RT-PCR enzyme mix, 10 μL 5× QIAGEN OneStep RT-PCR buffer, 400 μmol/L dNTP, 0.6 μmol/L of each primer, and 10 μL Q-solution in a final volume of 50 μL. The conditions used for the Gene Amp 97700 (Applied Biosystems, Streetsville, Ontario, Canada) thermocycler were as follows: 50°C for 30 min for reverse transcription, 95°C for 15 min for the activation of the HotStart DNA polymerase; then 35 cycles of 94°C for 20 s, 58°C for 30 s, 72°C for 4 min, followed by an extension of 10 min at 72°C. The PCR products were purified by using QIAquick PCR purification kit (QIAGEN) and sequenced on an ABI 377 Sequencer, using a fluorescent dye-terminator kit (Applied Biosystems). The DNA sequences were assembled and analyzed with SEQMAN, EDITSEQ, and MEGALIGN programs in Lasergene (DNASTAR, Madison, WI, USA). Phylogenetic trees were generated by the neighbor-joining method using the MEGA program (*11*).

Serologic Testing

Once it became evident that A/Canada/1158/2006 was closely related to swine influenza viruses, HI was performed on serum specimens collected from the index patient, the symptomatic sibling, and both parents 29 days after the hospitalization. To further investigate the spread of SIV to humans, approval was then granted by the Health Research Ethics Board of the University of Alberta to obtain information and serum specimens from other members of the communal farm. The study team visited the farm 3 months after the hospitalization of the index patient and explained the study to the occupants. Serum specimens were then collected from the other 4 siblings of the index patient and 46 other occupants who lived in a total of 17 households. Participants provided the following data: age, exposure to swine (none, <1 hour/week, or ≥1 hour/week), and history of influenza-like illnesses (ILI; defined as cough and fever) in the preceding year. Serum samples were tested by using an HI assay against the currently circulating human strains A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/2005 (H3N2), and the isolate from the index patient, A/Canada/1158/2006. HI titers were defined as the reciprocal of the highest dilution of serum that completely inhibited hemagglutination of a 0.7% solution of guinea pig erythrocytes. Specimens were considered seropositive for influenza virus at a titer of ≥32.

Swine Investigation

The purpose of these investigations was to determine the extent of recent swine influenza in swine on the farm and to look for evidence of infection with the SIV strain isolated from the index child. The history of influenza or unexpected respiratory illness in the swine on the farm was

obtained. Nasal swabs were obtained from grower pigs (4 to 16 weeks of age) and processed by RT-PCR for influenza A matrix gene. Serologic testing for influenza, using an ELISA for H1N1 and H3N2 strains and HI for A/Canada/1158/06, was performed on samples from grower-finisher pigs (12 weeks to 6 months of age). Five grower pigs that were doing poorly were killed and pulmonary autopsies were performed. All swine used in these investigations were on the farm at the time the index child was ill.

Results

Antigenic and Molecular Characterization of A/Canada/1158/06

Initial HI testing showed that the isolate was not inhibited by antiserum against recent (A/Wisconsin/77/2005 and A/New Caledonia/20/99) and past (A/Panama/2007/99 and A/Nanchang/933/95) human influenza A strains but was inhibited by antiserum against A/swine/Texas/4199–2/98 (H3N2) virus with HI titer of 128. These findings indicate that the A/Canada/1158/06 virus was antigenically related to SIV (Table 1). The results also indicate that the assay is specific because no cross-reactivity was observed between the human reference strain antiserum and the swine influenza viruses (Table 1). Nucleotide sequences of the full-length coding regions of all 8 RNA segments of the isolate further determined that it was most closely related to A/swine/Ontario/33853/2005 (H3N2) virus, which shares the same human/classic swine/avian triple reassortant genotype as the H3N2 subtype viruses that emerged in swine in the United States in 1998 (*8*). Sequence analysis showed that nucleic acid homology between A/Canada/1158/2006 and A/swine/Ontario/33853/2005 ranges from 98.4% (HA) to 100% (M1), and that amino acid (aa) identities range from 97.9% (HA) to 100% (NP, NS2, M1). A deletion of 4 aa at position 156–159 was observed in the HA1 region of the A/Canada/1158/2006 HA protein. Amino acid substitutions were found in the HA (HA1 domain: G7, K142, S162; HA2 domain: T77, Q139, M149, E150, N160), neuraminadase (NA) protein (P45, K74, N150, M349, L354), NS1 (M112), PB1 (K211, D738), PB2 (K368, S661, T722), and PA (V44, R99, I42) proteins. Phylogenetic analysis showed that all of the genes of A/Canada/1158/06 clustered with Canadian swine isolates from 2005 (*9*) (data not shown). Nucleic acid identity between the HA and NA genes of A/Canada/1158/06 and the current vaccine strain A/Wisconsin/67/05 was 90.9% and 94.6%, and the aa identities were 90.2% and 94.5%, respectively.

Serologic Testing

Seropositivity (HI titer ≥32) to A/Canada/1158/2006 was demonstrated in the index patient, the symptomatic sibling, 1 asymptomatic sibling, and both parents (Table 2, household A). Three other siblings were seronegative. Four children from 2 other households were also seropositive (Table 2, households B and C); the father from household B, 1 other child from household B, and the mother from household C were seronegative. The father from household C worked in the swine barn but was unavailable for testing. History of ILI within the preceding 12 months in seropositive participants was reported only for the index patient and for a 3-year-old girl from household C who was not hospitalized or tested for influenza virus during her illness. Seronegative results were obtained from another 20 adults (14 women and 6 men) and 19 children (8 girls and 11 boys) from 14 different households. For these households, swine exposure was reported as none for 9 adults and 7 children, <1 hour/week for 11 adults and 8 children, and ≥1 hour/week for 4 children including 3 teenagers who worked in the swine barns. When serum samples from the 54 participants in the study were tested for HA-specific antibodies to the current human influenza A virus H3N2 and H1N1 subtypes, one of the patients who was seropositive for SIV at a titer of 32 had an identical titer for A/Wisconsin/67/2005 (H3N2) (Table 2), and one of the adults who was seronegative for SIV had a titer of 32 for A/New Caledonia/20/99 (H1N1) (data not shown). All other persons tested were seronegative for the 2 human strains of influenza.

Table 1. Hemagglutination-inhibition reaction of A/Canada/1158/2006 isolates with reference antiserum against currently circulating human and swine viruses

	Antiserum (titers)				
Antigen	A/New Caledonia/20/99 (human H1N1)	A/Wisconsin/67/2005 (human H3N2)	A/Panama/2007/99 (human H3N2)	A/Nanchang/933/95 (human H3N2)	A/Swine/Texas/4199–2/98 (swine H3N2)
Control					
A/New Caledonia/20/99 (human H1N1)	320	<4	<4	<4	<4
A/Wisconsin/67/2005 (human H3N2)	<4	320	64	8	8
A/Ontario/RV1273/2005 (swine H3N2)	<4	<4	<4	<4	256
Patient					
A/Canada/1158/2006	<4	<4	<4	<4	128

Table 2. Clinical features and hemagglutination-inhibition reaction of positive antiserum from 9 members of 3 different households of a communal farm with recently circulating swine influenza (H3N2) virus A/Canada/1158/2006*

Household	Age, y	Sex	A/Wisconsin/ 67/2005 titer	A/New Caledonia/ 20/99 titer	A/Canada/ 1158/2006 titer	Swine exposure	Clinical features
A (index patient)	0.6	M	<4	<4	256	None	Hospitalization with ILI and isolation of swine influenza
A†	1	M	<4	<4	256	None	None (URI coincident with ILI in index case)
A	35	F	<4	<4	32	None	None
A	38	M	8	<4	32	<1 h/wk	None
A	8	M	<4	<4	64	<1 h/week	None
B	7	M	32	<4	32	<1 h/wk	None
C	8	M	4	<4	64	≥1 h/week	None
C	5	M	<4	<4	128	<1 h/week	
C	3	F	<4	<4	128	None	ILI 1 mo before index case

*URI, upper respiratory illness; ILI, influenza-like illness.
†Symptomatic sibling.

Swine Investigation

Influenza (H3N2) was last documented in the swine herd in September 2005. The herd received breeding animals from a Manitoba herd, where swine influenza of an unknown subtype had recently been documented. Nasal swabs collected from 25 grower pigs ≈3 weeks after the index child was ill were negative for SIV. Serum specimens obtained from 10 grower-finisher pigs were all negative by ELISA for swine influenza (H1N1), but 4 were positive for swine influenza (H3N2) strains, with 1 of these 4 strains being seropositive for A/Canada/1158/2006 by HI assay (HI titer 32). Results of the lung autopsies all showed evidence of subacute bronchointerstitial pneumonia, varying from mild to moderate. Lesions typical for swine influenza were not noted, but an initial insult due to SIV could not be excluded.

Discussion

We describe an infant with virologic and serologic evidence of infection with SIV (H3N2) and an ILI. Serologic evidence of infection with the same strain was found in 4 of 7 household members and in 3 of 46 nonhousehold contacts, with only 1 of the seropositive patients having a history of an ILI within the preceding year, which demonstrated unrecognized human infection with SIV. This relatively high seroprevalence is in contrast to a recent outbreak of avian influenza (H7N3) in which seropositivity was not documented in 91 persons exposed to infected poultry, including 2 poultry workers from whom the virus was isolated (*12*). The difference in the apparent incidence of infection may be explained in part by the fact that culling of infected poultry occurred immediately; in our study, infection of swine was not recognized and long-term human exposure may have occurred.

Infection of swine with human influenza viruses has been recognized for decades (*2*); in a recent US study, 22.8% of pigs were seropositive for human influenza viruses, although some may have had vaccine-induced immunity (*13*). Swine influenza (H3N2) emerged in 1998 in the United States, where subtype H1N1 viruses had predominated for 60 years (*2*). The isolate from this current study is closely related to triple reassorting genotype viruses that spread rapidly throughout the US swine population and have HA, NA, and RNA polymerase (PB1) genes of human influenza virus lineage; nucleoprotein, matrix, and nonstructural genes of classic swine influenza (H1N1) lineage; and RNA polymerase (PA and PB2) genes of North American avian virus lineage (*8*). However, triple reassortant SIV was not documented in swine in Canada until 2005 (*8*), which makes it unlikely that human cases occurred before that year and that seroreversion had occurred in any of the persons in the current serosurvey.

A previous study showed cross-reactivity in HI assay between the vaccine strain A/Panama/2007/99 reference antiserum and the triple reassortant A/swine/Minnesota/593/99, which is not unexpected since the HA gene of the triple reassortant viruses is a descendant of human viruses that circulated in 1995 (*14*,*15*). However, no cross-reactivity was observed between the reference human strain antiserum and the isolate from this study, which suggests that the seroconversion observed was indeed due to infection with swine influenza (H3N2) and not to cross-reactive antibody to human influenza (H3N2) infection. The low rate of seropositivity to recently circulating strains of human influenza in the study is likely explained by the fact that the farm is a relatively closed community. The child who was seropositive for both human and swine influenza viruses was likely exposed to both viruses. The HA protein of A/Canada/1158/2006 diverges significantly from the one of A/Wisconsin/67/2005, and antiserum against A/Wisconsin/67/2005 does not inhibit A/Canada/1158/2006 in HI assay.

Swine influenza (H3N2) has recently reassorted with H1N1 strains to produce H1N2 subtypes and has spread to turkeys in the United States (*16*) and Canada (*8*). A 4-aa deletion was found in the HA protein of A/Canada/1158/2006 when compared with similar swine influenza (H3N2)

strains currently circulating in North America. This region of the protein has been assigned to antigenic sites (*17*) and has been associated with adaptation to growth in eggs (*18*). Phylogenetic analysis showed that each of the 8 viral genes of A/Canada/1158/2006 clustered with A/swine/Ontario/33853/2005 (H3N2) and other swine/turkey Canadian isolates from 2005. Although the HA gene of these isolates were shown to be closely related to American viruses that were first isolated from pigs in 1999, they represent a new distinct cluster (*2*). The NA genes are phylogenetically distinct from the US swine isolates and are represented by human influenza (H3N2) isolates from Asuncion, Paraguay (2001), and New York (2003) (*2*).

A recent review described 50 cases of symptomatic human infection with SIV, documented in the literature through April 2006; 46 cases were infected with subtype H1N1 and 4 were infected with subtype H3N2 (*19*). The spectrum of pathogenicity of SIV infection ranges from asymptomatic infection (*6*) to death; 7 of these 50 patients died (*5*,*20*–*24*). Laboratory-confirmed swine influenza in humans may be "the tip of the iceberg." Diagnosis of the current case was serendipitous because typing was performed only because the case occurred outside of influenza season.

The mode of spread of SIV in humans is not established. Because of his young age, the index patient was not likely to have had unrecognized direct contact with swine. That aerosolization of influenza virus occurs is increasingly recognized (*25*), but the child was reportedly never in the barns that housed the swine. However, other members of the farm reported that infants were sometimes taken for walks through the barn. The child also may have acquired the virus from person-to-person spread or from fomites. All 13 patients in the Fort Dix outbreak and 15 of 37 previously reported civilian case-patients also had no swine contact (*19*,*20*).

The Fort Dix outbreak of SIV in humans lasted only 21 days and never spread outside the military base. The calculated basic reproductive rate (R_0) was only 1.1 to 1.2. This suggests that person-to-person spread of the implicated H1N1 strain was not efficient enough to produce a major epidemic (*26*). However, future strains of SIV could have a higher R_0, and documentation of a case of swine influenza (H3N2) in a child with unrecognized transmission within the community adds another possible mechanism by which major epidemics of influenza could arise. Swine influenza infection in humans most commonly results in either no symptoms or a self-limited illness (*6*). However, routine surveillance for cases among swine workers may enable early detection of a strain with the potential for person-to-person transmission, prompting institution of infection control measures and vaccine development.

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Swine Workers and Swine Influenza Virus Infections

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In 2004, 803 rural Iowans from the Agricultural Health Study were enrolled in a 2-year prospective study of zoonotic influenza transmission. Demographic and occupational exposure data from enrollment, 12-month, and 24-month follow-up encounters were examined for association with evidence of previous and incident influenza virus infections. When proportional odds modeling with multivariable adjustment was used, upon enrollment, swine-exposed participants (odds ratio [OR] 54.9, 95% confidence interval [CI] 13.0–232.6) and their nonswine-exposed spouses (OR 28.2, 95% CI 6.1–130.1) were found to have an increased odds of elevated antibody level to swine influenza (H1N1) virus compared with 79 nonexposed University of Iowa personnel. Further evidence of occupational swine influenza virus infections was observed through self-reported influenza-like illness data, comparisons of enrollment and follow-up serum samples, and the isolation of a reassortant swine influenza (H1N1) virus from an ill swine farmer. Study data suggest that swine workers and their nonswine-exposed spouses are at increased risk of zoonotic influenza virus infections.

Since 1997, numerous instances of avian influenza virus infection have been documented in humans (*1*). The latest of such viruses, strains of subtype H5N1, have rapidly spread among domestic bird species across several continents and caused disease in >330 humans since 2003 (*2*). Like the influenza (H5N1) viruses that are circulating today, a highly virulent avian virus subtype, H1N1, was responsible for the 1918–1919 pandemic. Coincident with the human pandemic, this virus also infected swine, caused large-scale epizootics of swine respiratory disease in the midwestern United States, and established itself among pigs as the "classical" swine influenza virus lineage of influenza (H1N1) viruses (*3*,*4*). It also apparently moved from swine to humans, causing illness among farmers (*3*). Anticipating that the next pandemic virus may similarly be readily transmitted among and between pigs and humans, we sought to prospectively study swine workers for risk factors for swine influenza virus infection.

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Methods

Study Population

After institutional review board approval, participants were recruited from the 89,658-person Agricultural Health Study (AHS) cohort (*5*) by using an informed consent process. The cohort, first assembled from 1993 through 1997, comprises primarily private pesticide applicators (predominately farmers) and their spouses living in Iowa and North Carolina. Through a stratified sampling scheme, participants living in Iowa were selected by previously reported exposures to swine or poultry, age group, sex, and proximity to the University of Iowa in Iowa City. Nonswine- and nonpoultry-exposed potential participants were similarly selected.

Potential AHS participants and their spouses were screened by telephone interviews and verified to be without immunocompromised conditions and without a history of accidental injection with swine influenza vaccines. They were then invited to participate in a 2-year prospective study of zoonotic influenza transmission. Enrollments were made through personal interviews held in 29 of the 99 counties in Iowa during the fall of 2004. After informed consent was obtained, each participant completed a questionnaire and permitted serum sample collection. Swine exposure was assessed by the participant's response to the enrollment question: "How many years have you worked

in swine production?" Participants who answered "never" were classified as nonexposed. Follow-up visits with similar questionnaires and phlebotomy were scheduled at 12 and 24 months. Upon enrollment and at 12 months, participants were given a first-class US Postal Service–ready kit with detailed instructions to complete another questionnaire and self-collect gargle and nasal swab specimens within 96 h of symptom onset if they met a case definition of influenza-like illness (fever ≥38°C and a cough or sore throat). The kit contained a freezer block that participants were asked to insert into the preaddressed shipping box before dropping off specimens and questionnaires with the US Postal Service. The US post office near the University of Iowa laboratory kept these boxes refrigerated until the study team picked them up on regular work days.

Data and serum samples from nonagricultural health study controls from a concurrent cross-sectional study (*6*) were included in population comparisons at enrollment. Study controls were generally healthy University of Iowa students, staff, and faculty who denied having swine or poultry exposures. They were not studied at 12 and 24 months after enrollment.

Laboratory Methods

Specimens

Gargle and swab specimens were transported to the University of Iowa by the US Postal Service in Micro Test M4RT Viral Transport Media (Remel, Inc., Lenexa, KS, USA) and preserved at –80°C. These specimens were studied with both culture in MDCK cells and R-Mix FreshCells (Diagnostic Hybrids, Inc., Athens, OH, USA) and with molecular techniques.

Hemagglutination-Inhibition (HI) Assay

Per our previous reports (*6,7*), serum samples were tested by using Centers for Disease Control and Prevention (CDC) HI assay protocol against 4 isolates of recently circulating swine and human influenza A viruses: A/swine/WI/238/97 (H1N1), A/swine/WI/R33F/2001 (H1N2), A/New Caledonia/20/99 (H1N1), and A/Panama/2007/99 (H3N2). Swine virus isolates were selected and provided by one of the authors (C.O.). A/swine/WI/238/97 (H1N1) is a classic swine (H1N1) virus (*8*). A/swine/WI/R33F/2001 (H1N2) is representative of reassortant (H1N2) viruses with classic swine virus HA, M, NP, and NS genes, human virus NA and PB1 genes, and avian virus PA and PB2 genes that first appeared among US pigs in 1999 (*9,10*).

The human viral strains and the A/swine/WI/238/97 swine strain were grown in embryonated chicken eggs; the A/swine/WI/R33F/2001 strain was grown in MDCK cells. Serum samples were pretreated with receptor destroying enzyme per CDC protocol. Prior to serum HI testing for the human strains, samples were hemabsorbed with guinea pig erythrocytes. A second aliquot of receptor-destroying enzyme-treated serum was hemabsorbed with turkey erythrocytes before HI testing of the swine strains. Titer results are reported as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination of a 0.65% (guinea pig) or 0.50% (turkey) solution of erythrocytes.

Molecular Studies

Real-time Reverse Transcription–PCR (RT-PCR)

RNA was extracted from 140 μL of each nasal swab and gargle sample using a QIAamp viral RNA extraction kit (QIAGEN Inc., Valencia, CA, USA) and screened by using a proprietary real-time RT-PCR protocol developed and provided by CDC. CDC's protocol is designed to first screen for influenza A, and then, through separate reactions, to rapidly determine influenza HA subtype. iScript One-Step RT-PCR Kit for Probes (Bio-Rad, Hercules, CA, USA) and the iQ Real-Time PCR Detection System (Bio-Rad) were used on a Bio-Rad iCycler real-time PCR platform for the real-time RT-PCR. Negative template controls and positive controls were included on each run. The human RNase P gene served as an internal control for human RNA. Clinical samples with negative results for the RNAse P gene were repeated. Samples positive by real-time RT-PCR for influenza A were further studied with RT-PCR and cDNA sequencing for phylogenetic analyses to confirm subtype and, in some cases, for further genotypic analyses, using previously described techniques and primers (*9–14*).

Cross-reactivity and Reliability

As we had previously identified partial serologic cross-reactivity between swine and human viral strains of the same hemagglutinin types (*6*), we adjusted for this potential confounding in each of the risk factor analyses by including human serologic results in the models. Regarding laboratory assay reliability, our previous study found 80% and 70% agreement (within 1 titer) for repeat swine influenza (H1N2) and (H1N1) virus testing, respectively (*6*).

Statistical Methods

We examined a number of potential risk factors for association with influenza virus infection outcomes: sex, age, influenza vaccination (human) history, seropositivity for human influenza viruses, years in swine production, days per week working with swine, use of personal protective equipment, recent swine exposure, number of pigs on the farm, and type of swine farm. HI test results from enrollment serum samples were first dichotomized with titers ≥40 considered as evidence of previous infection (*15,16*). The χ^2 statistic or 2-sided Fisher exact test was used to examine bivariate risk factor associations. Age was examined

by using analysis of variance. Geometric mean HI titers were calculated for each virus strain. Titer distribution was compared with potential risk factors by using the Wilcoxon rank-sum test with normal approximation. Afterwards, the distribution of antibody titer levels was examined for associations with multiple risk factors by using both unconditional logistic regression and proportional odds modeling (*17*). The score test was used to evaluate the proportional odds assumption. Final multivariable models were designed by using a saturated model including all potential risk factors and manual backwards elimination. Analyses were performed by using SAS software version 9.1 (SAS Institute, Inc., Cary, NC, USA).

We used bivariate and unconditional logistic regression to examine risk factors for evidence of influenza virus infection in 2 ways. First, using the classical approach, we examined risk factor associations for any 4-fold rise in HI titer (enrollment to 12 months, 12–24 months, or enrollment to 24 months) against the swine influenza viruses in a binary logistic regression model. Next, we examined risk factors for any increase in HI titer (using the participants' greatest increase in titers, enrollment to 12 months, 12–24 months, or enrollment to 24 months) to the swine viruses through examining the entire spectrum of HI titer increase (e.g., no increase, 2-fold rise, 4-fold rise, 6-fold rise and 8-fold rise) through proportional odds modeling. We have found the proportional odds method to have greater power to detect important risk factor associations than more commonly used binary (yes or no) outcomes (*18*).

Results

Among the 3,259 AHS persons contacted by telephone or mailing, 1,274 (39.1%) were considered eligible and were willing to participate. Among these, 803 (63.0%) attended enrollment sessions, granted informed consent, and were enrolled. After excluding 15 persons who self-reported accidental needle-stick with swine vaccine and another person with missing exposure information, 707 participants were classified as AHS swine-exposed and 80 as AHS nonswine-exposed. Enrollment data were compared with 79 nonswine-exposed University of Iowa controls (Table 1). More AHS swine-exposed participants were male than female and they also were older than those in the other 2 groups. The AHS nonswine-exposed participants were primarily women (96.3%); among these, 75.5% were spouses of AHS swine-exposed participants.

During the 24 months of follow-up, 6 of the enrolled study participants died and 4 withdrew from the study. Among the remaining 788 volunteers, 709 (90%) participated in the 12-month follow-up encounters (632 AHS swine-exposed and 77 AHS nonswine-exposed). Serum samples were drawn from 658. Similarly, among the 788 AHS participants, 714 (91%) participated in the 24-month follow-up encounter (638 AHS swine-exposed, 75 AHS nonswine exposed). Serum samples were drawn from 654. Overall, 756 (96%) of 788 persons participated in at least 1 follow-up encounter, and 726 (92.1%) consented and provided at least 2 serum specimens.

Self-Reported Exposures upon Enrollment

More than 50% of the participants reported receiving influenza vaccines during the 4 years before enrollment (Table 1). More than 90% of the AHS swine-exposed participants had worked with swine for >10 years, and 90.0% reported living on a swine farm for ≥10 years. Although AHS controls did not report direct swine exposure, 66.3% reported living on a swine farm, and 52.5% had done so for >10 years. Few participants had ever worked in the meat processing industry.

Seroprevalence Findings upon Enrollment

The distribution of HI titers against swine influenza virus subtypes H1N1 and H1N2 was different between groups. AHS swine-exposed participants had significantly higher titers against swine influenza subtypes H1N1 (geometric mean/percentage ≥40 = 9.7/12.4%, 6.5/5.0%, 5.1/0.0%) and H1N2 (geometric mean/percentage ≥40 = 12.9/20.2%, 7.5/6.3%, 5.6/1.3%), compared with AHS nonswine-exposed participants and university controls, respectively.

At enrollment, for both initial unconditional logistic regression (data not shown), and proportional odds modeling (Table 2), AHS swine-exposed and AHS nonswine-exposed participants had markedly higher distributions of antibody titers against both swine influenza viruses compared to university controls. For example, against swine influenza (H1N1), AHS swine-exposed persons had an adjusted odds ratio (OR) of 54.9. Interestingly, AHS nonswine-exposed persons also were at increased risk compared with university controls, with an adjusted OR of 28.2. Men had increased adjusted odds of elevated titers against both swine viruses compared with women. Receiving a flu shot in the past 4 years and having an antibody titer ≥40 against human influenza (H1N1) virus were important individual risk factors for elevated titers against swine influenza virus subtypes H1N1 and H1N2, respectively.

Self-Reported Exposures and Illness

Among the 726 study participants who provided serum samples in at least 1 follow-up encounter, 339 (46.7%) reported swine exposures during follow-up, 102 (14.0%) reporting never using gloves when working with animals, and 174 (24.0 %) worked with ≥400 pigs on a farm during follow-up (online Appendix Table, available from http://www.cdc.gov/EID/content/13/12/1871-appT.htm). During the 24 months of follow-up, an influenza-like illness developed in 66 participants; they submitted 74 sets of

self-collected nasal and gargle swab specimens. On average, specimens were collected within 2.8 days of symptom onset (range 0–7 days) and were received at the laboratory within 1.8 days of collection (range 1–5 days). Two of the study participants were culture positive for influenza B virus, and 22 were real-time RT-PCR and culture positive for influenza A virus. The hemagglutination genes of 21 of the 22 influenza A isolates were very similar to those from circulating human influenza (H3N2) viruses. However, complete genomic sequencing and phylogenetic analyses (data

Table 1. Characteristics of study participants at enrollment*

Variables	AHS swine-exposed, no. (%), n = 707	AHS nonswine-exposed, no. (%), n = 80	University controls, no. (%), n = 79
Sex†			
Male	455 (64.4)	3 (3.8)	26 (32.9)
Female	252 (35.6)	77 (96.3)	53 (67.1)
Age group, y			
24–45	71 (10.0)	19 (23.8)	56 (70.9)
46–54	179 (25.3)	22 (27.5)	13 (16.5)
55–89	457 (64.6)	39 (48.8)	10 (12.7)
Mean age‡	56	51.1	35.3
Received influenza vaccine in the past 4 y			
Yes	392 (55.5)	43 (53.8)	44 (55.7)
No/unsure	315 (44.6)	37 (46.3)	35 (44.3)
Swine influenza vaccine in 1976*			
Yes	62 (8.8)	4 (5.0)	1 (1.3)
No	506 (71.6)	53 (66.3)	78 (98.7)
Unsure	132 (18.7)	22 (27.5)	0
Missing	7 (1.0)	1 (1.3)	0
Currently work with nursery or finishing swine			
Nursery swine	18 (2.6)	0	–
Finishing swine	126 (17.8)	0	–
Both	168 (23.8)	0	–
No	391 (55.3)	80 (100.0)	–
Missing	4 (0.6)	0	
Years worked in swine production			
Never	0	76 (95.0)	–
<1	1 (0.1)	0	–
1–4	10 (1.4)	0	–
5–10	38 (5.4)	0	–
>10	650 (91.9)	0	–
Missing	8 (1.1)	4 (5.0)	
On average, how often do you see or touch swine, other than the swine on the farm where you work?			
Never	270 (38.2)	49 (61.3)	–
Rarely	344 (48.7)	24 (30.0)	–
Monthly	27 (3.8)	0	–
Weekly	27 (3.8)	0	–
Every day	14 (2.0)	1 (1.3)	–
Missing	25 (3.5)	6 (7.5)	–
How long have you lived on this or other swine farm?			
Never	15 (2.1)	18 (22.5)	–
<1 y	1 (0.1)	1 (1.3)	–
1–4 ys	4 (0.6)	2 (2.5)	–
5–10 y	18 (2.6)	8 (10.0)	–
>10 y	636 (90.0)	42 (52.5)	–
Missing	33 (4.7)	9 (11.3)	–
Work in a slaughterhouse or meat processing plant			
Yes	4 (0.6)	2 (2.5)	–
No	674 (95.3)	75 (93.8)	–
Missing	29 (4.1)	3 (3.8)	–

*AHS, Agricultural Health Study; AHS swine-exposed, participants from the A HS who reported working in swine production; AHS nonswine-exposed, participants from the AHS who denied ever working in swine production (96.3% female and among these females 75.5% were spouses of the AHS swine–exposed); university controls, faculty, staff, and students from the University of Iowa who denied ever working in swine production.
†Statistically significant considering a 95% confidence level by Fisher exact test for the 3 groups.
‡Statistically significant considering a 95% confidence level by analysis of variance test for the 3 groups.

Table 2. Odds ratios for elevated hemagglutination inhibition assay antibodies (enrollment sera) against swine influenza virus using proportional odds modeling

		Swine (H1N1)		Swine (H1N2)	
Variables	n	Unadjusted OR (95% CI)	Adjusted OR† (95% CI)	Unadjusted OR (95% CI)	Adjusted OR† (95% CI)
AHS swine-exposed	707	35.8 (8.7–146.8)	54.9 (13.0–232.6)	17.2 (7.9–37.7)	13.5 (6.1-29.7)
AHS nonswine-exposed	80	10.6 (2.4–47.5)	28.2 (6.1–130.1)	4.7 (1.9–11.4)	6.9 (2.8-17.2)
University controls	79	Ref	Ref	Ref	Ref
Age continuous	866	1.00 (0.99–1.01)	0.97 (0.96–0.98)	1.02 (1.01–1.03)	–
Sex					
Male	484	3.7 (2.8–4.9)	3.3 (2.4–4.5)	3.5 (2.7–4.5)	3.0 (2.3-4.0)
Female	382	Ref	Ref	Ref	Ref
Received flu shot in the past 4 y					
Yes	479	1.0 (0.8–1.3)	1.4 (1.1–1.9)	1.3 (1.0–1.7)	–
No/unsure	387	Ref	Ref	Ref	–
Human influenza (H1N1) (titer ≥40)					
Positive	347	1.1 (0.9–1.4)	–	1.6 (1.2–2.0)	1.8 (1.4-2.4)
Negative	519	Ref	–	Ref	Ref

*OR, odds ratio; CI, confidence interval; AHS, Agricultural Health Study; AHS swine-exposed, participants from the AHS who reported working in swine production; AHS nonswine-exposed, participants from the AHS who denied ever working in swine production, 94% were spouses of AHS swine-exposed; university controls, faculty, staff, and students from the University of Iowa who denied ever working in swine production.
†Final multivariable models were designed that used a saturated model including all potential risk factors (see methods) and manual backwards elimination.

not shown) of 1 isolate (A/Iowa/CEID23/05) showed that this virus was a "triple reassortant" influenza (H1N1) virus (GenBank accession nos. DQ889682-DQ889689), with H1 HA, N1 NA, M, NP, and NS genes of classic swine influenza virus lineage, PB1 gene of human influenza virus lineage, and PA and PB2 genes of avian influenza virus lineage. Viruses of this genotype emerged among US swine in the late 1990s (*19*) following prior emergence of related human/swine/avian triple reassortant H3N2 and H1N2 subtypes among American pigs (*9*–*11*,*20*,*21*).

Participant with Swine Influenza A Infection and Illness

The participant whose specimens yielded A/Iowa/CEID23/05 was a 50-year-old man who lived on a swine farm and was currently working with nursery and finishing swine. He self-reported having a sore throat, cough, runny/stuffed nose, and a measured oral temperature of 38.2°C at the time of culture. No headache, red/itchy eyes, body aches, chills, diarrhea, nausea/vomiting, or hoarseness were reported. He also reported exposure to sick swine (with symptoms of cough, runny nose, and/or poor food intake) during the 10 days before his illness. The isolation of A/Iowa/CEID23/2005, together with the prior recovery of genotypically related reassortant influenza (H1N1) and (H3N2) viruses from 2 people following apparent zoonotic transmission from pigs (*22*,*23*), indicates that viruses of human/swine/avian triple reassortant genotype can be human pathogens.

Evidence for Influenza Infections during Follow-up

Like the enrollment serum samples, the 12-month and 24-month follow-up samples showed geometric mean titers that were elevated for the AHS swine-exposed compared with the AHS nonswine-exposed participants against swine influenza (H1N1) viruses (12 months 10.05, 7.18; 24 months 16.60, 8.71) and (H1N2) (12 months 11.64, 7.84; 24 months 10.14, 7.21). Although study participants' sera were obtained at 12-month intervals and some infections were likely missed, we found considerable statistically significant evidence for recent influenza virus infection. Considering the 726 participants who donated serum at least twice and after examining each serum pair (enrollment to 12 months, 12 to 24 months, and enrollment to 24 months), 180 participants (25%) showed a ≥4-fold rise in antibodies against swine influenza (H1N1) virus, 37 (5%) against swine influenza (H1N2) virus, and 32 (4%) against human influenza (H1N1) virus at some time during the 24 months of follow-up (Table 3). There was more serologic activity against swine influenza (H1N1) during the 12- to 24-month follow-up period. However, among these same participants with rises in antibody titers, relatively few self-reported having influenza-like illness during the 24-month study period (Table 3).

After the paired serum samples were examined over time, AHS swine-exposed participants showed an increased risk for infection with swine influenza (H1N1) virus compared with AHS nonswine-exposed participants during the follow-up period (online Appendix Table; OR 2.6, 95% confidence interval [CI] 1.3–5.4). However, identifying the specific exposure during follow-up that caused this increase in risk was elusive. We examined glove use, direct swine exposure during follow-up, the number of pigs exposed to during follow-up, and the type of direct swine exposure (nursery and finishing), as well as a history of influenza (human) vaccination and serologic changes in antibodies against human H1 influenza viruses. Although there were

Table 3. Serologic evidence for influenza infections during the 24 months of follow-up

		≥4-fold increase					
		Swine influenza (H1N1)		Swine influenza (H1N2)		Human influenza (H1N1)	
Period	N	n	Reported ILI,* n (%)	n	Reported ILI,* n (%)	n	Reported ILI,* n (%)
Enrollment to 12-mo follow-up	658	26	3 (11.5)	17	7 (41.2)	10	1 (10)
12- to 24--mo follow-up	586	109	18 (16.5)	16	2 (12.5)	19	3 (15.8)
Enrollment to 24-mo follow-up	654	141	31 (22)	23	2 (8.7)	20	3 (15)
Any increase between pairs of serum samples†	726	180	38 (21.1)	37	9 (24.3)	32	4 (12.5)

*Percentage of the participants who demonstrated a ≥4-fold increase in titer who also self-reported an influenza-like illness (ILI) during follow–up.
†From enrollment to 12 mo, 12 to 24 mo, or enrollment to 24 mo, among participants who permitted serum sample collections at least 2 times during the study.

suggestions that these exposure variables were important, male sex was the strongest independent predictor of a 4-fold or any increase in titer over time. Similar analyses for increased titers against the swine influenza (H1N2) virus and stratifications of data by sex also failed to implicate a specific swine exposure as etiologic (data not shown).

Discussion

Humans, pigs, and avian species are inextricably linked in influenza transmission. The 1918, 1957, and 1968 pandemic influenza viruses all had structural components from an avian influenza virus (*24*). During the 1918 pandemic, a concomitant epizootic of swine influenza spread across the US Midwest (*4*). Numerous anecdotal accounts described influenza-like illnesses developing in farmers and their families after contact with ill swine and of swine developing symptoms of swine influenza after contact with ill farmers (*3*). Since the 1918 pandemic, human influenza viruses have infected swine (*25*,*26*) and swine influenza viruses have occasionally caused recognized disease among humans (*27*). Swine influenza transmission is known to occur nonseasonally and sporadically in the US swine population. Approximately 25%–33% of 6- to 7-month-old finishing pigs and 45% of breeding pigs have antibodies to the classic swine influenza (H1N1) virus (*28*,*29*). Anticipating that the next pandemic influenza virus may be efficiently transmitted from swine to swine and between swine and humans, we examined risk factors for previous and incident swine influenza virus infections in humans as surrogates for pandemic virus risk among those occupationally exposed to swine.

Study results suggest that swine workers are at markedly increased risk for swine influenza virus infections. Swine workers (AHS swine-exposed) had >50 times the odds of elevated antibodies against the classic swine influenza (H1N1) virus and remarkably, the AHS nonswine-exposed (mostly spouses of swine-exposed participants) also were at increased risk, with >25 times the odds of influenza (H1N1) infection compared with truly nonexposed controls (university controls). These ratios suggest that the AHS nonswine-exposed participants acquired infection either through indirect exposure to swine (e.g., handling dirty laundry or exposure to other fomites), misclassification (did not report direct contact with swine but did occasionally enter a swine barn), or exposure to their spouses who were shedding swine influenza viruses. Although the latter explanation is likely a rare event, even spouses who reported never living on a swine farm had increased odds of elevated antibody titers (data not shown). These findings should be tempered with the acknowledgment that laboratory-based evidence for human-to-human transmission of swine influenza viruses is sparse in medical literature.

Consistent with our previous report (*7*), among the significant unadjusted risk factors, we found exposure to nursery pigs was associated with an increase in antibody titer over time to swine influenza (H1N1) virus (online Appendix Table; OR 1.5, 95% CI 1.1–2.1), but being male was a stronger predictor. Among the participants who seroconverted to ≥1 of the swine viruses, <25% reported an influenza-like illness during the 2 years of follow-up, which suggested that most swine influenza virus infections are mild or subclinical. Among the 66 study participants with influenza-like illness who submitted 74 sets of gargle or nasal swab specimens through the US postal system, 22 cultures showed influenza A virus and 1 (4.5%) showed swine influenza virus.

This study has a number of limitations. Participation was voluntary, and participants might have been more likely to suffer zoonoses than their peers. Exposure data were collected through self-report, were unverified, and were subject to recall and other biases. University controls were younger than AHS participants and had substantially fewer years of life to come in contact with influenza viruses. Although age was selected in only 1 of the final multivariable models (Table 2), we checked for age difference confounding by forcing age in each of the other final multiviariate models, and the covariates presented in Tables 2 and 3 remained statistically significant (data not shown). As the study HI assays are strain dependent, a mismatch between circulating human or swine strains and those we used for the assays could have resulted in inaccurate estimates of risk.

Additionally, there was likely some confounding effect on antibodies against human influenza virus reacting in the HI assays against swine influenza virus. We attempted

to control for potential cross-reactivity through statistical adjustments. However, these and the other demographic risk factor adjustments could have been inadequate to isolate swine exposure risk factors. Further, our detection of incident influenza virus infections was suboptimal. Paired sera were collected 12 months apart, which likely permitted some influenza virus infection to be missed. Also, because of the wide dispersal of study participants, we relied upon self-identification of influenza-like illness, self-collection of nasal and gargle specimens, and shipping of specimens by the US postal system, all likely reducing the probability of identifying influenza virus infections. Even so, we detected both serologic and culture evidence of incident swine influenza virus infections. This study is unique in that a large cohort of rural farmers, many with swine exposures, were prospectively followed for influenza-like illnesses. The aggregate study data clearly documents increased occupational risk of swine influenza virus infection for these workers and their nonswine-exposed spouses.

As our study data suggest, swine influenza virus infections in humans are often mild or subclinical; however, when detected they can be quite serious. Myers et al. recently reviewed the 50 cases in the medical literature and found the overall case-fatality rate to be 14% (*27*). Human clinical morbidity and mortality rates would likely be increased if a pandemic virus's effect on rural communities were amplified by infection in swine herds. Thus, our data have important public health implications. With risk for infection so high and exposure so common, swine workers should be considered for special public health interventions (*1*). To our knowledge, there is no US national or state policy that offers swine workers priority access to annual influenza vaccines, pandemic vaccines, or influenza antivirals as part of influenza pandemic planning. These workers are also not considered a high priority for influenza surveillance efforts.

Protecting swine workers from influenza viruses will also benefit those with whom they have contact, namely family members, as well as the swine herds for which they care. Assuming an influenza virus may readily move among and between species, recent modeling studies have shown that such workers could accelerate an influenza epidemic among nonswine workers in their communities as much as 86% (*30*). Additionally, there is now extensive evidence for human influenza virus reassortment with swine and/or avian viruses in pigs (*9–11*,*19–21*,*25*,*26*). Encouraging swine workers to receive annual influenza vaccines will reduce their potential role in the genesis of novel influenza strains. Our study results corroborate the numerous arguments (*1*) that protecting swine workers from human and zoonotic influenza makes good public health sense.

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Epidemiology and Molecular Virus Characterization of Reemerging Rabies, South Africa

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The incidence of dog rabies in Limpopo Province, South Africa, increased from 5 cases in 2004 to 100 in 2006. Human rabies had last been confirmed in 1981, but investigations instituted after an index case was recognized in February 2006 identified 21 confirmed, 4 probable, and 5 possible human cases between August 5, 2005, and December 31, 2006. Twelve of these case-patients were identified retrospectively because the diagnosis of rabies was not considered: 6 of these patients consulted a traditional healer, 6 had atypical manifestations with prominent abdominal symptoms, and 6 of 7 patients tested had elevated liver enzyme activity. Molecular genetic analysis indicated that outbreak virus strains were most closely related to recent canine strains from southern Zimbabwe. Delayed recognition of the human cases may have resulted from decreased clinical suspicion after many years of effective control of the disease and the occurrence of atypical clinical presentations.

Despite the availability of effective human and animal vaccines against rabies, and other measures for its control, rabies continues to account for at least 55,000 human deaths each year, mainly in the developing countries of Africa and Asia (*1*,*2*). In these countries, most human rabies infections result from exposure to infected dogs, by bites, scratches, and mucosal exposures (*3*). Rabies vaccination of animals and postexposure prophylaxis (PEP) for humans is prohibitively expensive for most African governments, and it has long been contended that the effects of rabies are underestimated in Africa (*4*).

Typical furious rabies occurs as an encephalitis, often with characteristic features such as hydrophobia and salivation, following a brief, nonspecific, febrile prodrome. Less commonly, rabies may occur in the paralytic form in which characteristic clinical features may be absent (*3*,*5*).

Limpopo is the northernmost province in South Africa and shares borders with Zimbabwe and Botswana. To the East, Limpopo is flanked by the Kruger National Park and Mozambique (Figure 1). The climate is variable with temperate and subtropical areas, and most of the population live in rural villages and subsist by farming maize and livestock.

Since the 1970s, most human rabies cases in South Africa have occurred in KwaZulu-Natal Province, where the major animal vector is the domestic dog (*6*). Human rabies is much less common in areas such as Limpopo Province, where the major animal vectors are wild animals such as the black-backed jackal species (*Canis mesomelas),* because these animals are less likely to come into contact with humans. Before this report, the most recent 2 laboratory-confirmed human rabies cases in Limpopo Province occurred in 1980 and 1981 (R. Swanepoel, pers. comm.). We describe the epidemiologic, clinical, and viral molecular features of an outbreak of rabies in Limpopo Province, South Africa, in 2005–2006.

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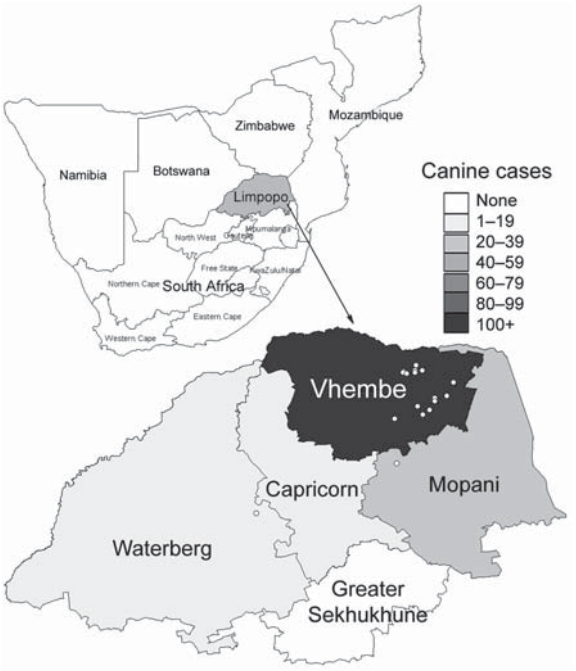

Figure 1. Provinces of South Africa, and neighboring countries. Inset shows a choropleth map of the number of confirmed dog rabies cases by district in Limpopo Province in 2005–2006 and the location of human cases (4 case-patients, for whom coordinates of place of residence were unavailable, were excluded).

Methods

Animal Rabies Surveillance

Brain specimens from all animals with suspected rabies in South Africa were submitted to the Rabies Reference Laboratory at Onderstepoort Veterinary Institute, Pretoria. Routine data collected included species, location of case-patient, and date of specimen collection. Archived data on confirmed animal rabies cases from Limpopo since January 1994 were reviewed. An animal case was defined as any case that was laboratory confirmed by fluorescent antibody test (FAT) (*7*) or virus isolation after specimen inoculation into suckling mice and monitoring for signs of rabies infection over 28 days.

Dog vaccine coverage was estimated as the number of doses of vaccine administered to dogs per year divided by the estimated dog population. A ratio of 7 persons to 1 dog was used to estimate the dog population based on unpublished survey data (E. Dyason, pers. comm.). Estimates of the human population by district were supplied by Statistics South Africa (Stats SA, Pretoria, South Africa).

Human Rabies Surveillance

Human rabies is notifiable in South Africa (Health Act No. 63, 1977); diagnostic specimens from suspected case-patients were submitted to the Special Pathogens Unit at the National Institute for Communicable Diseases, Johannesburg. The diagnosis of rabies was confirmed by using FAT on brain tissue (*7*); by using a heminested reverse transcriptase–PCR (RT-PCR) of saliva (*8*); or by isolating virus from brain, saliva, and cerebrospinal fluid (CSF) specimens as described above. Serum and CSF specimens received were tested for antirabies antibodies, and CSF specimens were tested for viral RNA by RT-PCR. Serologic testing was performed by using indirect immunofluorescence (*9*).

Epidemiologic Investigation of Human Cases

The study team visited hospitals in the outbreak area in February 2006. Potential cases of rabies (meeting the clinical case definition) in the previous 12 months were identified by clinician interviews, and prospective active surveillance was introduced for new suspected rabies cases.

Data were collected on a standardized data collection form and included demographic data, clinical and laboratory features, history of animal exposure, management of the initial bite exposure, and patient outcomes (Table). Data were obtained by review of clinical records and interview of attending clinicians. For 3 probable cases, no clinical records or laboratory results were available, and data were obtained only by interview of the attending clinician. Data on the cost and numbers of doses of vaccine and immunoglobulin distributed in Limpopo Province were obtained from relevant manufacturers.

Case Definitions

Clinical case. A clinical case-patient was defined as any person who died after January 1, 2005, and who resided in Limpopo Province before onset of illness with 1 of the following clinical symptoms—delirium, hydrophobia, salivation, acute psychosis, acute flaccid paralysis, muscle spasms, convulsion or respiratory paralysis—and with no other identified cause of death.

Possible case. A possible case-patient was defined as a person who met the clinical case definition, but whose case was not laboratory confirmed, and who had no documented history of animal exposure.

Probable case. A probable case-patient was defined as a person who met the clinical case definition, but whose case was not laboratory confirmed, and who had history of exposure to a suspected rabid animal.

Confirmed case. A confirmed case-patient was defined as a person who met the clinical case definition and had laboratory-confirmed rabies.

Molecular Analysis of Viruses Obtained from Animal and Human Rabies Case-Patients

After viral RNA underwent extraction and RT-PCR (*10*,*11*), the amplicons obtained were purified with a com-

Table. Clinical and laboratory features of confirmed, probable, and possible human rabies cases, Limpopo Province, South Africa, 2005–2006

Characteristic	No. confirmed cases/total (%)	No. probable cases/total (%)	No. possible cases/total (%)	Total
Clinical features				
Hypersalivation	19/21 (90)	4/4 (100)	1/2 (50)	24/27 (88)
Agitation	14/21 (67)	2/4 (50)	0/2 (0)	16/27 (59)
Weakness or paralysis	14/21 (67)	3/4 (75)	2/2 (100)	19/27 (70)
Fever	14/21 (67)	1/4 (25)	1/2 (50)	16/27 (59)
Hallucinations	11/21 (52)	4/4 (100)	1/2 (50)	16/27 (59)
Confusion	12/21 (57)	0/4 (0)	1/2 (50)	13/27 (48)
Hydrophobia	8/21 (38)	1/4 (25)	0/2 (0)	9/27 (33)
Alternating lucidity and confusion	8/21 (38)	0/4 (0)	1/2 (50)	9/27 (33)
Aggression	6/21 (29)	1/4 (25)	0/2 (0)	7/27 (26)
Vomiting	6/21 (29)	1/4 (25)	1/2 (50)	8/27 (30)
Spasms	5/21 (24)	0/4 (0)	0/2 (0)	5/27 (19)
Convulsions	5/21 (24)	0/4 (0)	1/2 (50)	6/27 (22)
Abdominal distension	4/21 (19)	1/4 (25)	0/2 (0)	5/27 (19)
Pain at the bite site	4/21 (19)	0/4 (0)	1/2 (50)	5/27 (19)
Diarrhea	2/21 (10)	1/4 (25)	0/2 (0)	3/27 (11)
Insomnia	2/21 (10)	1/4 (25)	0/2 (0)	3/27 (11)
Laboratory results				
Elevated leukocyte count (>10 x 10^9 cells/L)	9/19 (47)	4/4 (100)	0/2 (0)	13/25 (50)
Elevated urea (>7 mmol/L)	11/19 (61)	2/4 (50)	0/2 (0)	13/25 (50)
Elevated creatinine (>100 μmol/L)	1/19 (5)	1/4 (25)	0/2 (0)	2/25 (1)
Elevated total bilirubin (>21 μmol/L)	0/5 (0)	0/4 (0)	0/1 (0)	0/10 (0)
Elevated conjugated bilirubin (>6 μmol/L)	0/5 (0)	0/4 (0)	0/1 (0)	0/10 (0)
Elevated alkaline phosphatase (>120 IU/L)	3/5 (60)	3/4 (75)	1/1 (100)	7/10 (70)
Elevated gamma glucosyl transferase (>35 IU/L)	2/5 (40)	0/4 (0)	0/1 (0)	2/10 (20)
Elevated alanine transaminase (>40 IU/L)	1/5 (20)	2/4 (50)	0/1 (0)	3/10 (30)
Elevated aspartate transaminase (>40 IU/L)	2/5 (40)	2/4 (50)	0/1 (0)	4/10 (40)

mercial kit (Wizard SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA) and sequenced bidirectionally on an ABI377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) with the G/L primer set. A 592-bp nucleotide portion of the cytoplasmic domain of the glycoprotein and the G-L intergenic region of the virus isolates included in the study sample were aligned in ClustalW (*12*). A phylogenetic tree was constructed with the neighbor-joining method (*13*) in MEGA (Molecular Evolutionary Genetics Analysis) software version 2.1 (*14*), and 1,000 replications. The phylogenetic tree was visualized with TreeView (*15*).

All available isolates from humans and a panel of dog rabies virus isolates from Vhembe were selected (online Appendix Table, available from www.cdc.gov/EID/content/13/12/1879-appT.htm). Virus isolates from other provinces in South Africa and neighboring countries were also included in phylogenetic reconstruction of the molecular epidemiology.

Results

Animal Rabies Cases

From 1994 through 2004, 8 to 76 laboratory-confirmed animal rabies cases were identified from Limpopo Province annually. Most of these cases were in *C. mesomelas* (black-backed jackal) and in livestock (mainly cattle) (Figure 2). *C. mesomelas* case numbers increased to 12 in 2005 and 16 in 2006.

Fewer than 10 rabies cases per year were reported from Limpopo in domestic dogs (*Canis familiaris*) from 1994 through 2004 (Figure 2). The number of laboratory-confirmed dog rabies cases increased markedly from 5 in 2004 to 35 in 2005 and 100 in 2006 (Figure 3). Most dog cases (106/135, 79%) in 2005 and 2006 came from the Vhembe District. The mean estimated dog vaccination coverage in Vhembe District from 1997 through 2005 was 39%; annual coverage estimates fluctuated but ranged from 4% to 60% (E. Dyason, pers. comm.).

Human Rabies Cases

Detection of the Outbreak

Rabies was confirmed by RT-PCR testing on a saliva specimen from the index case-patient, a 10-year-old boy who was brought to the hospital on February 7, 2006 (Figure 3). Twelve patients with encephalitis that met the case definition were identified retrospectively, and rabies was confirmed by IFA for 2 of these patients for which brain tissue was available and by RT-PCR of saliva for a further

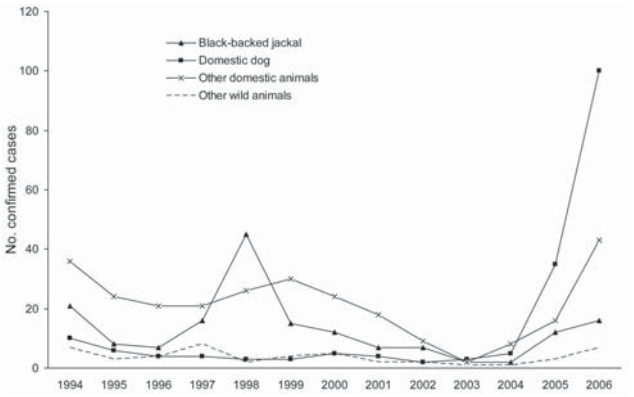

Figure 2. Laboratory-confirmed animal rabies cases, Limpopo Province, South Africa, 1994–2006.

2 patients. The earliest identified case-patient was a 9-year-old boy who was admitted in August 2005 with a history of dog bite. An admission CSF specimen, submitted for rabies diagnosis, was found to be negative by both RT-PCR and antibody testing.

Description of the Outbreak

From January 1, 2005, through December 31, 2006, 21 confirmed, 4 probable, and 5 possible human rabies cases were identified (Figure 3). The earliest identified case-patient became ill on August 5, 2005. The numbers of confirmed cases peaked in March 2006. Case numbers decreased from May 2006, but 1 to 2 cases per month continued to be reported until December 31. Of the 30 case-patients, 28 were from the Vhembe District (Figure 1).

Twenty-seven cases were in children 3–12 years of age (median 9 years). All case-patients were hospitalized. The median duration from admission to death was 4 days (range 1–25 days). All 4 patients who survived >10 days were admitted to intensive care units.

Clinical and Laboratory Features of Human Cases

The median incubation period was 8 weeks (range 3–28 weeks) for the 22 case-patients for whom the date of exposure was known. The most common clinical feature observed in patients with confirmed cases was salivation (19/21, 90%), followed by agitation (14/21, 67%), weakness (14/21, 67%), fever (14/21, 67%), and hallucinations (11/21, 52%) (Table).

The median period between when a person first experienced illness and when the person sought healthcare was 2 days (range 0–8 days) in the 19 patients for whom date of onset of symptoms was available. Lumbar puncture was performed on 14 patients. CSF findings were within normal limits for all 11 patients who did not have blood in the CSF specimen. Nine of 19 patients tested (47%) had an elevated leukocyte count (>10 $\times 10^9$/L), and 11 (61%) of 18 had elevated urea levels (>7 mmol/L). All 4 patients tested for HIV were HIV seronegative. No abnormalities were detected in hemoglobin level, platelet count, or erythrocyte sedimentation rate in any of the 19 patients.

The cluster of 11 case-patients who sought treatment before the index case-patient was identified (excluding the primary case-patient in whom rabies was suspected) was reviewed separately to identify possible reasons for the delayed diagnosis of rabies. Of these patients, 6 reported having consulted a traditional healer before visiting the clinic. Clinical and laboratory data were available for 8 patients: 6 exhibited prominent abdominal symptoms (including abdominal distension in 4, vomiting in 3, and diarrhea in 2), 3 of whom reported consulting a traditional healer. Liver function tests were performed for 7 case-patients; of these, 6 had elevated alkaline phosphatase enzyme levels (>120 IU/L). Clinicians' differential diagnoses of these cases included viral encephalitis, typhoid, pyrexia of unknown origin, epilepsy, panic attacks, poisoning or toxin exposure, and Guillain-Barré syndrome. Five patients were not asked about possible animal exposures.

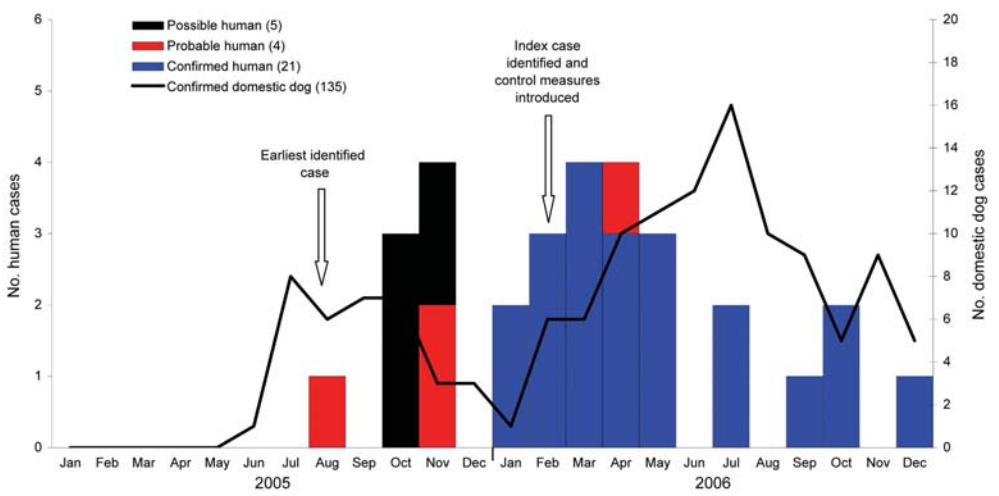

Figure 3. Numbers of possible, probable, and confirmed human cases and laboratory-confirmed domestic dog rabies cases by month of diagnosis, Limpopo Province, South Africa, 2005–2006.

For 7 case-patients reported in this outbreak, specimens were not submitted for rabies diagnosis. For all 21 confirmed case-patients, saliva specimens were positive by RT-PCR; for 7 of these case-patients, brain tissue specimens sampled on postmortem examination were also positive by IFA, and for 3 unvaccinated patients, serum specimens were positive for rabies antibodies. Virus isolation was attempted on 7 saliva specimens and was successful for 5. No virus isolation was attempted on brain specimens because all were received in formalin, despite guidelines for submission of samples in glycerol saline. All 7 CSF specimens were collected during the first week of illness and were negative for antirabies antibodies and by RT-PCR. A saliva specimen from 1 patient with typical rabies symptoms, who had been bitten by a dog with suspected rabies, tested negative by RT-PCR. He was classified as a probable case-patient; postmortem brain tissue could not be obtained.

Management of Exposures

All 24 case-patients who were asked about a history of animal exposure reported an exposure to a potentially rabid dog. All documented exposures were category 3 (high-risk) exposures, i.e., a bite or scratch that drew blood or a lick to mucous membranes or broken skin. Most patients (20/24, 83%) reported a bite, but 3 patients reported scratches only, and 1 reported that the dog had licked and nibbled at mucous membranes. For 22 case-patients with a known date of exposure, 15 (68%) exposures had occurred before the outbreak was identified and control measures were implemented.

Of 16 case-patients for whom site of exposure was reported, half of the exposures (8) were on the lower limb, but exposures were also reported to the upper limb (3), trunk (2), and head and neck (3). Most exposures were to unknown dogs, but 5 of 20 case-patients reported exposure to their own dog. Of the 18 case-patients able to give a history of the management of the original bite exposure, 12 (67%) did not report to a clinic at the time of exposure. All 6 case-patients who sought treatment at clinics received wound cleaning, but only 2 were vaccinated (1 received only 1 dose).

One case-patient, a 4-year-old boy who had been bitten on the left cheek by a dog on September 6, 2006, received antirabies immunoglobulin (Rabigam, National Bioproducts Institute, Pinetown, South Africa) in addition to vaccination with Verorab (Sanofi Pasteur, Lyon, France) within 12 hours of exposure. Details of wound cleaning are unclear, although the wound was not sutured. The patient received antirabies immunoglobulin at the recommended dose of 20 IU/kg, half injected into the wound site and half injected into the deltoid muscle, and rabies vaccine administered into the deltoid muscle on days 0, 3, 7, and 14. Whether this was the deltoid opposite to that used for the immunoglobulin was not known. Rabies developed in the patient on September 23, 2006 (17 days after exposure) and was confirmed by RT-PCR of brain tissue; the patient died on September 25, 2006. Vaccine and immunoglobulin batches were found to meet required potency standards (Z. Goondiwala, Sanofi Pasteur, pers. comm.; C. Rochat, National Bioproducts Institute, pers. comm.).

Figure 4. Neighbor-joining tree of canid rabies viruses from humans and animals from Limpopo (LP), Mpumalanga (MP), North West (NW), Free State (FS), Eastern Cape (EC), Northern Cape (NC), KwaZulu-Natal (KZN), and Western Cape (WC) Provinces of South Africa (SA) and neighboring countries of Zimbabwe, Mozambique, and Namibia. The Pasteur virus strain (PV) was used as the reference strain in the sequence alignment. Horizontal scales represent the evolutionary distance; vertical lines are for clarification purposes only. The scale bar indicates nucleotide substitutions per site. Viruses are identified by a laboratory reference number, source animal, locality of origin, and year of isolation. A–F represent virus lineages supported by bootstrap values of >70%; sublineages are indicated numerically. *Identical strains.

Molecular Epidemiology

Phylogenetic analysis of nucleotide sequences indicated that the viruses originating from humans in the Vhembe area of Limpopo were genetically indistinguishable from those obtained from domestic dogs in the same geographic area (Figure 4). Notably, this cluster represented a new phylogenetic group not previously encountered in Limpopo Province (*16*) and clearly distinct from the viruses isolated from *C. mesomelas* from Limpopo. Outbreak viruses were most closely related to viruses obtained from dogs and jackals across the border in southern Zimbabwe (sublineage A1). A second closely related sublineage (A2) was composed of viruses from southeastern Zimbabwe and western Mozambique, which suggests that a dog rabies cycle exists within South Africa, Zimbabwe, and Mozambique. The inclusion and analysis of rabies virus isolates from other provinces of South Africa and neighboring countries did not suggest any close link with the outbreak viruses.

Control Measures

Central-point dog vaccination campaigns in villages in the affected area were intensified after identification of the increased numbers of rabies cases in domestic dogs. A community awareness program related to the hazards of dog bites and the importance of timely visits to the clinic for rabies postexposure prophylaxis was established in February 2006. Furthermore, healthcare workers were educated regarding appropriate management of dog bites. Vaccine and immunoglobulin availability was improved by increasing the number of facilities providing the vaccine and by ensuring that patients did not have to pay for treatment. Registers of dog bite cases were implemented in clinics that did not have existing registers in March 2006. All registering staff emphasized the importance of documentation and follow-up for those not returning for all scheduled doses of rabies vaccine.

The combined number of doses of human rabies vaccine (human diploid cell [Mérieux Inactivated Rabies Vaccine, Aventis Sanofi, Lyon, France], purified Vero cell vaccine [Verorab, Aventis Pasteur], and inactivated chick embryo vaccine [Rabipor, Biovac, Johannesburg, South Africa]) used in Limpopo Province in the public sector increased from 3,000 in 2004 to 6,000 in 2005 and 56,000 in 2006 (R. Watson, Biovac, pers. comm.). Use of antirabies immunoglobulin (Rabigam, National Bioproducts Institute, Pinetown, South Africa) also increased over the same period with ≈100 doses given in 2004, increasing to 500 in 2005 and 2,500 in 2006 (C. Rochat, National Bioproducts Institute, pers. comm.). At a cost of 130 South African rand (R130; US $18) per vaccine dose and R300 (US $43) per immunoglobulin dose, total cost for biologics alone is estimated at 8 million R (≈US $1.1 million) for the year 2006. This figure would be substantially higher with the inclusion of patient costs and other indirect costs.

Discussion

We describe an outbreak of human rabies in a province of South Africa where rabies had been well controlled for >10 years. Late recognition of this outbreak resulted in delayed implementation of control measures. Although the clinical features of classic rabies have been described as unmistakable (*5*), the diagnosis may be missed due to low index of suspicion and variable clinical features (*17*), as occurred in this outbreak. Cases of rabies may be incorrectly attributed to other causes of pyrexia and confusion common to rural Africa, including cerebral malaria, bacterial infections, and infection with HIV (*18*,*19*).

In this outbreak, the clinical signs and symptoms of the initial case-patients may have been altered due to use of traditional medicines. Of 12 case-patients in whom the diagnosis of rabies was missed, 6 reported having visited a traditional healer before seeking treatment at a hospital. The use of traditional medicines is common in rural settings in South Africa (*20*,*21*) and may result in toxicities, including abdominal and psychiatric symptoms and abnormal liver function test results (*22*). These medicines could have contributed to the atypical manifestations in some cases. In addition, clinicians may have attributed some of the neurologic symptoms to herbal intoxication.

Nevertheless, rabies was in fact suspected in the primary case-patient, identified in August 2005. The diagnosis was not, however, confirmed because an inappropriate specimen (a CSF specimen taken on admission) was submitted. Anti-rabies antibodies in the CSF are not usually detected <1 week after the onset of clinical illness, and RT-PCR results for rabies RNA on CSF may be negative in rabies cases; thus, a negative CSF result does not exclude the diagnosis of rabies (*17*,*18*,*23*). It is therefore recommended that repeated saliva and serum specimens be submitted in addition to CSF and that a postmortem brain specimen be actively sought in all suspected rabies cases (*18*).

Four case-patients who sought treatment at a clinic before identification of the outbreak were not offered PEP, probably because the risk for rabies infection was not considered. Our case series includes 1 child in whom rabies developed despite the administration of seemingly adequate PEP. Possible contributing factors to the development of rabies in this case include the facial location of the wound, possible inadequate wound cleansing, and the fact that all of the immunoglobulin could not be infiltrated into the wound site. The full dose of immunoglobulin should be administered on the first day of PEP and should be infiltrated into the wound (*24*,*25*).

Rabies of the canid biotype has been endemic in *C. mesomelas* in Limpopo Province since the 1950s, with occasional spillover to cattle and domestic dogs. Since 1952, several attempts at control have been made, including destroying ≈22,000 dogs in that year, poisoning an estimated

3,900 jackals from 1951 through 1956, and vaccinating 181,414 dogs from 1952 through 1962 (*6*). Despite these efforts, a low incidence of dog rabies was observed in the province in the 1960s. Rabies became a serious problem again in cattle and jackals in the mid 1970s, likely following its reintroduction from Zimbabwe in 1974, and it has remained endemic in jackals with sporadic cases occurring in domestic dogs (*6*).

As in a classic situation, this outbreak in humans followed an outbreak in domestic dogs of the region. Increasing numbers of human rabies cases in Africa have been attributed to increasing numbers in animals, to the mobility of human and animal populations, and to deteriorating infrastructure and resources for rabies control (*4*,*26*). Reasons for the reemergence of canine rabies in Limpopo after many years of effective disease control are unclear. In Zimbabwe, dog rabies cases increased after 1990, after declining vaccination coverage associated with decreased resources and diversion of resources (*27*). Low vaccination coverage in domestic dogs in Limpopo over several years may have led to an accumulation of susceptible animals, which led to the reestablishment of transmission.

The reintroduction of canine rabies into northern KwaZulu-Natal Province in 1976 followed an influx of refugees from Mozambique (*6*). The possible contribution of increased immigration into Limpopo Province from Zimbabwe in recent years is difficult to quantify (*28*). Molecular genetic analysis indicates that the virus isolates from both humans and dogs in this outbreak were most closely related to those from southern Zimbabwe. This finding suggests that the outbreak may have extended across the border from Zimbabwe.

The number of human rabies cases in Limpopo Province decreased after May 2006; no further human cases had occurred as of June 30, 2007. This decrease is likely due to the introduction of coordinated control measures (including aggressive PEP). Although highly effective if administered correctly, PEP is much more costly than vaccination of domestic dogs (*29*,*30*). Unfortunately, dog vaccination is difficult in many developing countries because of high dog turnover rates, shortages of funding and personnel, and competing priorities (*26*,*31*).

The number of reported human rabies cases, particularly in Africa, greatly underestimates the true effects of the disease. Contributing factors include failure to seek treatment at healthcare facilities, failure to make a laboratory diagnosis, and failure to report the disease (*2*,*32*). Our attempts to conduct active case finding through clinician interviews at hospitals in Vhembe District encountered several problems. First, we were unable to review all hospital admissions records because of incomplete record keeping. We also recognize that at least some infected persons may not have visited hospitals and died at home. In addition, epidemiologic data were not available for all cases since several cases were identified retrospectively. An increased awareness of rabies after interventions for control may have contributed to increased case reporting after February 2006; this situation may have affected apparent trends in human case numbers and contributed to the delay in observed decline in dog cases.

This outbreak highlights the fact that rabies is a transboundary disease and can reemerge in areas where successful control programs have been active for many years. Clinicians should consider rabies in the differential diagnosis, especially in cases of fatal encephalitis and submit appropriate specimens for rabies diagnosis. Sustained awareness, together with political and economic commitment to animal and human rabies control programs, particularly the vaccination of dogs, is essential.

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Phenotypic Similarity of Transmissible Mink Encephalopathy in Cattle and L-type Bovine Spongiform Encephalopathy in a Mouse Model

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Transmissible mink encepholapathy (TME) is a foodborne transmissible spongiform encephalopathy (TSE) of ranch-raised mink; infection with a ruminant TSE has been proposed as the cause, but the precise origin of TME is unknown. To compare the phenotypes of each TSE, bovine-passaged TME isolate and 3 distinct natural bovine spongiform encephalopathy (BSE) agents (typical BSE, H-type BSE, and L-type BSE) were inoculated into an ovine transgenic mouse line (TgOvPrP4). Transgenic mice were susceptible to infection with bovine-passaged TME, typical BSE, and L-type BSE but not to H-type BSE. Based on survival periods, brain lesions profiles, disease-associated prion protein brain distribution, and biochemical properties of protease-resistant prion protein, typical BSE had a distint phenotype in ovine transgenic mice compared to L-type BSE and bovine TME. The similar phenotypic properties of L-type BSE and bovine TME in TgOvPrP4 mice suggest that L-type BSE is a much more likely candidate for the origin of TME than is typical BSE.

Transmissible mink encephalopathy (TME) is a rare prion disease in ranch-raised mink (*Mustela vison*) in North America and Europe (*1–4*). Six outbreaks have been reported from 1947 through 1985 in North America, and several have been linked to contaminated commercial feed (*1*). Although contamination of feed with scrapie-infected sheep parts has been proposed as the cause of TME, the origin of the disease remains elusive. The idea that scrapie in sheep may be a source of TME infection is supported by findings that scrapie-infected mink have a similar distribution of vacuolar pathologic features in the brain and the same clinical signs as mink with natural and experimental TME (*5*). However, mink are not susceptible to scrapie infection following oral exposure for up to 4 years postinoculation, which suggests that either the scrapie agent may not be the source of natural TME infection or that only specific strains of the scrapie agent are able to induce TME (*6,7*).

Epidemiologic investigations in the Stetsonville, Wisconsin, outbreak of TME in 1985 suggested a possible cattle origin, since mink were primarily fed downer or dead dairy cattle but not sheep products (*8*). Experimental transmission of Stetsonville TME into cattle resulted in transmissible spongiform encephalopathy (TSE) disease with an incubation period of 18.5 months. Back passage of bovine TME into mink resulted in incubation periods of 4 and 7 months after oral or intracerebral inoculation, respectively, which was similar to that found following inoculation of Stetsonville TME into mink by these same routes (*8*). These findings indicated that cattle are susceptible to TME, and that bovine-passaged TME did not result in a reduced pathogenicity for mink. These studies raised the question as to whether an unknown TSE in cattle was the source of TME infection in the Stetsonville outbreak. Several additional TME outbreaks in the United States have been associated with mink diet that contained downer or dead cattle (*9*). These TME outbreaks happened before bovine spongiform encephalopathy (BSE) was identified in Europe or before 3 cases of BSE had occurred in the United States after

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2003. Despite a potential link of TME with a cattle TSE, clinical and histologic studies indicate that mink inoculated with BSE have features that distinguish this disease from natural and experimental TME (*7*,*8*,*10*).

Recent studies demonstrate, on the basis of the molecular features of the protease-resistant prion protein (PrP^{res}), that BSE has at least 3 different phenotypes in cattle (*11*). The cattle TSEs include: a) typical BSE, which is the prion strain identified during the BSE epidemic in Europe during the 1980s; b) H-type BSE, which is an uncommon type that was originally found in France (*12*); and c) L-type BSE, also called bovine amyloidotic spongiform encephalopathy (BASE), which is a rare form of BSE first identified in Italy (*13*). More recently, the H-type and L-type BSEs have been shown to differ from typical BSE with respect to incubation periods, vacuolar pathologic changes in the brain, and biochemical properties of PrP^{res} in mice on transmission into wild-type or transgenic mice that express the bovine prion protein gene (*14–18*). The origin of these BSE cases is unknown, but researchers have proposed that they represent a spontaneous form of TSE in cattle that is distinct from typical BSE; alternative hypotheses have also been considered, for example, cross-species contamination by another TSE source such as scrapie (*15*). If TME were to be due to infection with a cattle TSE, then these unusual forms of BSE are potential candidate agents since they may have a more widespread geographic distribution and were likely to precede the appearance of BSE in the USA.

In previous reports (*19–22*), to compare typical BSE and scrapie strains ruminants TSEs were transmitted into transgenic mice (TgOvPrP4) that overexpress the ovine prion gene (*19–22*). In the current study, we investigated the possible origins of TME by transmission of the bovine-passaged Stetsonville TME agent into TgOvPrP4 mice, to compare the molecular and histopathologic features to those of typical and atypical BSE types. Our findings indicate that bovine TME is distinct from typical BSE and H-type BSE but shares many features with L-type BSE. The implications of these findings with respect to the origin of TME are discussed.

Materials and Methods

Cattle TSE Isolates

Cattle TSE isolates used in these studies included: a) the Stetsonville TME isolate experimentally-passaged into cattle as previously described (*8*); b) an L-type BSE isolate (02-2528) (*11*,*13*,*23*,*24*); c) a H-type BSE isolate (03-2095) (*12*,*14*,*17*); and d) a typical BSE isolate (01-2281), for which transmission in TgOvPrP4 ovine transgenic mice was previously described (*20*). The typical, L-type, and H-type BSE cases were diagnosed after active surveillance by rapid tests at rendering plants; the animals were 4, 8, and 12 years of age at death, respectively. For a comparison of BSE transmission without a species barrier in ovine transgenic mice, a BSE isolate passaged in sheep (SB1) was also included in mouse transmissions studies (*20*,*21*).

Mouse Lines and Experimental Infections

The TgOvPrP4 mouse line expresses the ovine prion protein gene ($A_{136}R_{154}Q_{171}$ genotype) in a PrP null mouse background as previously described (*25*). The ovine prion protein gene is 2–4× more important in a sheep brain of the same PrP genotype. Mice were cared for and housed according to the guidelines of the French Ethical Committee (decree 87-848) and European Community Directive 86/609/EEC. Experiments were performed in the biohazard prevention area (A3) of the author's institution with the approval of the Rhône-Alpes Ethical Committee for Animal Experiments.

Female mice, 4 to 6 weeks of age (6–12 animals per experimental group), were inoculated intracerebrally with 10% (wt/vol) cattle brain homogenates in 5% glucose (20 μL per animal). Brain specimens from TgOvPrP4 mice in which a TSE developed were subsequently passaged by intracerebral inoculation of a 1% (wt/vol) homogenate into a second group of transgenic mice. Mice were sacrificed at the terminal stage of disease, and the brains were collected and either analyzed for PrP^{res} by Western blot or fixed in buffered 4% paraformaldehyde for histopathologic studies. Statistical analyses of survival periods were performed by using the log-rank test and the R software package (version 2.4.1); p values <0.05 were considered statistically significant.

Western Blot Analyses of PrP^{res}

PrP^{res} was extracted from bovine brain stem samples by using the TeSeE Western blot Bio-Rad kit (Bio-Rad, Marnes-la-Coquette, France; Ref 355 1169) following the manufacturer's instructions. Briefly, 250 μL of 20% brain homogenates were incubated with an equal volume of reconstituted proteinase K solution (reagent A + PK) at 37°C for 10 min. After addition of 250 μL of reagent B, samples were centrifuged at 15,000× *g* for 7 min. The pellets were resuspended in 50 μL of denaturing buffer (TD4215) (4% sodium dodecyl sulfate [SDS], 2% β-mercaptoethanol, 192 mmol/L glycine, 25 mmol/L Tris, and 5% sucrose), heated for 5 min at 100°C, then centrifuged at 12,000× *g* for 15 min. The pellets were discarded, and the supernatants were run on a 15% SDS–polyacrylamide gel (SDS-PAGE) before transfer to nitrocellulose membrane and immunoblotting with anti-PrP antibodies. Western blot methods used to identify and characterize PrP^{res} in TgOvPrP4 mice have been previously described (*19*,*20*). Briefly, PrP^{res} was obtained after mouse brain homogenates were treated with proteinase K (Roche, Meylan, France) (10 μg/100 mg brain

tissue for 1 h at 37°C) and concentration by ultra-centrifugation (100,000 *g* for 2 h on a 10% sucrose cushion). After denaturation in TD4215 buffer, PrP^{res} was separated in 15% SDS-PAGE, electroblotted to nitrocellulose membranes, then detected on the membrane by using anti-PrP monoclonal antibodies.

PrP^{res} was detected by using anti-PrP Bar233 monoclonal antibody (ascitic fluid 1/10,000, provided by J. Grassi) or Sha31 (1/10 from kit TeSeE sheep/goat Bio-Rad) against the ovine 152-FGSDYEDRYYRE-163 and 156-YEDRYYRE-163 PrP sequences, respectively. Quantitative studies of PrP^{res} polypeptide molecular mass and glycoforms proportions were performed by using Quantity One (Bio-Rad) software analysis of chemiluminescent signals following ≥3 independent runs of the samples from ≥3 different mice per experimental group. Glycoforms ratios were expressed as mean percentages (± standard deviations) of the total signal for the 3 PrP^{res} glycoforms and the apparent molecular masses were evaluated by comparison of the positions of the PrP^{res} bands with a biotinylated marker (B2787; Sigma, St. Quentin Fallavier, France).

Histopathologic Analyses

Mouse brain fixed in buffered 4% paraformaldehyde was treated for 1 h at room temperature with formic acid (98%–100%) before being embedded in paraffin blocks (Thermo Electron, Cergy-Pontoise, France). Tissue sections, 5 μm thick, were cut from paraffin blocks, placed on treated glass slides (Starfrost, Medite Histotechnic, Burgdorf, Germany), and dried overnight at 55°C. Once dewaxed, slides were stained for either histopathologic or immunohistochemical examination. Amyloid deposits were identified with a Congo red stain, and vacuolar lesions were observed on slides stained with hematoxylin-eosin (HE), according to Fraser's lesion profile analyses (*26*). Lesion profiles were measured by using a computer-assisted method (*27*). Brain slices were immunostained for the presence of disease-associated prion protein (PrP^d) by using 2 μg/mL of anti-PrP SAF84 monoclonal antibody (SPI Bio, Massy, France). Recently described pre-treatments designed to enhance PrP^d detection were also applied (*28*). These consisted of a 10-min formic acid (98%) bath at room temperature, 20 min of hydrated autoclaving at 121°C (Prestige Medical, AES Laboratories, Blackburn Lane, UK), and digestion at 37°C with proteinase K (Roche Diagnostics, Meylan, France) at a concentration of 20 μg/mL for 15 min, with an additional incubation with streptomycin sulfate at 8.75 mmol/L for 1 h. Endogenous peroxidase activity was also blocked. A peroxidase-labeled avidin-biotin complex (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA, USA) and a solution of diaminobenzidine intensified with nickel chloride (DAB-Ni, Zymed, Montrouge, France) to give black deposits were used to amplify and visualize binding of the disease-associated form of PrP (PrP^d). Final detection was achieved with a solution of diaminobenzidine intensified with nickel chloride (DAB-Ni, Zymed) to give black deposits. The specificity of PrP^d immunolabeling was also assessed by using uninfected brain samples. Finally, the slides were counterstained with aqueous hematoxylin, dehydrated, mounted by using Eukitt mounting medium (VWR International, Limonest, France) and observed under a light microscope BX51 (Olympus, Rungis, France) coupled to an image analysis workstation (MorphoExpert Software, Explora Nova, La Rochelle, France).

Results

Transmission of TSE Isolates in TgOvPrP4 Mice

To determine the ability of the 4 bovine TSE isolates (experimental bovine TME, L-type BSE, H-type BSE, and a typical BSE natural isolate) to cause a TSE in a common host species, they were inoculated into TgOvPrP4 mice (Table). The shortest survival period was observed in mice infected with the typical BSE isolate (421 ± 48 days), compared with 436 ± 77 days or 627 ± 74 days in TgOvPrP4 mice inoculated with bovine TME or L-type BSE, respectively. At second serial passage of bovine TME, L-type BSE, and typical BSE into TgOvPrP4 mice, the survival periods were shortened for all 3 sources, but especially for L-type BSE (202 ± 26 days) and bovine TME (234 ± 27 days), although survival periods were statistically different for these 2 groups of mice ($p = 0.0095$). The survival period for

Table. TSE sources transmitted to TgOvPrP4 mice*

		First passage		Second passage	
TSE sources	Nature	Survival periods (mean ± SD dpi)	No. PrP^d positive mice/total†	Survival periods (mean ± SD dpi)	No. PrP^d positive mice/total†
Experimental isolates					
TME	TME	436 ± 77	9/11	234 ± 27	9/9
SB1	Ovine BSE	296 ± 46	19/19	365 ± 36	11/12
Natural isolates					
02-2528	L-type BSE	627 ± 74	9/10	02 ± 26	9/9
03-2095	H-type BSE	692 ± 129	0/8	ND	ND
01-2281	Typical BSE	421 ± 48	10/10	354 ± 48	10/10

*TSE, transmissible spongiform encephalopathy; dpi, days postinoculation; PrP^d, disease-associated prion protein; TME, transmissible mink encephalopathy; BSE, bovine spongiform encephalopathy; ND, not done.
†Results obtained by Western blot or by immunohistochemical test.

typical BSE (354 ± 48 days) was significantly longer than that found for L-type BSE and bovine TME ($p<0.0001$). For the ovine BSE isolate, the incubation period was 296 ± 46 days at first passage, but the period increased to 365 ± 36 days at second passage. The survival period at second passage of ovine BSE was not significantly different from that of typical BSE ($p = 0.814$). Most of the mice inoculated with the 3 bovine TSE isolates were positive (28/31 at first passage) for PrP^{res} by Western blot or PrP^{d} by immunohistochemical testing. In contrast, all (8/8) the transgenic mice inoculated with the H-type BSE isolate, which had a survival period of 692 ± 129 days, were negative for PrP^{res} by either Western blot or PrP^{d} by immunohistochemical analyses.

PrP^{res} Molecular Features in Cattle and in TgOvPrP4 Mice

Western blot analysis of PrP^{res} from bovine TME, L-type BSE, H-type BSE, and typical BSE used for inoculation into TgOvPrP4 mice was performed to compare the molecular features of PrP^{res}. The bovine TME and L-type BSE isolates had similar molecular masses for the 3 PrP^{res} polypeptides (Figure 1). The unglycosylated PrP^{res} polypeptide has a similar molecular mass (≈18.5 kDa) in L-type BSE, bovine TME, and in typical BSE, whereas a difference in molecular mass (≈0.5–0.8 kDa lower) was found for the diglycosylated band in both L-type BSE and bovine TME compared to typical BSE. A comparison of PrP^{res} glycoform ratios also showed similar proportions of the diglycosylated and monoglycosylated isoforms of PrP^{res} in bovine TME and L-type BSE, both of which had lower levels of diglycosylated PrP^{res} than in typical BSE (Figure 2, panel A).

On transmission of the bovine TSE isolates to TgOvPrP4 mice, the L-type BSE differed from typical BSE in its lower proportion of diglycosylated PrP^{res}, but the molecular masses of the unglycosylated PrP^{res} polypeptides were similar between these isolates (Figure 2, panel B, and Figure 3, panel A). In contrast, Western blot analysis of PrP^{res} showed indistinguishable patterns in transgenic mice infected with the L-type BSE or bovine TME, with respect to the molecular mass of the 3 PrP^{res} polypeptides (Figure 3, panel B) and the ratio of these PrP^{res} glycoforms (Figure 2, panel C). These comparable features were maintained in both L-type BSE and bovine TME at second passage in TgOvPrP4 mice (Figure 2, panel D, and Figure 3, panel C), and these were distinct from typical BSE, on the basis of the ratio of the 3 PrP^{res} glycoforms.

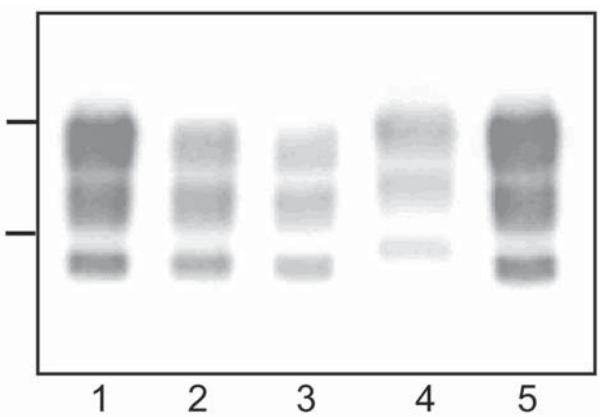

Figure 1. Western blot analyses of protease-resistant prion protein from proteinase K–treated brain homogenates from cattle transmissible spongiform encephalopathies (TSEs). Typical bovine spongiform encephalopathy (BSE) (lanes 1, 5), L-type BSE (lane 2), transmissible mink encephalopathy (TME) in cattle (lane 3), H-type BSE (lane 4). Bars to the left of the panel indicate the 29.0- and 20.1-kDa marker positions.

Histopathologic Features of TME in Cattle and L-type BSE in TgOvPrP4 Mice

To further examine the phenotypes of the bovine TSE agents, the distribution of vacuolar lesions and the distribution and features of PrP^{d} were investigated at standardized brain levels of TgOvPrP4 mice (Figure 4). TgOvPrP4 mice infected with L-type BSE at first passage showed low vacuolar lesion intensity but PrP^{d} accumulation was strongly detected, which was characterized by PrP^{d} aggregation into plaques. These plaques were not amyloid based on an absence of Congo red birefringence (data not shown). At the second passage, L-type BSE agent induced a lower degree of PrP^{d} accumulation than in the first passage; the type of deposition was fine powdery to granular, and no plaques were observed. Although the brain lesion profile showed a higher degree of vacuolation than in the first passage, the PrP^{d} mapping was similar at first and second passage in transgenic mice. At second passage, some additional sites had PrP^{d} accumulation, including the septal areas and the midbrain. The L-type BSE remained distinct from typical BSE, in terms of lesion profiles and types of PrP^{d} deposition. In the brain of TgOvPrP4 mice infected with typical BSE and ovine BSE, numerous florid plaques containing PrP^{d} were seen, and these were amyloid, based on Congo red staining. The florid plaques were prominent in the cortical regions of the brain in transgenic mice infected with typical BSE but were not found in any of the mice infected with L-type BSE at either first or second passage. At second passage, vacuolar changes were more intense in the dorsal medulla nuclei, hypothalamus, and hippocampus in mice infected with typical BSE; in L-type BSE-infected mice, lesions were more pronounced in the colliculus, thalamus, and cerebral cortex.

The distribution of vacuolar changes and PrP^{d} in the brain of TME-infected mice was similar to that observed in mice infected with L-type BSE, although minor differences in the PrP^{d} immunolabeling intensities were found in some brain regions, including the absence of the fine, powdery PrP^{d} in the caudate putamen of bovine TME-infected mice.

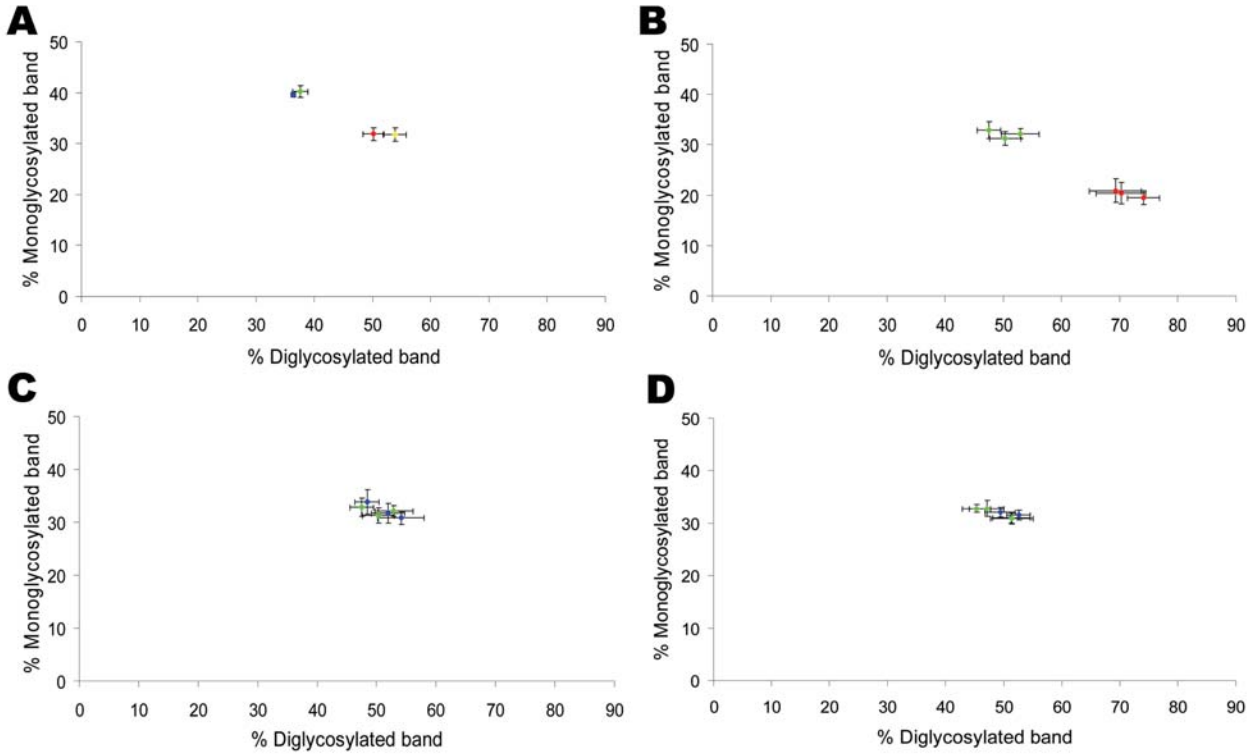

Figure 2. Glycoforms proportions (means ± standard deviations) of protease-resistant prion protein detected by using Sha31 antibody. A) Cattle transmissible spongiform encephalopathies (TSEs). B) First passage of L-type and typical bovine spongiform encephalopathy (BSE) into TgOvPrP4 mice. C) First passage of transmissible mink encephalopathy (TME) in cattle and L-type BSE into TgOvPrP4 mice. D) Second passage of TME in cattle and L-type BSE into TgOvPrP4 mice. Results from 3 individual mice from each experimental group are shown. Typical BSE in red, H-type BSE in yellow, L-type BSE in green, and TME-in-cattle in blue.

It is noteworthy that the PrP^{d} distribution was also comparable in transgenic mice infected with bovine TME and typical BSE; however, no florid plaques were detected in any of the mice infected with bovine TME.

Discussion

Comparison of TME and 3 distinct types of BSE in a common host species was prompted by previous observations that TME, whose origin is unknown, did not reduce its pathogenicity for mink after passage into cattle (*8*), and transmission of TME into cattle resulted in a low-molecular-mass PrP^{res} profile (*29*). A similar low-molecular-mass PrP^{res} profile has been observed in L-type BSE or BASE (*11*,*13*,*16*), and in the current study, these similar PrP^{res} molecular properties between bovine TME and L-type BSE were demonstrated in TgOvPrP4 mice. These 2 distinct bovine TSE sources were both readily transmitted into TgOvPrP4 mice (illustrating the usefulness of transgenic mouse models for prion agent strain typing when transmission to a common wild-type rodent is not possible) and had several common features including survival periods, PrP^{res} molecular features, and the distribution of vacuolar pathologic changes. This combination of biochemical and phenotypic properties indicates that they have similar biologic properties in TgOvPrP4 mice and could represent independent isolation of the same TSE strain, although some subtle histologic differences between L-type BSE and bovine TME were observed on second passage in TgOvPrP4 mice. Additional serial passages from these cattle TSE sources are required for further characterization, especially since the possibility cannot be excluded that transmission of a cattle TSE into mink can modify its biologic properties.

In contrast, transmission of L-type BSE and typical BSE into TgOvPrP4 mice resulted in different incubation periods, PrP^{res} molecular properties, and histopathologic features during 2 serial passages. The survival period at second passage for L-type BSE was considerably reduced and significantly shorter (>120 days) than typical BSE after 2 passages into TgOvPrP4 mice. Although florid plaques were found in transgenic mice infected with typical BSE, they were not observed in mice following first or second passage of L-type BSE in TgOvPrP4 mice. Analysis of PrP^{res} properties also found differences between L-type and typical BSE with respect to the relative proportion of diglycosylated PrP^{res} polypeptides. The lower molecular mass of the unglycosylated PrP^{res} polypeptide in L-type BSE was

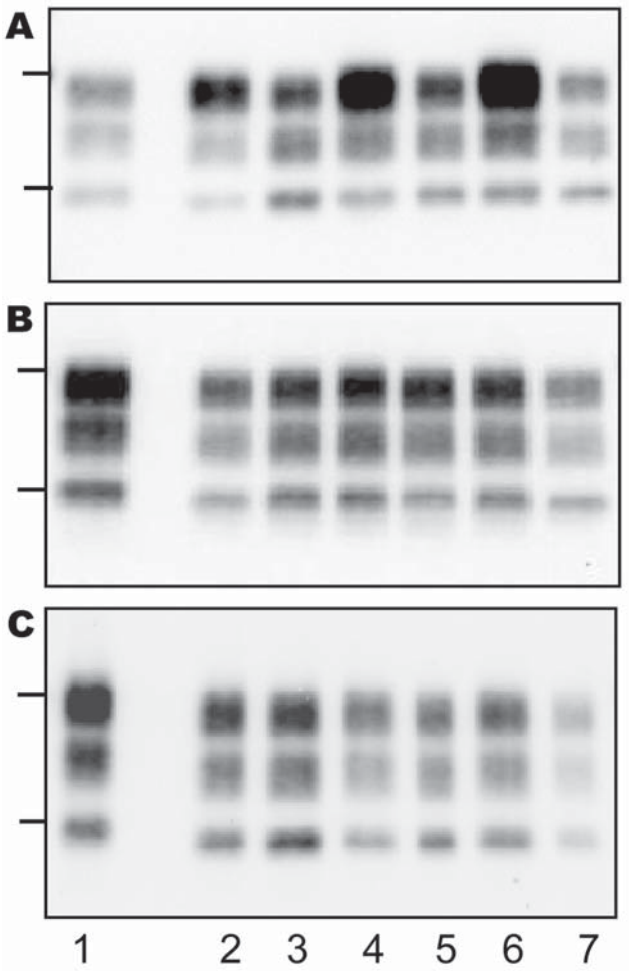

Figure 3. Western blot of protease-resistant prion protein from TgOvPrP4 mice after proteinase K digestion and immunodetection with anti-PrP Sha31 antibody. A) First passage of typical bovine spongiform encephalopathy (BSE) (lanes 2, 4, and 6) and L-type BSE (lanes 3, 5, and 7). B) First passage of TME in cattle (lanes 2, 4, and 6) and L-type (lanes 3, 5, and 7). C) Second passage of TME in cattle (lanes 2, 4, and 6) and L-type BSE (lanes 3, 5, and 7). Each lane shows PrP^{res} from a distinct individual mouse from each experimental group. Bars to the left of the panel indicate the 29.0- and 20.1-kDa marker positions. Lane 1, PrP^{res} control from a scrapie-infected TgOvPrP4 mouse (C506M3 strain).

maintained in TgOvPrP4 mice as well as in typical BSE. In cattle, the size of the unglycosylated PrP^{res} polypeptide in L-type BSE is within the range of that found in typical BSE (*16*). Notably, our findings did not produce evidence for modification of the phenotypic features of these cattle TSEs on passage into TgOvPrP4 mice, which was recently described after transmission of L-type BSE into wild-type (*18*) or tg338 ovine transgenic (*24*) mice.

The current and previous studies demonstrate that when typical BSE and L-type BSE are transmitted into TgOvPrP4 mice, key features of these cattle TSEs were maintained (*19–21*). However, these transgenic mice were not susceptible to the H-type BSE, as confirmed with 4 other isolates (data not shown). This finding is in contrast to a previous study that demonstrated transmission of H-type BSE into a different transgenic mouse line expressing the ovine prion protein gene (*15*). Possible explanations for this discrepancy are the lower level of the cellular prion protein in TgOvPrP4 mice compared with tg338 mice (2- to 4-fold vs. 8- to 10-fold greater than ovine brain), different cellular patterns of expression due to the use of different promoters, or the different sequence of the prion gene ($V_{136}R_{154}Q_{171}$ in tg338 mice) (*20*,*30*). These findings on the transmission of H-type into transgenic mice provide further evidence for the distinct biologic properties of this cattle TSE compared to L-type BSE and typical BSE.

The Stetsonville isolate of TME that was experimentally passaged into cattle was also readily transmitted into TgOvPrP4 mice and resulted in a TSE phenotype that shared common biologic features with L-type BSE, but not typical BSE, in these transgenic mice. Transmission of TME from temporally and geographically different outbreaks into cattle showed that bovine TME is similar among the different isolates but all of them are phenotypically distinct from typical BSE (*7*). Histopathologic studies showed more severe spongiform changes, especially at rostral levels, in cattle infected with TME (*7*) than infected with typical BSE, and these changes were also observed in TgOvPrP4 mice infected with L-type BSE at second passage. Conversely, mink infected with typical BSE had a greater degree of spongiform change in the brainstem than mink infected with TME (*10*). Similar changes were also observed in TgOvPrP4 mice infected with typical BSE. This preferential involvement of rostral brain regions in L-type BSE has also been described in cattle (*13*) and on transmission into bovine transgenic mice (*18*,*24*). Transmission of TME or typical BSE into wild-type mice (*31*,*32*) or hamsters (*33*,*34*) also resulted in distinct transmissibility between these TSEs in these host species. Based on these findings, we conclude that typical BSE is not a likely source for TME in mink; however, if TME were to be due to infection with a cattle TSE, the most likely candidate is L-type BSE.

L-type BSE has not been reported in the United States, although importation of cattle or cattle products with a TSE infection cannot be excluded as a potential source. Recently, 2 BSE cases identified in cattle born in the United States had unusual PrP^{res} properties similar to those described for H-type BSE (*11*,*12*,*35*,*36*), which raised the possibility that atypical TSEs in cattle may be a source for TME infection. In Europe, the prevalence of the H-type or L-type BSE is estimated to be very low (in France the L-type BSE occurs at a prevalence of 1 case found PrP^{res} positive in the brain stem per 3 million cattle tested per year) (*23*). The very rare

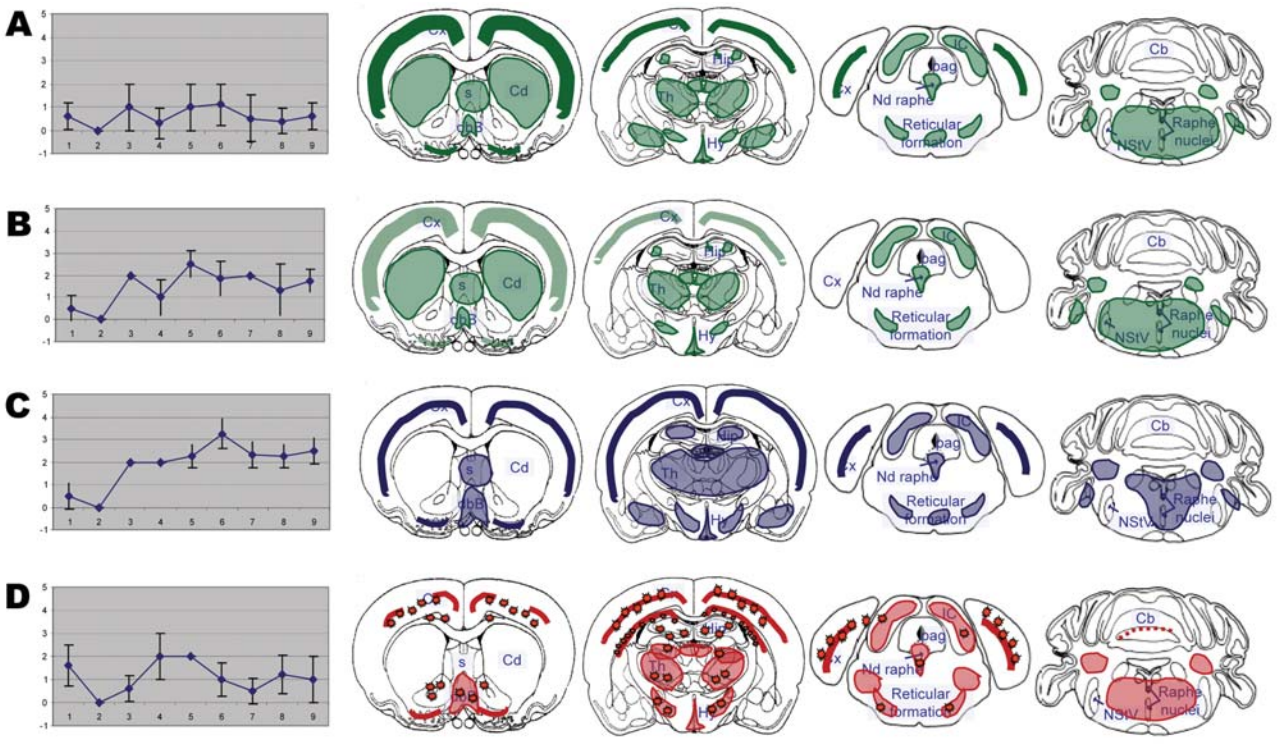

Figure 4. Brain lesion profiles (left panels) and protease-resistant prion protein brain distribution (right panels) observed in the brain of TgOvPrP4 mice infected with L-type bovine spongiform encephalopathy (BSE), at first (A, n = 5) and second (B, n = 4) passages; TME in cattle (C, n = 5); or typical BSE (D, n = 4) at second passage. Brain vacuolation was scored (means ± standard deviations) on a scale of 0–5 in the following brain areas: 1) dorsal medulla nuclei, 2) cerebellar cortex, 3) superior colliculus, 4) hypothalamus, 5) central thalamus, 6) hippocampus, 7) lateral septal nuclei, 8) cerebral cortex at the level of thalamus, and 9) cerebral cortex at the level of septal nuclei. In right panels, showing the PrP^d distribution, stars indicate the presence of florid plaques.

prevalence of TME may be partially due to the rare occurrence of cattle TSEs that enter the mink diet (*8*,*29*).

Conclusion

These studies provide experimental evidence that the Stetsonville TME agent is distinct from typical BSE but has phenotypic similarities to L-type BSE in TgOvPrP4 mice. Our conclusion is that L-type BSE is a more likely candidate for a bovine source of TME infection than typical BSE. In the scenario that a ruminant TSE is the source for TME infection in mink, this would be a second example of transmission of a TSE from ruminants to non-ruminants under natural conditions or farming practices in addition to transmission of typical BSE to humans, domestic cats, and exotic zoo animals (*37*). The potential importance of this finding is relevant to L-type BSE, which based on experimental transmission into humanized PrP transgenic mice and macaques, suggests that L-type BSE is more pathogenic for humans than typical BSE (*24*,*38*).

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Changing Epidemiology of Human Brucellosis, Germany, 1962–2005

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Trends in the epidemiology of human brucellosis in Germany were investigated by analyzing national surveillance data (1962–2005) complemented by a questionnaire-based survey (1995–2000). After a steady decrease in brucellosis incidence from 1962 to the 1980s, a persistent number of cases has been reported in recent years, with the highest incidence in Turkish immigrants (0.3/100,000 Turks vs. 0.01/100,000 in the German population; incidence rate ratio 29). Among cases with reported exposure risks, 59% were related to the consumption of unpasteurized cheese from brucellosis-endemic countries. The mean diagnostic delay was 2.5 months. Case fatality rates increased from 0.4% (1978–1981) to a maximum of 6.5% (1998–2001). The epidemiology of brucellosis in Germany has evolved from an endemic occupational disease among the German population into a travel-associated foodborne zoonosis, primarily affecting Turkish immigrants. Prolonged diagnostic delays and high case fatality call for targeted public health measures.

Brucellosis is one of the most common zoonotic diseases worldwide (*1*). The disease is caused by *Brucella* spp. and is transmitted from its animal reservoirs to humans by direct contact with infected animals or, more often, through the consumption of raw animal products such as unpasteurized milk or cheese. Four of 6 nomen species of the genus *Brucella* are pathogenic for humans, i.e., *B. melitensis* (transmitted from sheep and goats), *B. abortus* (from cattle and other bovidae), *B. suis* (from pigs), and *B. canis* (from dogs) (*2*).

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In Germany, human brucellosis was highly endemic from the 1950s well into the 1980s, with up to 500 cases reported annually. Most of these cases were related to occupational exposure associated with calf breeding and dairy farming, leading to a predominance of *B. abortus* infections (*3*,*4*). Because of successfully established eradication and control programs for animal brucellosis, the number of human cases decreased steadily. In 2000, Germany was declared "officially free from ovine/caprine and bovine brucellosis" (*5*). Human brucellosis cases, however, continued to occur in Germany. Although limited case series from Germany and Denmark indicate that human brucellosis could be associated with travel to and immigration from disease-endemic areas (*6*,*7*), there are no population-based or nationwide studies on epidemiologic characteristics of the disease in northern and central Europe.

The objective of our study was to describe trends in laboratory-confirmed human brucellosis in Germany over the past 40 years by analyzing national surveillance data. To provide background information, which may be useful for targeting public health measures, we focused on geographic origin and source of infection, modes of transmission, risk factors, and regional distribution of the disease.

Methods

In the former German Democratic Republic (East Germany), human brucellosis became a reportable disease in 1951. From 1947 through 1961, in the former Federal Republic of Germany (West Germany) only *B. abortus* infections were reported. After 1962, brucellosis (independent of the disease-causing species) became a reportable disease according to the West German Federal Communicable Disease Act, which was the applicable law after the reunification in 1990.

Detailed data about brucellosis patients were compiled from 1995 through 2005 on demographics (age, sex, nationality, and current residence), onset of symptoms, clinical signs (fever, night sweats, fatigue, lack of appetite or weight loss, headache, arthralgia), contact dates with the treating physician, hospitalization, death, laboratory diagnosis, bacterial species, geographic origin, and possible vehicle of infection. The data collected from 1995 through 2000 are based on a standardized questionnaire, which was sent to local health departments for every reported case of brucellosis (*8*). Since 2001, similar information has been available from an improved surveillance system implemented for mandatory case reporting of infectious diseases. Fatal brucellosis cases documented on death certificates (1962–2005) were obtained from the Information System of Federal Health Monitoring, Germany (www.gbe-bund.de); population data were provided by the Federal Statistical Office, Germany (www.destatis.de).

Both clinical signs (the occurrence of an acute febrile illness or 2 other clinical signs) and laboratory confirmation (positive culture, only 1 significant titer, or an increase in the titer in the follow-up serum sample) were required to meet the case definition for brucellosis (*9*). From 1995–2005, isolates suspected to be *Brucella* spp. were sent from various microbiologic laboratories throughout Germany to the former German Reference Center for Human Brucellosis at the Federal Institute for Risk Assessment in Berlin. Standard microbiologic methods were used for further identification (*10*).

To assess temporal trends, mean annual incidences and case-fatality ratios were calculated for 4-year intervals starting from 1962 through 2005. Statistical tests for trend were performed by using the Cochrane-Armitage test (*11*); 95% confidence intervals were calculated according to Wilson (*12*). The Mann-Whitney test was used for comparative analysis of continuous variables. We tested for significance of incidence rate ratios (IRRs) using a Poisson regression model. Odds ratios (ORs) were tested for significance by using the χ^2 test. Data were analyzed with EpiInfo version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA, USA) and Stata version 9.0 (StatCorp., College Station, TX, USA). A p value <0.05 was considered significant.

Results

From 1962–2005, 6,269 human brucellosis cases were reported in Germany. During this 44-year period, the annual number of cases generally declined (Figure 1). The mean annual incidence decreased from 0.6/100,000 population (1962–1965) to the lowest observed incidence of 0.03/100,000 population during 1998–2001 (Figure 2). A total of 58 deaths were caused by brucellosis (overall case-fatality rate 0.9%). The lowest case-fatality rate was 0.4% in 1978–1981. From then on, a significantly increasing trend (p<0.01) reaching a maximum of 6.5% in 1998–2001 was observed, which subsequently dropped to 2.1% in 2002–2005.

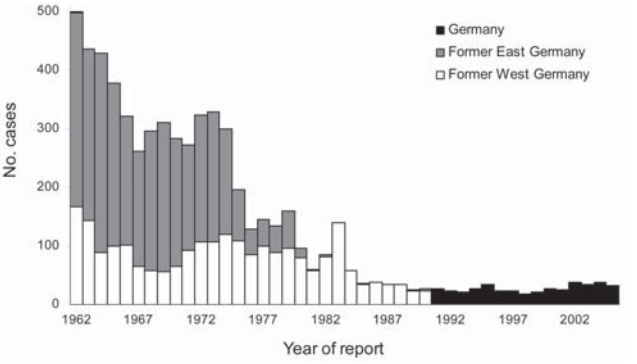

Figure 1. Reported brucellosis cases, Germany, 1962–2005.

Through 1974, most of the brucellosis cases were reported from East Germany, with a maximum of 82% in 1969. After 1974, the relative number of cases reported in East Germany decreased compared with those in West Germany. Since 1981, brucellosis has been rarely reported in East Germany; the West-East divide was still present after reunification (Figure 3).

From 1995 to 2005, a total of 290 brucellosis cases were reported, of which 245 (84%) met the case definition and were included in this analysis. Area of residence, sex, age, clinical symptoms, and laboratory findings were known for all 245 case-patients. Most cases were reported in the federal states of North Rhine Westphalia (49), Baden-Württemberg (45), Bavaria (39), and Hesse (23); in the cities of Berlin (19), Hamburg (8) and Bremen (4); and in large conurbations, e.g. Munich (10) and Ludwigshafen (8). The spatial distribution of brucellosis cases was associated with the immigrant density in the administrative districts (Figure 3).

Both sexes were almost equally represented among brucellosis patients (54% male vs. 46% female). In patients <30 years of age and >59 years of age, male sex predominated (60% and 73%, respectively); in persons 30–59 years

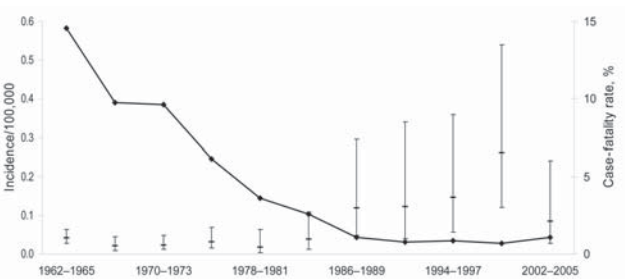

Figure 2. Incidence (per 100,000 inhabitants) and case-fatality rate for brucellosis, Germany, 1962–2005. Error bars indicate 95% confidence intervals.

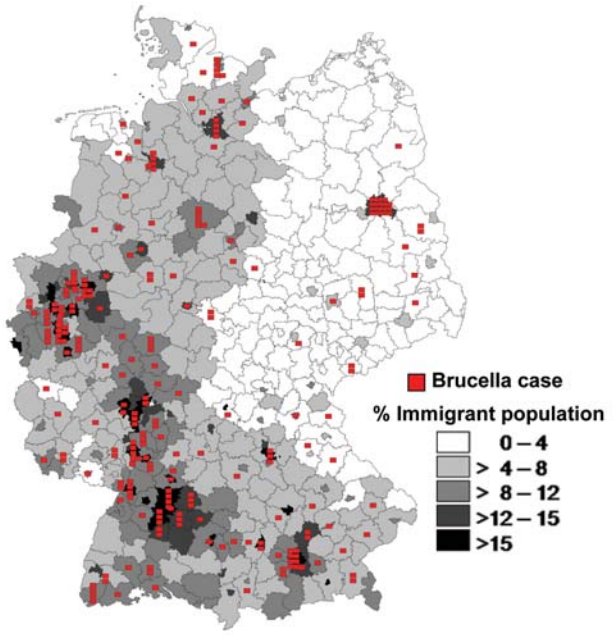

Figure 3. Regional distribution of brucellosis cases and percentage of immigrants per county, Germany, 1995–2005.

of age, 56% were female. The age-specific incidence was highest for persons 60–69 years of age, with a mean annual incidence of 0.05/100,000, and lowest for children <10 years of age, with a mean annual incidence of 0.02/100,000 (Figure 4).

Detailed data about the nationality of patients were available for 106 (43%) of the 245 cases. A total of 58 (55%) were non-Germans, of which 62% (36) were Turkish. Four were Italian, 3 each were Greek and Iraqi, 2 were Kazakh, and 1 each were Bosnian, Kosovar, Portuguese, Syrian, Arabian, Indian, Pakistani, Yemeni, and Somali; in 1 case, a non-German status was reported without nationality. The incidence rate was 0.3/100,000 in Turks and 0.01/100,000 in Germans (IRR 29, p<0.01).

The country where the infection had been contracted was known for 234 (96%) of 245 cases. In 172 cases (74%), the origin of infection was likely to be outside Germany. Of these, 137 (80%) were associated with travel to disease-endemic countries surrounding the Mediterranean Sea, e.g. Turkey (94), Italy (13), and Spain (9). Possible origins of infection were the Balkans for 5 cases, African countries for 7 cases, Middle Eastern countries (not bordering the Mediterranean Sea) for 6 cases, Minor Asian countries for 9 cases, former Soviet Union countries for 5 cases, and the Czech Republic for 1 case. Two patients were infected overseas (Peru and New Zealand). In 62 cases (26%), the origin of infection was assumed to be Germany.

In 102 (42%) of 245 patients, ≥1 probable source of infection could be identified. Fifty-six (55%) had only 1 exposure risk, whereas the other 46 patients (45%) mentioned various combinations. Twenty-seven patients consumed unpasteurized milk, 65 patients ate unpasteurized cheese or other dairy products, and 7 patients ate raw meat. Foodborne infections were almost equally distributed among Turks and Germans (31% vs. 35%). Direct contact with cattle, sheep and goats was reported by 16, 24, and 16 patients, respectively. Most of the people infected by direct animal contact were Turks (49%); only 29% were Germans.

In 18 cases (18%), a possible occupational exposure was reported. Among these, 7 infections were laboratory-acquired, exclusively in German cases. The other work-related cases were linked to direct contact with animals or animal products outside Germany. Two shepherds, 2 persons working on a sheep breeding farm, 2 farmers, 4 butchers and 1 veterinarian were affected. In 84 cases (82%), no occupational exposure risk was observed.

Ten minor outbreaks were reported during 1995–2005. Four cases were epidemiologically linked to *Brucella* infections observed in friends and relatives living in disease-endemic countries, i.e., Turkey, Italy, and Bosnia. In 7 cases, the patient was related to at least 1 other person living in Germany who also had *Brucella* infection. One laboratory-acquired infection and its index case were also reported as an outbreak.

The date of onset for symptomatic disease was reported for 207 (84%) of 245 cases. In most cases, the onset of disease was in August or September (31%). Another smaller peak comprising 23 cases (11%) occurred in June (Figure 5). In 85 cases, more detailed information about the incubation period was available. The period between presumed infection and onset of symptomatic disease varied extremely, ranging from a few days to 24 months (median 4 weeks).

The major symptom in 215 (88%) of 245 patients was fever, which was significantly associated with hospitalization of the patient (OR 4.1; p<0.01). A total of 121 (49%) patients reported fatigue; 105 (43%) reported arthralgia,

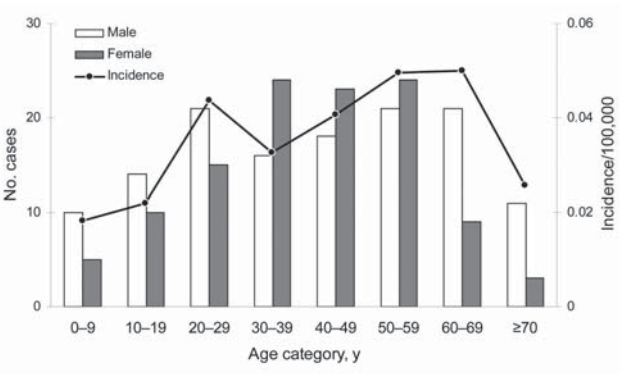

Figure 4. Age and sex distribution of brucellosis cases (n = 245), Germany, 1995–2005.

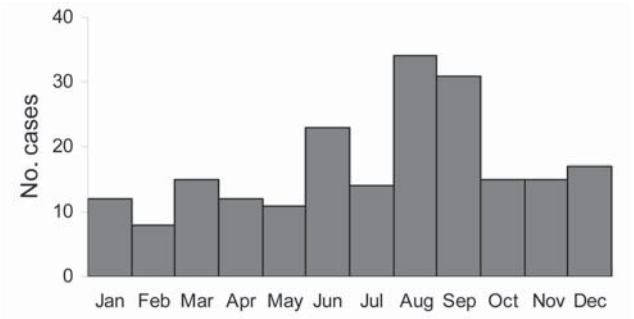

Figure 5. Seasonal distribution of brucellosis cases (n = 207), Germany, 1995–2005.

101 (41%) reported headaches, 66 (27%) reported massive sweating, 30 (12%) reported loss of appetite, and 31 (13%) reported loss of weight.

The delay between onset of disease and definite laboratory-confirmed diagnosis was reported for 175 cases. The mean diagnostic delay was 2.5 months, with no differences between ethnic groups. In 77 patients (44%), brucellosis was diagnosed within 4 weeks. For 89 patients (51%), diagnostic delay ranged from 1 to 6 months. In the remaining 9 patients (5%), brucellosis was definitely diagnosed >6 months after onset of symptoms.

In 24 cases, the period between the first presumptive diagnosis and the final laboratory confirmation was reported; the mean period was 6 days. Sixty-three (26%) patients were treated as outpatients, while 181 (74%) were hospitalized. In 1 case, this information was not available.

From 1995–2005, a total of 134 cases was culture-proven at the former German Reference Centre for Human Brucellosis at the Federal Institute for Risk Assessment in Berlin. Standard microbiologic tests identified 131 *B. melitensis* isolates (98%), 1 *B. suis* strain, and 2 *B. abortus* strains. Of the 245 total cases, 164 (67%) were diagnosed by serologic tests, 113 (69%) by serum agglutination test (SAT), 3 (2%) by complement fixation (CFT), and 15 (9%) by ELISA. In 31 cases (19%), a positive SAT was confirmed by CFT or ELISA. In 2 cases, the serologic tests used could not be identified. Among the cases with serologic confirmation (n = 164), 1 strongly elevated titer was reported for 81 patients, while seroconversion was shown in 11 patients. In 72 cases, no data on the type of serologic confirmation was available.

Discussion

Up to the mid-1980s, a substantial decrease in the incidence of human brucellosis was observed in Germany. However, national surveillance data demonstrated a persistent level of reported cases in recent years. Our study indicates that these infections are primarily related to persons with a migrational background. Taking into account that <10% of *Brucella* infections are recognized and reported because of unspecific clinical symptoms (*13*,*14*), our results strongly suggest that human brucellosis has emerged as an important and probably neglected health problem among immigrants in Germany. The present epidemiology of brucellosis in Germany mirrors the reemergence of the disease in Turkey. An increase in brucellosis incidence has also been reported from several other countries in the Middle East and the Balkans (*1*), which emphasizes the magnitude of the problem and its potential to accelerate in the future. Immigrants from these regions form an increasing proportion of the German population.

The current status of brucellosis in Germany is the result of continuous changes in the epidemiologic characteristics of the disease during the past 40 years. The number of reported autochthonous human cases continuously decreased in parallel to the decreasing prevalence of infected animals. At the same time, the number of immigrants, especially from Turkey, increased considerably. In 1960, only 1% of the German population was foreign born and only 2,700 Turkish residents lived in Germany. In 2004, ≈8.8% of the population was foreign born, and Turks formed the largest foreign nationality group, with 1,764,318 immigrants (24% of all foreigners).

Our results indicate that the exposure risk of Turkish immigrants to *Brucella* spp. continues after immigration to Germany, with a brucellosis incidence (0.3 cases/100,000 Turkish immigrants) falling between the incidence in the German population as a whole (0.01 cases/100,000 Germans) and the incidence observed in Turkey (26.2 cases/100,000 population) (*1*). The continuing risk may be attributed to more frequent exposures during summer recreational activities in disease-endemic countries, e.g., when visiting friends and relatives in rural areas. In brucellosis-endemic regions, the peak for human brucellosis is in June and July (*15*–*17*). Onset of disease occurs in August and September, just after the end of the German summer holiday season, in most reported cases. In addition, *Brucella* spp. may survive for several days up to months in contaminated food products privately imported from disease-endemic countries (*18*), which may contribute to infections contracted in Germany. An association of brucellosis with the immigrant population has previously been reported from Denmark and the United States (*7*,*19*). In the United States, Hispanic ethnicity, recent travel to disease-endemic areas in Mexico, and ingestion of nonpasteurized dairy products are major risk factors for *Brucella* infections (*13*,*19*–*22*).

Brucellosis was traditionally more prevalent in German states with a high degree of agricultural activity. Our results demonstrate a fundamental shift of brucellosis from a rural disease into an infection of urban and suburban residents. Because most immigrants live in the centers of in-

dustry, most cases were reported from cities and areas with high-density populations in Germany. The pronounced West-East divide we observed mirrors the much higher proportion of foreign-born people in western Germany compared with eastern Germany (10.1% versus 2.4%).

Regarding the age distribution in our study group, only 16% of the reported cases were <20 years of age. The age-specific incidence was highest in persons 60–69 years of age. These persons were mainly first-generation immigrants who keep in closer contact with family members still living in their homelands. A similar age-specific incidence distribution has been described in studies from other countries not endemic for brucellosis, whereas in brucellosis-endemic countries, cases of this disease do not cluster in a particular age class (*16*). We did not observe a male predominance in the working age group as in countries where brucellosis is strongly related to occupational exposure risks. In Germany, brucellosis has evolved into a foodborne disease, and unpasteurized goat cheese is the most frequently reported vehicle of exposure in our study population; thus, there is no reason to expect gender predominance. From 1995–2005, 2.9% of the cases reported were associated with *Brucella* infections in family members. The serologic screening of household members of brucellosis patients may therefore help to detect these frequently unrecognized cases with identical risk factors (*23*).

In Germany, ≈7% of the infections with a known source were laboratory acquired. *Brucella* spp. are among the most commonly recognized causes of laboratory-transmitted infections worldwide, but only 2% of all human cases are actually laboratory-acquired (*13*,*24*). This discrepancy may reflect that microbiologists in German laboratories are not aware of brucellosis as a possible case of fever of unknown origin because the disease is very rare in Germany. A low index of suspicion and misidentification of the organism may lead to a higher proportion of laboratory-associated infections.

Consistent with the literature, fever >38.5°C was the leading symptom in most (88%) of our patients. Osteoarticular manifestations are known to be the most frequent focal complications (*17*,*25*,*26*) and were reported in 43% of our cases. Key results of our study are the extensive diagnostic delay in brucellosis and the exceptionally high case-fatality rate. The degree of illness in patients with fever of unknown origin is directly related to the diagnostic delay. In 56% of the cases reported in Germany, symptoms lasted >4 weeks before diagnosis, and the mean diagnostic delay was 2.5 months. It is well documented that the number of focal complications increases with a diagnostic delay of >30 days and the risk for an unfavorable clinical course is much higher in patients with focal complications (*25*). In disease-endemic areas, the index of suspicion is high, and the duration of symptomatic disease before hospital admission is <2 weeks in 72% of the cases (*27*). In part, the increase in deaths observed in our study may be caused by a lack of suspicion by medical professionals. In addition, language barriers may hinder obtaining detailed medical histories from immigrants (*28*).

Most human brucellosis cases worldwide are caused by *B. melitensis* (*29*), which is also true in Germany (98% of all isolates). Most *B. melitensis* strains isolated in Germany are of the East-Mediterranean genotype (*30*), which is consistent with the epidemiologic data presented.

Conclusions

In Germany, brucellosis has emerged as a disease among Turkish immigrants. In this population group, the infection is associated with major diagnostic delays, possibly resulting in treatment failures, relapses, chronic courses, focal complications, and a high case-fatality rate. Because of a lack of knowledge on the changing epidemiology of the disease, many physicians may not be able to act efficiently as first responders recognizing natural or artificial outbreaks. Public health programs should therefore focus on educating the Turkish segment of the German population about the risks of consuming animal products imported from Turkey or unpasteurized cheese and other dairy products during visits to Turkey. In addition, healthcare providers should be informed about the disease, especially if they work in areas with a large Turkish population.

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Dr Al Dahouk is a physician at RWTH Aachen University, Germany. His main research interests are diagnosis, epidemiology, and pathogenesis of bacterial zoonotic diseases.

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Viable Newcastle Disease Vaccine Strains in a Pharmaceutical Dump

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To assess the viability of discarded and buried vaccine strains, we examined vaccines that had been buried for >20 years in an industrial waste dump in the city of Milan, Italy. Viability results showed potential biological risk associated with uncontrolled burial of pharmaceutical industry waste, including some live vaccines.

During most of the 20th century, biopharmacologic products, including vaccines, prophylactic serum, blood flasks, and animal-origin waste, were buried ≤1.5 m deep in the 12,000-m^2 waste dump of a pharmaceutical research institute in Milan (Istituto Sieroterapico Milanese [ISM]). ISM was founded in 1886 but bankrupt by 1994. This dump area was recently reclaimed after nearly a decade of abandonment.

Some of the material with potential biological risk, such as animal carcasses, has been decomposing in direct contact with the soil. Other material, including vaccines against human and animal diseases such as rabies, poliomyelitis, anthrax, and Newcastle disease (ND), were recovered in hermetically sealed vials, so their contents might have been totally or partially preserved. Our aim in this preliminary study was to assess the viability of the unearthed vaccines.

The Study

The reclamation procedure was performed under strict safety conditions. All operations were conducted under biocontainment tents that had air exchangers and extractors with filters. Full personal protective equipment, including biohazard suits, gas masks, and gauntlets, was provided to all workers. The amount of biopharmacologic waste removed was impressive (35,764 tons). In particular, large quantities of ND vaccines were recovered, in liquid and in lyophilized form; the estimated net weight of biological material was 20–25 kg.

Four vaccine types against ND (2 in liquid and 2 in lyophilized form) were unearthed from the dump. A review of documentation found that all 4 types had been produced by ISM from 1975 through 1988. Because the exact composition of the vaccines was unknown, the vial contents were subjected to viability tests in culture and to molecular characterization assays. The viability evaluations were performed by inoculation of the vial contents onto a confluent monolayer of Vero cells (African green monkey kidney), followed by observation of the cell culture for 7 days and assessment of any cytopathic effect (*1*). Each assay was performed twice. The positive control was a commercially available ND vaccine (Izovac; IZO S.p.A, Brescia, Italy), containing >10^6 50% embryo infectious dose live attenuated viruses.

As a result of bacterial contamination, no virus could be isolated from the 2 types of liquid vaccine. However, virus was isolated from the 2 lyophilized ND vaccine strains and caused cytopathic effect, which was further confirmed by hemoadsorbing assay (Figure 1). These lyophilized strains,

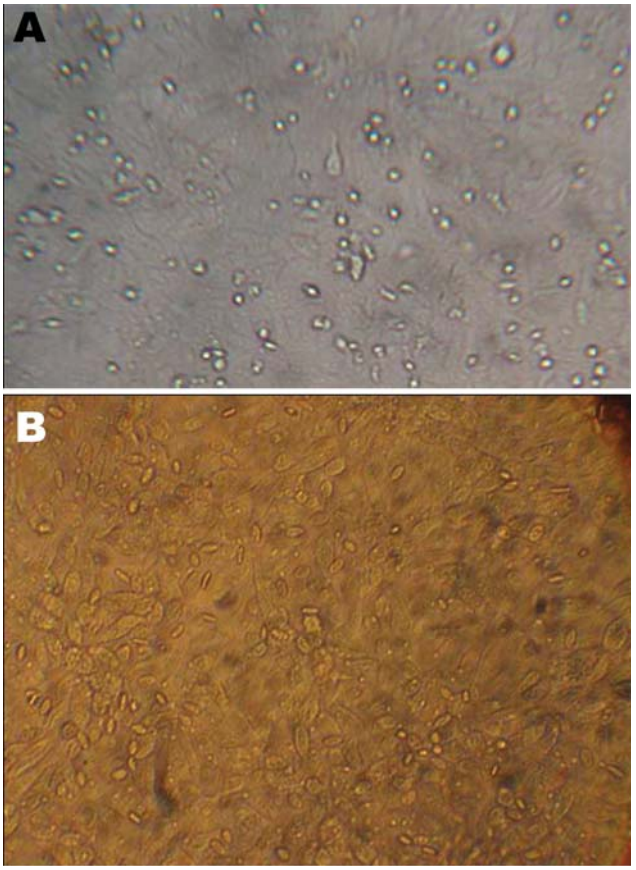

Figure 1. A) Cytopathic effect of lyophilized Newcastle disease virus (NDV) vaccine strains on Vero cells. B) Hemagglutination test. Presence of tear-shaped erythrocytes confirms the specificity of the cytopathic effect of NDV on Vero cells.

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which showed vitality in Vero cells, were then propagated in embryonated chicken eggs, the preferred substrate for ND virus (NDV) growth. Briefly, samples were inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs and further incubated at 36°C for 5 days. Eggs were subsequently chilled to 4°C, and the allantoic fluid was tested for hemagglutination (HA) activity to confirm the presence of viral replicaion (*2*). A 512-fold increase of HA titer was observed after infection (i.e., from 4 HA units in the whole, resuspended vaccine to 2.048 HA units in the allantoic fluid).

An additional part of the original vial content of the unearthed vaccines was used for molecular characterization. Viral RNA extracted from all 4 vaccine types (liquid and lyophilized) was subjected to sequencing of the genomic region, including the cleavage site, which is the determinant of virulence for NDV strains (*3*,*4*). For this purpose, vial content was subjected to genic amplification of a 615-bp fragment (*5*) of the F gene, which encodes for the fusion protein. The nucleotide sequence obtained was aligned with sequences in online databases by using ClustalX software (ftp://ftp.ebi.ac.uk/pub/software/unix/clustalx). The amino acid sequence was then predicted with BioEdit software (www.mbio.ncsu.edu/bioedit/bioedit.html), and a detailed analysis was performed on the cleavage site (Table). The phylogenetic tree of the F gene fragment (Figure 2) provided the vaccine strain's classification. The lyophilized samples contained La Sota–like strains classified as lentogenic strains. The liquid samples contained genomic sequences of the cleavage site characteristic of Herts/33-like strains (velogenic strains).

Conclusions

Vast areas that were once used as uncontrolled dumps for pharmaceutical industry waste still exist in many countries. One example is the waste dump of the ISM, which was 1 of the leading Italian companies in the field of research and development of vaccines against the infectious diseases most prevalent at the time, including diphtheria, smallpox, tetanus, anthrax, rabies, and poliomyelitis.

Table. Comparison of Newcastle disease virus sequences

Classification*	Amino acid sequence
Lentogenic	^{112}(G/E)(R/K)Q(G/E)RL^{117}
Velogenic	^{112}(R/K)RQ(R/K)RF^{117}
Strains†	**Nucleotide/amino acid sequence**
ISM-1, ISM-2	GGG AGA CAG GGG CGC CTT ^{112}G R Q G R L^{117}
ISM-3, ISM-4	AGG AGA CAG AGA CGG TTT ^{112}R R Q R R F^{117}

*Amino acid sequences at the F protein cleavage site of lentogenic and velogenic strains of Newcastle disease virus.
†Nucleotide/amino acid sequences of the samples recovered from a dump at Istituto Sieroterapico Milanese (ISM), Milan, Italy, and analyzed in this study. ISM-1 and ISM-2 (GenBank accession nos. EU082818 and EU082819, respectively) were recovered in lyophilized form; ISM-3 and ISM-4 (GenBank accession nos. EU082820 and EU082821, respectively) were recovered in liquid form. The cleavage site sequences of ISM-1 and ISM-2 are typical of lentogenic strains; the cleavage site sequences of ISM-3 and ISM-4 are typical of velogenic strains.

Figure 2. Phylogenetic analysis of Newcastle disease virus (NDV) vaccine strains unearthed from Istituto Sieroterapico Milanese (ISM), showing the phylogenetic placement of ISM-1 (EU082818), ISM-2 (EU082819), ISM-3 (EU082820), and ISM-4 (EU082818) based on partial F gene necleotide sequences. Sequences determined in this study are in **boldface.** ISM-1 and ISM-2 belong to La Sota–like cluster; ISM-3 and ISM-4 belong to Herts/33-like cluster. Sequence alignment was achieved with ClustalX version 1.81 (ftp://ftp.ebi.ac.uk/pub/software/unix/clustalx), with sequences from other NDV strains retrieved from GenBank (accession nos. indicated). The phylogenetic study was conducted by using MEGA version 3.1 (www.megasoftware.net). The phylogenetic tree was constructed with the neighbor-joining method.

Because of this unusual line of production, the reclamation activities of the ISM area took into consideration the potential biological risks caused by the buried and unprocessed waste. This first attempt in Italy to reclaim such an area yielded large quantities of biological material, including well-preserved vaccines and by-products of their manufacturing processes. Because data about the manufacturing and disposal procedures used at the time are missing, the recovery of such biological materials raises concerns about persistent biological activity. Moreover, veterinary vaccines, especially those in lyophilized form, may represent a relevant biological risk because they are often prepared with strains that have been attenuated for the target animals but not for humans.

This preliminary study considered the residual pathogenic potential of ND vaccines. ND is a viral infection of poultry, caused by an avian paramyxovirus serotype 1 (*6*), which may cause human disease and may pose a hazard to exposed workers (*7*,*8*). ND infections usually cause unilateral or bilateral reddening and edema of the eyelids, excessive lacrimation, conjunctivitis, and subconjunctival hem-

orrhage. ND infections are usually transient with no corneal involvement (*9*); however, severe complications leading to lasting vision impairment have been described (*10*).

The viability data of lyophilized strains of live NDV vaccines, conserved in hermetically sealed vials and buried for >20 years, showed that the strains had persisting replication ability in Vero cells and in embryonated chicken eggs. This residual vitality implies that manipulation of discarded vaccines may involve risk for infection. Molecular characterization of the F gene classified the lyophilized vaccines as derived from lentogenic strains. On the contrary, the cleavage site of the liquid vaccines contained genomic sequences characteristic of velogenic strains. That the liquid vaccine originally consisted of inactivated NDV or of velogenic strains (used in the past) attenuated by several passages in culture systems is possible.

This study indicates the existence of biological risk deriving from the uncontrolled burial of vaccines and their byproducts and underlines the absence of worldwide-accepted criteria defining the extent and persistence of biological risk–related biopharmacologic waste materials. The results of the study support the need to plan and perform rational reclamation operations in abandoned biopharmaceutical waste areas, implementing biocontainment strategies and personal and environmental safety measures. These measures are particularly necessary in those situations in which the buried material could contain highly infectious and pathogenic agents such as pox and anthrax, which were largely used by leading vaccine producers in the past century.

In conclusion, further studies are needed to fill knowledge gaps regarding disposed biological material. Such studies are warranted to evaluate the extent and the persistence of the infectious risk brought about by buried vaccines.

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Parachlamydia spp. and Related *Chlamydia*-like Organisms and Bovine Abortion

Nicole Borel,* Silke Ruhl,* Nicola Casson,† Carmen Kaiser,* Andreas Pospischil,* and Gilbert Greub†

Chlamydophila abortus and *Waddlia chondrophila* cause abortion in ruminants. We investigated the role of *Parachlamydia acanthamoebae* in bovine abortion. Results of immunohistochemical analyses were positive in 30 (70%) of 43 placentas from which *Chlamydia*-like DNA was amplified, which supports the role of *Parachlamydia* spp. in bovine abortion.

Chlamydiae are implicated in a wide variety of clinically and economically important diseases in livestock and companion animals. *Chlamydophila pecorum* has been associated with abortion, conjunctivitis, encephalomyelitis, enteritis, pneumonia, and polyarthritis in ruminants, and *Cp. abortus* infection is the most common cause of abortion in sheep and goats (*1*). *Cp. abortus* also causes zoonotic infection in humans, which in pregnant women, can result in spontaneous abortion (*2*,*3*).

During the past decade, new *Chlamydia*-like organisms have been discovered and now emerge as possible public health threats. *Simkania negevensis* is considered a possible emerging agent of pneumonia (*4*), and evidence supports the role of *Parachlamydia acanthamoebae* as an agent of pneumonia (*5*,*6*). *Waddlia chondrophila* is another *Chlamydia*-like organism initially isolated from lung, liver, and other tissues of an aborted bovine fetus in the United States (*7*). This organism is now considered an abortigenic agent with a worldwide distribution in cattle, as shown by a recent report of *Waddlia*-related abortion in Germany (*8*).

The role of *Chlamydia*-like organisms in bovine abortion is further supported by results of a study of abortion in cattle in Graubünden, Switzerland (*9*). Analysis of placental specimens by PCR showed that 43 (18.3%) of 235 placentas contained DNA from *Chlamydia*-like organisms (*9*). Of these 43 specimens, 8 showed sequence similarity to *P. acanthamoebae* (95%–99%). Identification was not possible in the remaining 35 specimens because of their strong sequence similarity with uncultured chlamydial DNA sequences (Table). These 35 specimens were referred to as *Chlamydia*-like organisms. None of these 35 specimens was positive by immunohistochemical analysis with antibodies against *Chlamydiaceae*. This finding indicates that routine diagnostic approaches based on chlamydial lipopolysaccharide would not detect most *Chlamydia*-like infections (*9*). To confirm the role of these novel chlamydiae in bovine abortion, we analyzed these placental samples from cattle in Switzerland by using a new specific immunohistochemical protocol and transmission electron microscopy.

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The Study

Formalin-fixed and paraffin-embedded placenta specimens were analyzed by using histopathologic and immunohistochemical techniques. Hematoxylin and eosin–stained histologic sections of all placenta specimens (n = 235) were examined for the type and degree of placentitis or vasculitis. Paraffin-embedded sections of specimens positive for *Chlamydia*-like organisms by 16S rRNA PCR (n = 43) were analyzed for *Parachlamydia* spp. and *Waddlia* by using specific mouse polyclonal antibodies as described (*10*). Optimization experiments for immunohistochemical analysis were performed by using infected amebal and infected HEp-2 cell pellets. Briefly, *Acanthamoeba castellanii* cultures were infected with *P. acanthamoebae* strain Hall coccus and *W. chondrophila* strain ATCC 1470. HEp-2 cell monolayers were infected with *Cp. abortus* strain S26/3. Uninfected cells were used as negative controls. Amebal and cell pellets were prepared as described (*11*). Optimization of the immunohistochemical protocol for experimentally infected amebal pellets showed the species specificity of mouse antibodies to *P. acanthamoebae* and *W. chondrophila*. We did not observe cross-reactivity of both antibodies with *Cp. abortus*–infected HEp-2 cell pellet (data not shown).

To test placental specimens, we used mouse polyclonal antibody against *P. acanthamoebae* and *W. chondrophila* at dilutions of 1:1,000 and 1:2,000, respectively. Antigen detection was performed with the ChemMate Detection Kit (Dako, Glostrup, Denmark) according to the manufacturer's instructions. Briefly, paraffin-embedded sections were deparaffinated in xylene and rehydrated through graded ethanol to water. Antigen was detected by using repeated microwave heating (750 W for 10 min) in citrate buffer, pH 6.0 (Target Retrieval Solution, Dako). Specimens (slides) and primary antibodies were incubated for 1 hour. Negative and positive controls of each section were included as described (*9*).

Histopathologic lesions such as purulent or necrotizing placentitis were observed in 149 (63.4%) of 235 specimens. Placentitis was observed in 5 of 8 specimens positive for *P. acanthamoebae*, and vasculitis was observed in 1 of 8 specimens (Table). Positive antigen labeling was observed

in 6 of 8 specimens for *Parachlamydia* spp., but antigen labeling was negative in all specimens for *Waddlia* (Table). The Figure, panel A shows positive immunohistochemical labeling in 1 of these specimens. Among the 35 placentas positive by PCR for *Chlamydia*-like organisms other than *P. acanthamoebae*, 28 (82.3%) showed obvious purulent or necrotizing placentitis by histologic analysis. Four of the 28 specimens with placentitis also had vasculitis. A total of 24 (68.6%) of 35 specimens were positive when tested with antibody against *P. acanthamoebae*, and all 35 specimens were negative when tested with antibody against *W. chondrophila*.

Two placental specimens positive for *Parachlamydia* spp. by immunohistochemical analysis and 16S rRNA

Table. Results of histologic, 16S rRNA sequence, and immunohistochemical analyses for 43 placentas positive for *Chlamydia*-like DNA by a 16S rRNA PCR*

Specimen	Histology		16S rRNA sequence†		Immunohistochemistry	
no.	Placentitis	Vasculitis	Species	% Similarity	*Parachlamydia* spp.	*Waddlia*
1	N	Yes	*Parachlamydia*	99	+	–
2	N	No	*Parachlamydia*	97	+	–
3	P/N	No	*Parachlamydia*	98	+	–
4	P/N	No	*Parachlamydia*	97	–	–
5	P/N	No	*Parachlamydia*	97	–	–
6	A	No	*Parachlamydia*	96	+	–
7	A	No	*Parachlamydia*	96	+	–
8	A	No	*Parachlamydia*	97	+	–
9	P/N	Yes	*Chlamydia*-like	92	–	–
10	P/N	Yes	*Chlamydia*-like	92	–	–
11	P/N	Yes	*Chlamydia*-like	93	–	–
12	P/N	Yes	*Chlamydia*-like	91	+	–
13	P/N	No	*Chlamydia*-like	82	+	–
14	P/N	No	*Chlamydia*-like	91	+	–
15	P/N	No	*Chlamydia*-like	92	+	–
16	P/N	No	*Chlamydia*-like	92	+	–
17	P/N	No	*Chlamydia*-like	92	+	–
18	P/N	No	*Chlamydia*-like	92	+	–
19	P/N	No	*Chlamydia*-like	92	+	–
20	P/N	No	*Chlamydia*-like	92	+	–
21	P/N	No	*Chlamydia*-like	93	+	–
22	P/N	No	*Chlamydia*-like	94	+	–
23	P/N	No	*Chlamydia*-like	95	+	–
24	P/N	No	*Chlamydia*-like	100	+	–
25	P/N	No	*Chlamydia*-like	93	–	–
26	P/N	No	*Chlamydia*-like	93	–	–
27	P/N	No	*Chlamydia*-like	95	–	–
28	P/N	No	*Chlamydia*-like	96	–	–
29	N	No	*Chlamydia*-like	85	+	–
30	N	No	*Chlamydia*-like	88	+	–
31	N	No	*Chlamydia*-like	88	+	–
32	N	No	*Chlamydia*-like	91	+	–
33	N	No	*Chlamydia*-like	91	+	–
34	N	No	*Chlamydia*-like	95	+	–
35	P	No	*Chlamydia*-like	91	+	–
36	P	No	*Chlamydia*-like	94	+	–
37	A	No	*Chlamydia*-like	91	+	–
38	A	No	*Chlamydia*-like	92	+	–
39	A	No	*Chlamydia*-like	92	+	–
40	A	No	*Chlamydia*-like	91	–	–
41	A	No	*Chlamydia*-like	92	–	–
42	A	No	*Chlamydia*-like	93	–	–
43	A	No	*Chlamydia*-like	95	–	–

*When partial 16S rRNA sequence showed a similarity ≥95% with a recognized species (i.e., *Parachlamydia acanthamoebae*), the corresponding genus was reported (i.e., *Parachlamydia* spp.). Conversely, when the sequence showed a best BLAST (www.ncbi.nlm.nih.gov) hit with uncultured or uncharacterized *Chlamydia*-related organisms, the sequence was designated as being similar to a *Chlamydia*-like organism. N, necrotizing; +, positive; –, negative; P, purulent; A, autolysis.
†A 278-bp fragment was amplified and sequenced (*9*).

PCR were further investigated by transmission electron microscopy for ultrastructural evidence of *Chlamydia*-like organisms. Briefly, placental tissue specimens were fixed with glutaraldehyde and osmium tetroxide and embedded in Epon resin. Ultrathin sections (80 nm) were mounted on gold grids (Merck Eurolab, Dietlikon, Switzerland), contrasted with uranyl acetate dihydrate (Fluka, Buchs, Switzerland) and lead citrate (lead nitrate and tri-natrium dehydrate, Merck Eurolab), and analyzed with a Philips (Eindhoven, the Netherlands) CM10 electron microscope. Both placentas showed *Chlamydia*-like structures (Figure, panel B).

Conclusions

To our knowledge, this is the first description of *Parachlamydia* spp. in bovine abortion. The organism was detected by PCR (*9*) and within placental lesions by immunohistochemical analysis by using an antibody specific for *Parachlamydia* spp. and electron microscopy. All specimens were negative for *Waddlia* by immunohistochemical analysis. Isolation of *Parachlamydia* spp. from aborted bovines is necessary to confirm that this agent causes bovine abortion. *Parachlamydia* spp. may be involved in lower respiratory tract infections in humans (*5*,*6*) and may replicate within both pneumocytes (*13*) and human macrophages (*14*). Thus, caution should be taken when handling bovine abortion material because of the potential zoonotic risk.

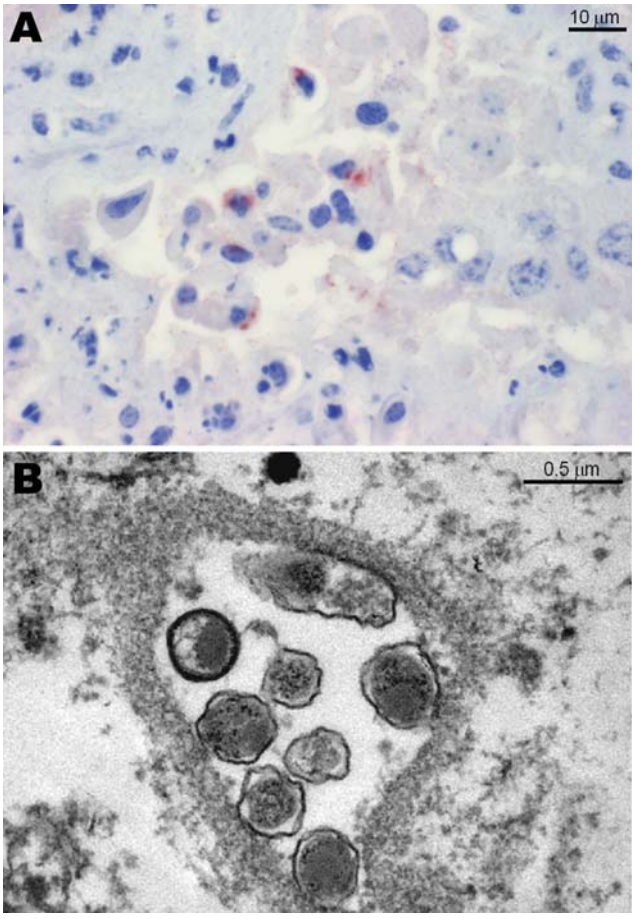

Figure. A) Immunohistochemical analysis of a bovine placenta positive by PCR for *Parachlamydia acanthamoebae*, showing a positive brown-red granular reaction within trophoblastic epithelium. Antigen detection was conducted with a polyclonal antibody against *Parachlamydia* spp. (3-amino-9-ethylcarbazole/peroxidase method, hematoxylin counterstain). B) Transmission electron micrograph of bovine placenta positive by PCR and immunohistochemical analysis for *P. acanthamoebae*, showing 7 cocci-shaped bacteria in an inclusion with morphologic features similar to those of *Chlamydia*-like organisms (*12*).

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Escherichia coli O157:H7 in Feral Swine near Spinach Fields and Cattle, Central California Coast[1]

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We investigated involvement of feral swine in contamination of agricultural fields and surface waterways with *Escherichia coli* O157:H7 after a nationwide outbreak traced to bagged spinach from California. Isolates from feral swine, cattle, surface water, sediment, and soil at 1 ranch were matched to the outbreak strain.

Recent experimental and epidemiologic studies suggest that domestic pigs are biologically competent hosts and a potential reservoir of *Escherichia coli* O157:H7 (*1*,*2*). Cattle are considered the primary reservoir of *E. coli* O157, but fecal shedding by other domestic livestock and wildlife has been described (*3*,*4*). *E. coli* O157 was isolated from a wild boar in Sweden, but there is limited information on its occurrence in feral swine in the United States (*5*). We report findings from an environmental and laboratory investigation after a nationwide spinach-associated outbreak of *E. coli* O157 in which the outbreak strain was isolated from feral swine and other environmental samples.

The Study

In September 2006, an outbreak of *E. coli* O157 was linked to consumption of fresh, bagged, baby spinach, with 26 states and Canada reporting 205 cases of illness and 3 deaths (*6*). Contaminated product was traced to 1 production date (August 15, 2006) at 1 processing plant and fields located on 4 ranches on the central California coast (*7*). The outbreak strain was isolated initially from cattle feces collected on September 27, 2006, ≈1 mile from an implicated spinach field on a ranch (ranch A) where numerous free-roaming feral swine were observed. We investigated potential involvement of feral swine in *E. coli* O157 contamination of spinach fields and surface waterways.

Feral swine were live-captured in traps or hunted and humanely killed during October–November 2006. Two feral swine corral traps were placed 1.4 km apart, and 1.7 km (trap 1) and 1.2 km (trap 2), respectively, from the implicated spinach field (Figure 1). Photographs from digital infrared remote-sensing cameras (Recon Outdoors, Huntsville, AL, USA) were used in combination with sightings and live-capture to ascertain the minimum number of individual feral swine present on the ranch (*8*). The average population density was calculated on the basis of an estimate of the area sampled by both traps and the estimated mean home range (1.8 km) for feral swine in mainland California by using ArcView version 9.2 (Environmental Systems Research Institute, Redlands, CA, USA) (*8*).

Colonic fecal samples were collected from 40 feral swine (31 live-captured, 9 hunted); buccal swabs, rectal-

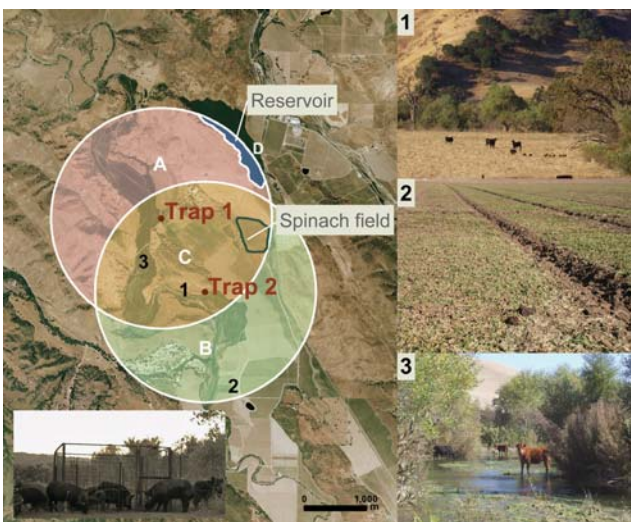

Figure 1. Left: aerial (2 m) photograph of ranch A showing overlapping circular buffer regions around feral swine trap 1 and trap 2 (San Benito Crop Year 2006; Image Trader, Flagstaff, AZ). The radius for the buffer (1.8 km) is the circumference of the mean home range for feral swine in mainland California (*8*). Estimated density = 4.6 swine/km^2 and total area = (A + B + C) – D = 14.8 km^2. Areas A, B, and C, combined with counts of individual feral swine from October through November 2006, were used to calculate the average population density. Bottom left: digital infrared photograph of feral swine at trap 1. Right: potential risk factors for *Escherichia coli* O157:H7 contamination of spinach at ranch A: 1) Feral sow and piglets sharing rangeland with cattle; 2) feral swine feces, tracks, and rooting in a neighboring spinach field; 3) cattle in surface water.

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anal swabs, and tonsils were analyzed from a subset of 8 animals (Table 1). Additionally, feces from domestic animals (cattle, dog, goat, horse, sheep) and wildlife (bird, coyote, deer, feral swine), surface water and sediment, soil, and well/irrigation water were analyzed. *E. coli* O157 was cultured by using an extended enrichment–immunomagnetic separation protocol (*9*,*10*). PCR analysis was used to confirm the presence of *E. coli* O157 and virulence factors (*9*,*10*). Genotypes of isolates from environmental samples were compared by using 10-loci multilocus variable number tandem repeat analysis (MLVA) and pulsed-field gel electrophoresis (PFGE) after digestion with *Xba*I and *Bln*I by using the PulseNet protocol (*10*–*13*).

E. coli O157 was cultured from 45 (13.4%) of 335 samples, including cattle and feral swine feces, feral swine colonic feces from necropsy, surface water and sediment, and pasture soil (Table 1). The *eaeA*, *hlyA*, and *stx2* genes were present in all strains, and the *stx1* gene was found in only 1 sample (subtype 5; Table 2, Figure 2). Isolates from 28 environmental samples at ranch A were indistinguishable from the major spinach-related outbreak strain by PFGE (Table 1). In contrast, *E. coli* O157 isolates from 3 other ranches implicated by traceback did not match the outbreak strain. Molecular typing by MLVA provided higher resolution discrimination between environmental strains (Figure 2). Three major MLVA clusters from ranch A and the surrounding watershed were identified. The cluster containing the outbreak strain (subtype E) is shown in Figure 2, and 16 other highly related subtypes were indistinguishable by PFGE (Table 2).

Ranch A is located in the central coast foothills of San Benito County, where the dominant habitat is coastal oak woodland interspersed with dense riparian vegetation near seasonal waterways (Figure 1). Approximately 2,000 range cattle were grazed on the ranch. Spinach and other leafy green vegetables were grown on a leased portion of the property that was separated from cattle pastures by wire mesh fence. Well water was used for irrigation. No evidence of cattle manure–based fertilizer application, runoff from cattle pastures, or flooding from surface waterways (based on topography) onto the implicated spinach field was found during the investigation (*7*).

Feral swine were the most abundant wildlife observed on ranch A, and evidence of intrusion, including tracks, rooting, or feces in crop fields and adjacent vineyards, was documented (Figure 1). Birds, black-tailed deer, cottontail rabbits, coyotes, and ground squirrels also were observed, but the population density of these species appeared lower, and their activity was confined mostly to rangeland areas according to visual observations. Swine visited the traps almost continuously from dusk until dawn with peak activity between 5:00 PM and midnight. An average of 3.6 swine/trap/night were live-captured. The estimated population density was 4.6 swine/km^2 (95% confidence interval [CI] 3.8–5.9), and the actual number of feral swine on ranch A was estimated to be 149 animals (95% CI 124–192) (Figure 1). Feral swine used livestock rangelands and gained access to adjacent crop fields through gaps formed at the base of the fence by erosion and rooting. Cattle and feral swine had access to and congregated at surface waterways on the ranch (Figure 1).

Table 1. *Escherichia coli* O157:H7 isolated from environmental samples collected at ranch A, California, September–November 2006

Sample type	No. tested	No. positive (%)	No. matches*
Cattle feces	77	26 (33.8)	15
Cattle water trough	10	0	NA
Compost (chicken pellets)†	1	0	NA
Feral swine			
Necropsy	40	2 (5)	2
Buccal swab	8	0	NA
Colonic feces	40	2 (5)	2
Rectal-anal swab	8	0	NA
Tonsil	8	0	NA
Feces from ground	47	11 (23.4)	6
Subtotal	87	13 (14.9)	8
Other animal specimens‡	26	0	NA
Surface water§	79	3 (3.8)	2
Soil/sediment	37	3 (8.1)	3
Well/irrigation water¶	18	0	NA
Total	335	45 (13.4)	28

*No. samples indistinguishable from the major spinach-related outbreak strain by pulsed-field gel electrophoresis (*Xba*I-*Bln*I PulseNet profile EXHX01.0124-EXHA26.0015). NA, not applicable.
†Commercial, heat-treated chicken manure.
‡Included feces from coyote (n = 1), deer (n = 4), dog (n = 1), horse (n = 2), sheep/goat (n = 3, composite), waterfowl (n = 2), unknown species (n = 11), and owl (n = 2).
§Surface water (rivers, streams, ponds) was sampled by collection of 100-mL grab samples or placement of a modified Moore swab for 4–5 d.
¶Well water was sampled from 3 wells or sprinkler heads by collection of 100-mL or 1,000-mL grab samples or by concentration of 40,000 mL to 500 mL by using ultrafiltration (*7*).

Table 2. Unique alphanumeric MLVA types of *Escherichia coli* O157:H7 isolated from environmental samples collected at ranch A and an upstream watershed, California, September–November 2006*

Sample type	No. samples	No. isolates	MLVA type
Reference (human stool, bagged spinach)	NA	NA	**E**
Cattle feces	26	34	**A**, **C**, **E**, **F**, I, **J**, L, **M**, P, Q, **R**, **S**, **T**, W, X, Z
Feral swine feces	11	14	**A**, **B**, **C**, **E**, L, O, P, X, 5, 6
Feral swine colonic feces (necropsy)	2	10	**A**, **C**, **D**, **G**, **H**, **K**, L, **U**, V, Y
Sediment (river)	2	8	**A**, **C**, L, **M**, **N**, W, **3**
Soil (cattle pasture)	1	1	**A**
Surface water	3	6	**A**, **C**, L, P, 4
Surface water Moore swab†	2	3	1, 2

*MLVA, multilocus variable number tandem repeat analysis; NA, not applicable. Samples indistinguishable from the major spinach-related outbreak strain by pulsed-field gel electrophoresis (*Xba*I-*Bln*I PulseNet profile EXHX01.0124-EXHA26.0015) are shown in **boldface**.
†Isolates collected from surface water (river) ≈32 km upstream of ranch A.

Conclusions

We describe the first, to our knowledge, isolation of *E. coli* O157 from feral swine in the United States. The percentage of specimens positive for *E. coli* O157 among feral swine (14.9%) and cattle (33.8%) and the density (4.6 swine/km^2) were high compared with results of previous ecologic studies (Table 1) (*2–5,8,14,15*). Molecular typing of isolates by PFGE and MLVA showed possible dissemination and persistence of the outbreak strain in multiple environmental samples as long as 3 months after the outbreak (Tables 1, 2). MLVA is more reproducible than PFGE and better at discriminating between closely related *E. coli* O157 isolates (*10,12,13*). Recovery of related *E. coli* O157 subtypes by both methods suggested swine-to-swine transmission, interspecies transmission between cattle and swine, or a common source of exposure such as water or soil (Table 2, Figure 2).

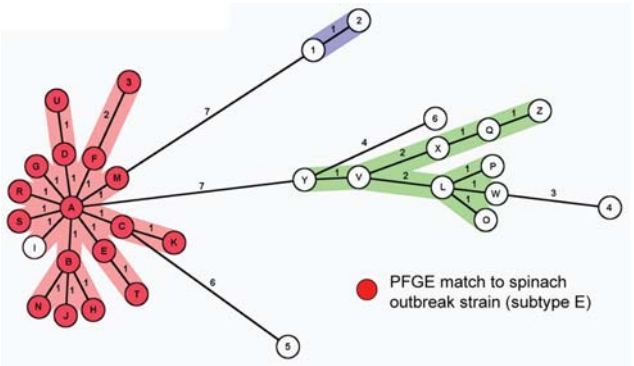

Figure 2. Minimum spanning tree analysis of multilocus variable number tandem repeat analysis (MLVA) data of 76 *Escherichia coli* O157:H7 strains typed from 47 samples compared with the spinach-related outbreak strain (subtype E). A categorical coefficient and the BURST priority rule of the highest number of single-locus changes were used for the clustering (Bionumerics software version 4.601, Applied Maths, Austin, TX, USA). Circles representing unique MLVA types are designated by an alphanumeric value (Table 2). Numbers between circles represent summed tandem-repeat differences between MLVA types (*10*). The shaded areas (red, green, and blue) denote genetically related clusters with MLVA differences <3. Red circles indicate types comprising isolates that were indistinguishable from the spinach-related outbreak strain (subtype E) by pulsed-field gel electrophoresis (PFGE).

Mechanisms of in-field contamination of leafy greens for this and previous outbreaks remain unclear, but hypotheses have emerged. A relatively high density of feral swine near cattle and spinach fields could represent a risk factor for *E. coli* O157 contamination. Wildlife may be sentinels for *E. coli* O157 in the produce production environment, or they may be vectors involved in the contamination of plants directly by fecal deposition or indirectly by fecal contamination of surface waterways or soil. Notably, baby spinach is harvested with a lawn mower–like machine that could pick up fecal deposits in the field and thereby contaminate large volumes of product during processing. Fecal loading of surface waterways by livestock and wildlife with subsequent contamination of wells used for irrigation represents another possible route of transmission to plants in the field. Although *E. coli* O157 was not detected in irrigation water, older agriculture wells at ranch A appeared vulnerable to contamination by surface water (R. Gelting, pers. comm.). Unrecognized environmental and management practices during preharvest and postharvest processing also could have contributed to amplification and dissemination of *E. coli* O157 in raw spinach.

In summary, *E. coli* O157 contamination of spinach and other leafy greens is likely a multifactorial process. Additional research is needed to develop and implement effective risk assessment and management practices. For example, studies are needed to determine colonization potential of and levels of fecal shedding by feral swine, and the importance of interspecies transmission to other vertebrate or invertebrate (e.g., flies) populations near agricultural fields.

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Crow Deaths Caused by West Nile Virus during Winter

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In New York, an epizootic of American crow (*Corvus brachyrhynchos*) deaths from West Nile virus (WNV) infection occurred during winter 2004–2005, a cold season when mosquitoes are not active. Detection of WNV in feces collected at the roost suggests lateral transmission through contact or fecal contamination.

In the northern United States, West Nile virus (WNV) is thought to overwinter in hibernating mosquitoes (*1*). Because reports of birds dying of WNV infection during the winter are rare, we investigated the cause of crow deaths in New York during the winter of 2004–2005.

The Study

Dead crows from a roost were reported to the Dutchess County Department of Health in December 2004 (Figure). The roost was located in coniferous and deciduous trees at the east end of the Mid-Hudson Bridge, Poughkeepsie, New York, USA. Because winter surveillance in Poughkeepsie had not previously confirmed WNV, the crows were not collected for testing.

However, after the third dead crow in January was reported, ground surveillance of the roost was initiated (Figure). Thereafter, carcasses were collected 4–5 times per week at a radius of 1/4 mile around the roost and were transported for necropsy to the New York State Department of Environmental Conservation. On March 1, 2005, the roost, culverts, and areas under the bridge were examined for overwintering mosquitoes. Temperature data from December 1, 2004, to March 31, 2005, were obtained from the National Oceanic and Atmospheric Administration, Silver Spring, Maryland, USA.

Oral swabs were collected from carcasses and screened by using VecTest (Medical Analysis Systems, Freemont, CA, USA) and Rapid Analyte Measurement Platform (RAMP; Response Biomedical Corp, Burnaby, British Columbia, Canada) (*3*,*4*). Brain tissue was submitted to the New York State Department of Health (NYSDoH) for testing by TaqMan reverse transcription–PCR (RT-PCR) and standard RT-PCR (*2*,*5*). When possible, blood clots were collected from heart chambers for antibody testing by ELISA (*6*). Ectoparasites were collected from some carcasses before necropsy and tested for WNV by TaqMan RT-PCR (*2*).

To characterize this WNV genotype, RNA was extracted from the homogenate of a WNV-positive crow kidney (strain 05000918) by using RNeasy (QIAGEN, Valencia, CA, USA). The envelope coding region was amplified in 3 overlapping fragments by using QIAGEN One-Step RT-PCR core kit. DNA was sequenced at the Wadsworth Center Molecular Genetics Core facility by using ABI 3100 or 3700 automated sequencers (Applied Biosystems, Foster City, CA, USA). We generated the sequence (GenBank accession no. DQ823132) by using the SeqMan module within Lasergene (DNASTAR, Madison, WI, USA) and compared it with previously characterized North American strains by using MegAlign within Lasergene.

We collected 45 fecal specimens from 12 sampling points in the roost and 10 from beneath 2 carcasses. Specimens were tested for WNV RNA by using TaqMan and standard RT-PCR (*2*) with minor modifications; 100 mg of each specimen was diluted in 1.0 mL of BA-1, homogenized, centrifuged, and sterile filtered. RNA was extracted from the filtrate by using RNeasy (QIAGEN), and RT-PCR was conducted.

From February 10 to March 29, 98 carcasses were collected from the roost area; of these, 12 (12.2%) were WNV-positive according to VecTest and RAMP and 13 (13.3%) were positive according to TaqMan RT-PCR (Figure). The crow isolate was characterized as the WN02 genotype (*7*).

Necropsy and histopathologic findings on WNV-positive crows (n = 13) were consistent with previously reported pathologic findings (*8*). Necropsy findings included low body weight (84.6%), enlarged spleen (23.1%), and enlarged liver (30.8%); histopathologic findings included slight to moderate encephalitis with mild, diffuse gliosis and occasional small foci of necrosis in the gray matter of the brain. Meningoencephalitis, characteristic of WNV-positive birds (*8*), was not observed. WNV-negative crows (n = 85) died from traumatic injuries (51.8%), predation (16.5%), avian pox (14.1%), pneumonia (11.8%), and poisoning (5.9%). Two pools of >20 lice (*Philopterus* spp.: *Mallophaga*) from 6 WNV-positive birds and 1 pool from 1 WNV-negative bird were tested; 6 positive pools were detected from 4 positive birds.

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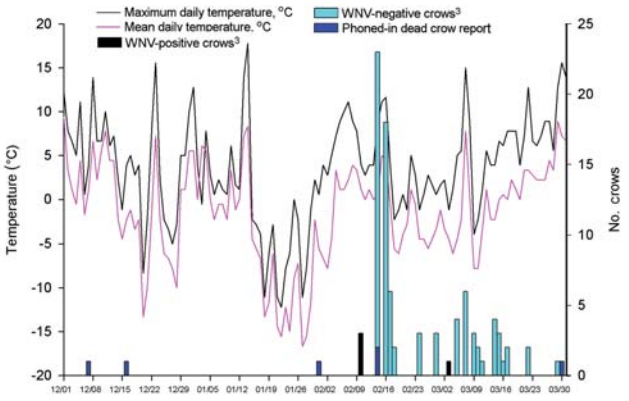

Figure. Crow deaths associated with West Nile virus (WNV) infection and maximum and mean temperatures for Poughkeepsie, New York, USA (December 1, 2004–March 31, 2005). Roost area was checked for crow carcasses at least every 48 hours after February 10, 2005. Temperature data were obtained from National Oceanic and Atmospheric Administration, Silver Spring, Maryland, USA. All 98 crow carcasses were tested for WNV by reverse transcription–PCR (RT-PCR) (*2*), VecTest, and Rapid Analyte Measurement Platform (*3,4*). Twelve were positive by all 3 tests; 1 crow collected on March 7, 2005, was positive by RT-PCR only.

All 56 blood clots collected were seronegative by ELISA for flavivirus antibodies. Of the 45 fecal samples, 3 were WNV-positive; 2 of these (1 collected from beneath a WNV-positive crow; 1 from a random roost sampling point) had >800 pfu/mL, according to extrapolation from TaqMan RT-PCR.

No mosquito hibernacula were located in the areas examined, and no mosquito activity was observed by field workers. Maximum daily temperatures were ≥10°C for 6 days in December, 4 days in January and February, and 5 days in March; mean temperatures were <10°C throughout the epizootic (Figure).

Conclusions

How WNV crow infections occurred during winter in New York when mosquito activity would have been limited is unclear (Figure). Reporting of crow carcasses can be as low as 10%; therefore, additional carcasses may have been observed and not reported before ground surveillance began (*9*). Initial crow infections could have occurred in November, when mean monthly temperature was ≥10°C and mosquito infection was more probable. Maximum daily temperatures ≥10°C occurred sporadically from December through March. However, mean temperatures remained at <10°C (Figure) and photoperiods at <12 h/day. Laboratory studies of wild-captured *Culex pipiens* L. females, the primary WNV vector in the northeastern United States, have shown that *Cx. pipiens* are unlikely to terminate diapause with photoperiods of <12 h/day and temperatures <10°C (*10*). Field studies in New York have shown that *Cx. pipiens* remain in overwintering locations until mid-April, at which time photoperiods are ≥12 h/day and mean temperatures ≥10°C (C. Drummond, NYSDoH Arbovirus Laboratories, unpub. data).

These winter deaths suggest a pattern of crow-to-crow transmission. WNV has been detected in blood–feather pulp of crows (*3*), and WNV-positive lice (*Philopterus spp.*) were collected from 4 WNV-positive crows. Research is needed on the risk for bird-to-bird viral transmission posed by ectoparasites, particularly to roost mates and nestlings. Scavenging of infected birds as a risk factor is supported by laboratory studies demonstrating WNV infection in crows after they ingested infected house sparrows (*Passer domesticus*) (*11*) and by chronic WNV infection in house sparrows and other bird species (*12*). Chronic infection in crows is unlikely given that laboratory studies have demonstrated 100% mortality rates within 5 days of infection (*11*).

Crow-to-crow transmission of WNV is supported by laboratory findings of fecal-shed WNV and contact transmission (*11*,*13*) and by WNV-positive results from oral and cloacal swabs used in VecTest and RAMP (*3*,*4*). In laboratory studies, crows shed WNV fecal titers as high as $10^{8.8}$ pfu/g (*13*). Our study provides the first evidence of fecal-shed WNV in the wild. In Illinois, healthy and WNV-infected crows roosted communally in summer (*14*); however, no additional evidence linked viremic crows and subsequent crow infections. Further study is needed on the role of summer and winter roosts and feces in the WNV transmission cycle. No human cases are known to be related to exposure to crow feces, although avoiding feces and wearing gloves when handling live or dead birds are recommended.

The role of birds in arbovirus overwintering and dissemination during migration has been suggested but is poorly understood. The last WNV-positive crow in this study was collected on March 29 as the roost was dispersing. Additional crows could have been infected before migrating to home territories. Radio-marked crows infected with WNV have traveled up to 4 km per night during the 5 days before they died (*14*). Thus, infected birds could transport the virus to new areas with active mosquitoes and contribute to the beginning of the WNV transmission cycle. We recommend additional study of winter WNV activity in crows and other bird species to determine their potential roles in arbovirus overwintering and the initiation of transmission when mosquitoes become active.

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Seroconversion in Wild Birds and Local Circulation of West Nile Virus, Spain

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A serosurvey for neutralizing antibodies against West Nile virus (WNV) in common coots (*Fulica atra*) was conducted in Doñana, Spain. Antibody prevalence was highest in 2003, intermediate in 2004, and lowest in 2005. Some birds seroreverted <1 year after first capture. Seroconversion of birds suggests local circulation of the virus.

In western Mediterranean countries, the frequency of outbreaks of West Nile virus (WNV) infection has increased in recent decades. Evidence for WNV circulation in Spain has remained elusive, although WNV foci have recently been identified in 3 neighboring countries (Morocco, Portugal, and France) (*1*–*3*). Recent WNV activity in Spain has been shown by serologic screening in humans, with detection of WNV-specific immunoglobulin M (*4*) and identification of the first clinical case in 2004 (*5*). In avian hosts, WNV-neutralizing antibodies have been found in chicks of wild migratory birds in southern Spain (*6*). However, interpretation of serologic data is not straightforward because antibodies in chicks may be the result of maternal transmission through eggs (*7*). To ascertain local circulation of WNV in Spain, we designed a capture-recapture study in which serum samples from wild birds were obtained at different times.

The Study

We focused on the partially migratory common coot (*Fulica atra*) because of its high seroprevalence for WNV detected during a preliminary screening of 72 bird species (J. Figuerola et al., unpub. data). Reasons for this high seroprevalence remain unclear, although preference of this bird for mosquito-rich habitats and its relative size (weight ≈800 g) might be involved in this pattern. Birds were captured in Doñana (37°6′N, 6°9′W) in a walk-in trap in October 2003 (3 capture sessions) and from September through February in 2004–2005 (12 sessions) and 2005–2006 (14 sessions). Overall, 853 captures of 515 different birds were conducted (1–7 captures/bird).

Blood was obtained from the tarsal vein and allowed to clot, and serum was stored at –20°C. All birds were marked with numbered metal rings. Age was determined by plumage characteristics before the birds were released. Neutralizing antibody titers for WNV (strain Eg101) were determined by using a micro-virus neutralization test as described (*6*). Only birds that showed neutralization (absence of a cytopathic effect) at dilutions ≥1:20 were considered seropositive. Controls for cytotoxicity in the absence of virus were included for every sample at a 1:10 dilution. Cytotoxic samples were excluded from the analysis.

Seroconversion was defined as a bird that was seronegative when first captured and became seropositive at recapture with an antibody titer that had increased 4-fold (*8*). Seroreversion was defined as a seropositive bird whose antibody titer decreased below the cut-off value of 20 at recapture. The interassay coefficient of variation of titers, expressed as $\log_{10}$ (calculated using an internal control repeated in 5 different assays, mean 2.56, standard deviation 0.35) was 13.67%. This variation is similar to that observed in individual samples and repeated in different assays. In a series of 27 samples tested twice, the mean fluctuation observed was 0.29 $\log_{10}$ units (≈2-fold). To obtain accurate measurements of titers, particularly when assessing seroconversion/seroreversion, we analyzed samples at least twice, and when results differed, they were assayed again until a consistent result was obtained. Specificity of the test was assessed by parallel neutralization against Usutu virus (strain SAAR 1776), a flavivirus found in wild birds that belongs to the same serogroup as WNV, with a panel of sera positive for WNV by micro-virus neutralization test. All titers were higher for WNV than for Usutu virus; 93.6% were ≥4× higher (Table 1). These results suggested that the neutralizing antibody response was generated by WNV or an antigenically related WNV-like virus.

Comparisons between years were restricted to data from October, the only month sampled in all 3 years. For analysis of variation in antibody prevalence within seasons, data were grouped into 2-month intervals. Prevalence was analyzed by generalized linear models with binomial distributed error, logit link, and randomly choosing 1 observation per bird.

Table 1. Antibody titers against West Nile virus (WNV) and Usutu virus in 47 serum samples from common coots, Doñana, Spain

	Usutu virus titer					
WNV titer	Negative	20	40	80	160	320
20	11	–	–	–	–	–
40	12	2	1	–	–	–
80	10	3	–	–	–	–
160	2	1	1	–	–	–
320	2	2	–	–	–	–

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Prevalence of WNV-neutralizing antibodies was highest in October 2003, intermediate in October 2004, and lowest in October 2005 (χ^2 22.80, df 2, p<0.0001, p<0.05 for all pairwise comparisons) (Figure 1). Juvenile (≤1 year of age) birds had lower antibody prevalences than adults in October (χ^2 7.14, df 1, p = 0.008). Antibody prevalence increased throughout the 2004–2005 season (χ^2 8.45, df 2, p = 0.02), but not during the 2005–2006 season (χ^2 1.10, df 2, p = 0.58) (Figure 1).

Of 95 birds captured in 2 consecutive years, 59% had no detectable antibodies in either year, 21% seroreverted, 6.3% seroconverted, and 13.7% had antibodies in both years. Seroconversion confirms that WNV circulation is present in the study area, and seroreversion indicates that antibody titers decreased. Antibodies persisted for >1 year in some birds, although whether this was caused by reinfection, which would stimulate the antibody response, is uncertain.

Of 54 birds captured at least twice in 2004–2005, 16.7% seroconverted (Table 2), 3.7% seroreverted, 46.2% never had any detectable antibodies, and 33.3% had antibodies whenever captured. This high rate of seroconversion, together with the few seroreversions observed, resulted in high seroprevalence, which reflects high WNV activity during this period. In 2005–2006, of 114 birds, 8.8% seroconverted, 15.8% seroreverted, 65.8% never had any detectable antibodies, and 2.6% had antibodies whenever captured. Antibody prevalence decreased in 2005–2006 (Figure 1), and antibody titers decreased to values near the cut-off point (Figure 2), which made changes in antibody status difficult to interpret. However, the most likely reason for these changes were fluctuations in titers (from undetectable to 10 to 20) (Figure 2) because 7% of the birds showed changes in titers (from 10 to 20) at recapture.

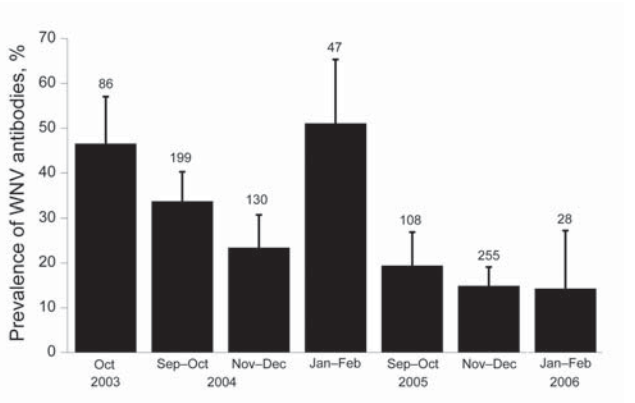

Figure 1. Prevalence of common coots with neutralizing antibodies against West Nile virus (WNV), Doñana, Spain, 2003–2006. Numbers above bars indicate sample size for each period. Error bars show 95% confidence intervals.

Conclusions

We provide evidence for local circulation of WNV in our study area during 2004–2005. This evidence was obtained just a few months after a reported outbreak of WNV that affected humans in Algarve, Portugal, ≈100 km west of our study area. However, no increase in clinical signs or mortality rates was observed in the common coot population during the study period. The high prevalence of antibodies in juvenile birds in September–October 2003 (37.5%) and 2004 (28.8%) also suggests that WNV may have been circulating during summer and autumn of 2003 and 2004.

WNV circulation decreased to low levels or was absent during the 2005–2006 winter season. There are several nonexclusive explanations for this pattern. First, the virus may not easily overwinter in Spain and thus needs to be reseeded each spring by migratory birds arriving from Africa. Nevertheless, climatic conditions probably enable the virus to survive winter because mosquitoes are present year round in the area (Servicio de Control de Mosquitos, unpub. data), and seroconversion in common coots occurred by midwinter. Second, in 2005, a severe drought reduced habitat for both mosquitoes and waterbirds. Third, high seroprevalence at the end of the winter of 2005 would have resulted in effective herd immunity, which may have reduced the number of available hosts in 2006 and transmission intensity. Although this negative loop is only valid if the rate of host population turnover is low (*9*), the scarcity of immunologically naive juvenile birds during 2005–2006 makes this a reliable alternative.

Table 2. Seroconversion results for antibodies to West Nile virus in 9 common coots, Doñana, Spain, 2004–2005

Bird ring no.	Age	Date of capture before seroconversion	Date of recapture (antibody titer)	No. days between captures
7060424	Juvenile	2004 Sep 30	2005 Feb 17 (40)	130
7060486	Adult	2004 Dec 2	2005 Feb 1 (640)	51
7069114	Juvenile	2004 Nov 19	2005 Feb 17 (80)	80
7069137	Juvenile	2004 Nov 19	2005 Jan 20 (20)	52
7069177	Adult	2004 Dec 15	2005 Jan 20 (160)	37
7073621	Juvenile	2004 Dec 2	2004 Dec 15 (640)	14
7073622	Juvenile	2004 Oct 29	2004 Dec 2 (640)	35
7073647	Juvenile	2004 Nov 4	2005 Feb 17 (40)	95
7081027	Adult	2004 Dec 15	2005 Jan 20 (320)	37

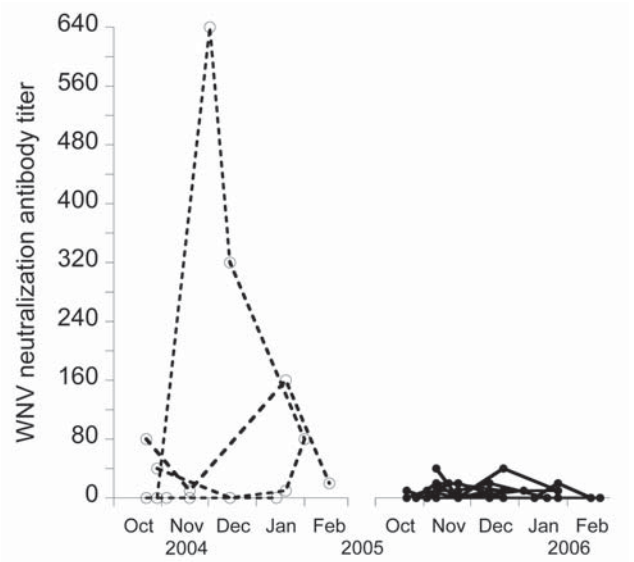

Figure 2. Evolution of West Nile virus (WNV) antibody titers in common coots captured on ≥4 occasions in the same winter, Doñana, Spain. Open circles and dashed lines indicate birds captured during 2004–2005, and solid circles and continuous lines indicate birds captured during 2005–2006.

Additional studies are needed to evaluate the role of these 3 mechanisms in the dynamics of WNV in Spain. Combining serologic results for common coots and vector sampling for virus detection may provide information needed to address these issues.

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Risk Factors for West Nile Virus Neuroinvasive Disease, California, 2005

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In 2005, 880 West Nile virus cases were reported in California; 305 case-patients exhibited neuroinvasive disease, including meningitis, encephalitis, or acute flaccid paralysis. Risk factors independently associated with developing neuroinvasive disease rather than West Nile fever included older age, male sex, hypertension, and diabetes mellitus.

Since the first identification of West Nile virus (WNV) in North America in New York, New York, in 1999, the virus has spread rapidly westward across the United States. In 2004 and 2005, California was the national epicenter of WNV activity, with 779 and 880 cases, respectively. The aim of this study was to identify potential risk factors for developing West Nile neuroinvasive disease among the WNV case-patients reported in California.

The Study

WNV human surveillance in California is conducted through several different mechanisms. Local clinicians are asked to refer patients with evidence of WNV disease, including encephalitis, aseptic meningitis, acute flaccid paralysis, or illness compatible with West Nile fever, for testing which is performed by 33 local public health laboratories and the state Viral and Rickettsial Disease Laboratory (VRDL). Persons with suspected cases are also tested through the California Encephalitis Project (*1*), which provides enhanced diagnostic testing for several viral agents that cause encephalitis, including WNV. In addition, Kaiser Permanente laboratories screen patients with suspected cases and forward positive specimens to VRDL for further testing, while commercial reference laboratories forward positive test results. Blood collection centers forward reports of WNV-positive donors, and local health departments perform follow-up investigations to identify donors in whom clinical disease later develops.

Local health departments use a standardized case history form to collect demographic and clinical information about patients who meet the clinical and laboratory criteria for WNV infection. Patients are classified as having West Nile fever if they exhibit symptoms of WNV infection (e.g., fever, headache, or muscle weakness) without development of neurologic manifestations (e.g., encephalitis, meningitis, or acute flaccid paralysis). The case history form includes questions about hypertension and diabetes.

The 880 case-patients identified were reported from 40 of 58 counties in California, with illness onset ranging from May through November 2005. The median age of all case-patients was 50 years (range 2–95 years), compared to a median of 78 years for the 19 WNV patients who died (range 56–92 years; $p<0.0001$); 55% of all patients were male. Of the 880 cases, 534 cases were classified as West Nile fever and 305 as WNV neuroinvasive disease. Not surprisingly, a greater proportion of the patients with neuroinvasive disease were hospitalized (90%) and required intensive care (27%) compared with the West Nile fever patients (31% and 2%, respectively; $p<0.0001$). The neuroinvasive disease patients also reported a greater frequency of severe symptoms such as altered mental status (54%) and seizures (7%) than did the West Nile fever patients (15% and 0.7%; $p<0.0001$ and $p<0.001$, respectively). Rash was reported among 22% of neuroinvasive disease patients compared with 51% of West Nile fever patients ($p<0.0001$), possibly because those with more severe disease are less able to mount an inflammatory response (*2*) (Table 1).

A greater proportion of neuroinvasive disease patients (46%) reported hypertension as an underlying medical condition than did the West Nile fever patients (29%; $p<0.001$). Thirty-three percent of the neuroinvasive disease patients reported having diabetes mellitus, compared with 11% of the West Nile fever patients ($p<0.001$).

In response to an open-ended question about past medical history, 193 (22%) of all case-patients reported other underlying illnesses. Coronary vascular disease was the most common underlying condition in both neuroinvasive disease and West Nile fever patients (23% and 17%, respectively). Other medical conditions reported for patients with neuroinvasive disease included renal insufficiency (10%) and chronic obstructive pulmonary disorder (8%); cancer (10%) and asthma (9%) were more frequently reported for West Nile fever patients.

Using SAS version 9.1 software (SAS Institute, Inc., Cary, NC, USA), we conducted a univariate analysis to compare the characteristics of patients with neuroinvasive disease to those with West Nile fever (Table 2). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by using Cochran-Mantel-Haenszel statistics. Patients with neuroinvasive disease were twice as likely to have hypertension (95% CI 1.44–3.01) and 4 times more likely to have diabetes (95% CI 2.63–6.55) than West Nile fever patients. Other risk factors for neuroinvasive disease included age

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Table 1. Characteristics of WNV cases reported in California, 2005*

Characteristic	WNND, no./total (%)	WNF, no./total (%)	95% Confidence interval	p value†
Sex, M	188/305 (62)	270/534 (51)	1.18–2.09	<0.01
Age, y				
<18	12/305 (4)	19/534 (4)	0.53–2.32	0.85
18–44	73/305 (24)	180/534 (34)	0.45–0.85	<0.01
45–64	121/305 (40)	240/534 (45)	0.61–1.07	0.15
65–74	51/305 (17)	61/534 (11)	1.09–2.33	0.03
≥75	48/305 (16)	33/534 (6)	1.78–4.53	<0.0001
Symptom				
Fever	254/283 (90)	315/456 (69)	2.54–6.04	<0.0001
Headache	202/262 (77)	387/483 (80)	0.58–1.20	0.33
Rash	54/244 (22)	239/469 (51)	0.19–0.39	<0.0001
Muscle pain/weakness	215/274 (78)	393/485 (81)	0.59–1.23	0.40
Seizures	11/153 (7)	2/313 (0.6)	2.64–55.06	0.001
Altered mental status	148/275 (54)	68/463 (15)	4.77–9.61	<0.0001

*WNV, West Nile virus; WNND, West Nile neuroinvasive disease; WNF, West Nile fever.
†Fisher exact test, 2-tailed.

>64 years (OR = 2.24, 95% CI 1.62–3.11) and male sex (OR = 1.57, 95% CI 1.18–2.09). Because of its collinearity with diabetes, hypertension was dropped from the logistic regression model. Age >64 years (p = 0.03), male sex (p<0.01), and diabetes (p<0.0001) were independently associated with neuroinvasive disease.

Conclusions

To our knowledge, this report summarizes the epidemiologic and clinical characteristics of the largest number of WNV case-patients to date. In contrast to most previous studies, our study included all patients identified with WNV illness, regardless of severity (i.e., inpatients and outpatients). Among the case-patients identified in our surveillance, univariate analysis identified older age and male sex as significant independent predictors of developing neuroinvasive disease, as seen in national surveillance data (*3*). Hypertension and diabetes were also identified as risk factors for developing neuroinvasive disease rather than West Nile fever. Notably, the frequency of diabetes is higher in patients with West Nile neuroinvasive disease than among the general population. The 2001 California Health Interview Survey, a population-based, standardized telephone health survey of >55,000 households throughout California, found that 1,225,000 (11%) persons in California ≥45 years of age reported ever having received a diagnosis of diabetes (*4*). In contrast, 44% of WNV neuroinvasive disease patients ≥45 years of age in our study had diabetes mellitus.

Others have cited diabetes and hypertension as possible risk factors for progression to West Nile neuroinvasive disease or death. In a study of 59 patients hospitalized with WNV infection in New York City (*5*), diabetes was an independent risk factor for death (age-adjusted relative risk = 5.1; 95% CI 1.5–17.3). A history of hypertension or hypertension-inducing drugs was a significant risk factor for encephalitis among 90 hospitalized patients in Houston (OR = 2.93; 95% CI 0.97–8.89) (*6*). Additionally, both diabetes and hypertension were predictors of severe illness in 656 WNV patients reported in Colorado in 2003 (*7*). Most recently, in a review of 221 persons hospitalized with WNV infection, West Nile encephalitis was 4 times more likely to develop in patients with diabetes (*8*).

Different mechanisms have been proposed to explain how diabetes and hypertension might promote the development of WNV neuroinvasive disease. Diabetes and its role in impairing immune status may lead to an increase in the magnitude and duration of WNV viremia, while hypertension may cause disruption of the blood-brain barrier, thereby promoting viral entry into the central nervous system (*9*).

This study had several limitations. Local health departments collected case history forms for all reported cases, but only 58% were complete. Case history forms for the severely ill neuroinvasive patients may have been more thoroughly completed than those for West Nile fever patients. Because this surveillance was not designed to specifically evaluate the contribution of diabetes to WNV disease, the

Table 2. Univariate analysis of potential risk factors for developing WNND versus WNF*

Characteristic	WNF, no. (%)	WNND, no. (%)	Odds ratio	95% Confidence interval
Diabetes	39 (11)	60 (34)	4.15	2.63–6.55
Age >64 y	94 (18)	99 (32)	2.24	1.62–3.11
Hypertension	105 (29)	83 (46)	2.08	1.44–3.01
Male Sex	270 (51)	188 (62)	1.57	1.18–2.09

*WNND, West Nile neuroinvasive disease; WNF, West Nile fever.

information collected on the case history form was limited. However, given the similar findings of other studies, the conclusions from the data are convincing.

In summary, underlying diabetes, as well as older age and male sex, appears to be a significant risk factor for development of WNV neuroinvasive disease. Continued investigation of the role of diabetes, including the degree of severity of diabetic disease, as measured by markers such as degree of end-organ damage (e.g., insulin requirements, presence of diabetic retinopathy, or end stage renal disease), is needed to better identify high-risk patients and target prevention messages.

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Host-Feeding Patterns of *Culex* Mosquitoes in Relation to Trap Habitat

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Mosquito feeding patterns identify vertebrate species potentially involved in the amplification of West Nile virus. In New York, northern cardinals (*Cardinalis cardinalis*) were the predominant hosts in most habitats. Crow (*Corvus* sp.) blood meals were most frequently identified from sewage treatment plant and storm water catch basin habitats.

In the northeastern United States, *Culex pipiens* and *Cx. restuans* are the most important vectors of West Nile virus (WNV), according to the frequency of viral detection, vector competence, and their largely ornithophilic feeding habits (*1*–*4*). Mosquito feeding studies suggest that American robins (*Turdus migratorius*) are the preferred avian hosts that support enzootic transmission in the Northeast (*4*,*5*). A shift in hosts from birds to mammals, including humans, as robins begin fall migratory movements is hypothesized to be responsible for the seasonal rise in human WNV cases (*5*). We identified blood meals of *Culex* mosquitoes collected in New York and found feeding patterns unlike those previously reported (*4*,*5*). We suggest caution in applying findings for epidemiologic purposes across different habitats and large geographic areas.

The Study

We determined host species of *Cx. pipiens* and *Cx. restuans* mosquitoes collected by mosquito surveillance programs in 2001 and 2002 in Nassau (89 identified/100 tested), Orange (66/87), Rockland (83/96), and Westchester (20/20) counties and in 2005 and 2006 in Tompkins County (46/52) (*1*). Dry ice–baited CDC light traps and gravid traps were used to capture host-seeking and ovipositing females, respectively. Seventy percent of mosquitoes were collected from traps located in public places such as parks, preserves, woodlots, cemeteries, and golf courses (hereafter, parks and preserves). The remaining 30% were collected on residential properties, near storm water basins and sewage treatment plants, and at a university composting facility and dairy barn. *Culex* species were identified molecularly with taxon-specific primers (*6*).

Genomic DNA was extracted from each mosquito by using DNAzol-BD (Molecular Research Center, Cincinnati, OH, USA) or DNeasy Blood & Tissue Kits (QIAGEN, Germantown, MD, USA). Blood meals were initially identified by PCR-heteroduplex assays (*7*). We subsequently used DNA sequencing with cytochrome b primers as follows: Cyt F 5′-GCHGAYACHWVHHYHGCHTTYTCHTC-3′ and Cyt H 5′-CCCCTCAGAATGATATTTGTCCTCA-3′, in which W = A or T, H = A, C, or T, Y = C or T, and V = A, C, or G. Cycling conditions were 94°C for 2 min, followed by 55 cycles at 94°C for 45 s, 50°C for 50 s, and 72°C for 1 min with a final extension at 72°C for 7 min. PCR amplifications were conducted by using Taq PCR Core Kits (QIAGEN). Expected 300-bp PCR products were purified with an exonuclease-alkaline phosphate kit (Exo SAP-IT, USB Corporation, Cleveland, OH, USA). Samples were sequenced at the Biotechnology Resource Center (Cornell University, Ithaca, NY, USA) with a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were identified by using BLASTn searches in the GenBank database to compare fragments (*8*).

We identified host species in 183 *Cx. pipiens* and 119 *Cx. restuans* (online Appendix Table, available from www.cdc.gov/EID/content/13/12/1921-appT.htm). *Cx. pipiens* fed on birds (n = 171, 92.9% of *Cx. pipiens* blood meals), mammals (n = 12, 6.5%), and a northern brown snake (*Storeria d. dekayi*) (n = 1, 0.5%). *Cx. restuans* fed exclusively on birds. Avian host species were similar to those previously reported (*3*,*4*), except that northern cardinals (*Cardinalis cardinalis*), not American robins, were the principal hosts throughout the season, and feeding patterns differed somewhat, depending on the habitat of the trap site. Mosquitoes trapped in parks and preserves fed on 32 species of birds. Northern cardinal, gray catbird (*Dumetella carolinensis*), American robin, and blue jay (*Cyanocitta cristata*) accounted for 64% of the identifications. On residential properties, 52% of the blood meals were from cardinals. American robin blood meals accounted for only 12% of the blood meals and were found only in parks, preserves, and residential and storm water catch basins habitats. Crows (*Corvus* spp.) accounted for 26% of the blood meals from storm water catch basins and sewage treatment plant sites but only 2% of the collections from parks and preserves. No crow blood meals were identified

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from other habitats. American crows (*C. brachyrhynchos*) and fish crows (*C. ossifragus*) are found where crow-fed mosquitoes were collected. Mammalian blood meals were identified in June (human, white-tailed deer, and raccoon), July (deer and Virginia opossum), and August (human, 3 white-tailed deer, and 2 eastern gray squirrels). The percentage of northern cardinal and American robin blood meals was relatively constant throughout the summer ($p = 0.261$, Fisher exact test) (Table). The proportion of gray catbird–derived blood meals increased somewhat late in the summer ($p = 0.668$, Fisher exact test).

The avifauna was not censused in Nassau, Orange, Rockland and Westchester Counties. However, data on breeding bird communities were available for mosquito trap locations, and all species detected in blood meals were known to be present there (*9*,*10*). In Tompkins County, 10-min point-count censuses within a 50-m radius of mosquito traps were conducted 2–3 times each month from June through September 2006, totaling 140 counts. Sites included 8 residential properties, a university composting facility, and a wooded area. Of the 84 avian species recorded, the most frequent were northern cardinal (n = 110), black-capped chickadee (*Poecile atricapillus*) (n = 109), American robin (n = 103), blue jay (n = 102), and American crow (n = 100). Although the relative frequency of northern cardinals and American robins was approximately the same at Tompkins County sites, northern cardinals were 7.7 times more likely than American robins to be selected at those sites.

Conclusions

We found northern cardinals, rather than American robins, to be the predominant hosts of *Cx. pipiens* and *Cx. restuans* in all habitat types except storm water catch basins, where crows were identified most frequently. Robin-derived blood meals were less common than reported elsewhere (*4*,*5*). No seasonal decline in robin-fed *Cx. pipiens* or shift to other birds or mammals was found. We found that 7% of *Cx. pipiens* fed on mammals, similar to findings of a study in Connecticut (*4*).

The infrequent identification of crow-derived blood meals relative to their local abundance is an enigma (*3*,*4*). However, the spatial and temporal distribution and social behavior of crows have never been considered. Unless mosquito traps are located where crows are present at dusk or sleeping, the probability of collecting a crow-fed mosquito in the area sampled is low. This caveat is particularly relevant for mosquitoes with relatively short flight ranges such as *Cx. pipiens* and *Cx. restuans,* which presumably would not travel far to find suitable hosts.

Why American robins were the predominant hosts found in host-feeding studies in Connecticut, Maryland, and Washington, DC (*4*,*5*) and why northern cardinals were the preferred hosts in our study are not clear. At the Tompkins County, New York, sites, the relative abundance of cardinals and robins was comparable throughout the season. Thus, host abundance does not explain the frequency of cardinal-derived blood meals, at least at those sites. Cardinals and robins are common, share similar habitats, and are capable of amplifying WNV (*11*,*12*). WNV seroprevalence rates in northern cardinals, American robins, and other birds differ across geographic areas from year to year (*13*). Whether host-feeding patterns parallel those findings is not known.

Cx. pipiens fed on humans in June and August and on deer throughout the summer. In areas experiencing recurrent human WNV infection, future blood meal analyses should focus on peridomestic populations of *Culex* spp. to better understand their predilection for avian and/or mammalian feeding and the spatial and temporal dynamics of their host-feeding activities.

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Table. Monthly prevalence of predominant avian hosts of *Culex pipiens* and *Cx. restuans,* New York

Month	Total no. blood meals identified	Northern cardinal			American robin			Gray catbird		
		Cx. pipiens	*Cx. restuans*	% of total	*Cx. pipiens*	*Cx. restuans*	% of total	*Cx. pipiens*	*Cx. restuans*	% of total
May	7		4	57.1		1	14.3			
Jun	83	5	13	21.7		11	13.3	1	4	6
Jul	99	22	17	39.4	7	*3*	10.1	3	1	4
Aug	82	21		25.6	11		13.4	14	1	18.3
Sep	18	7		38.9	1		5.6	6	1	38.9
Oct	1	1		100						

include the transmission dynamics of arboviruses and ecology of vectorborne diseases.

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Human and Animal Infections with *Mycobacterium microti*, Scotland

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During 1994–2005, we isolated *Mycobacterium microti* from 5 animals and 4 humans. Only 1 person was immunocompromised. Spoligotyping showed 3 patterns: vole type, llama type, and a new variant llama type.

Naturally occurring mycobacteria that are part of the *Mycobacterium tuberculosis* complex include *M. tuberculosis, M. bovis, M. caprae, M. africanum, M. microti,* and *M. pinnipedii*. Although these species show remarkable genetic homology, there are notable phenotypic differences, particularly in their relative pathogenicity for different mammalian species.

Tuberculosis in wild rodents was first studied in 1937 as part of an investigation of cyclical changes in the population density of voles (*1*). Field voles, bank voles, wood mice, and shrews are particularly susceptible to infection with *M. microti* (*2*). However, other small mammals such as guinea pigs, rabbits, mice, and rats are resistant to *M.microti* infection, even at high doses of infection. More recently, sporadic cases have been described in larger mammals (*3*–*6*).

There have been only 6 published reports of human infections, comprising 13 patients in total (*7*–*11*). Salient information from these reports is summarized in Table 1.

M. microti has been used in extensive trials to assess its efficacy and safety as a vaccine. Percutaneously administered *M. microti* vaccine was found to be safe but no more effective than *M. bovis* BCG (*12*). The low virulence and poor immunogenicity are due to several key genetic deletions, resulting in the inability to produce the strongly immunogenic T-cell antigens ESAT-6 and CFP-10 (*13*).

Several genotypes of *M. microti* have been recognized by spacer oligotyping (spoligotyping). The llama-type (presence of spacers 4–7, 23, 24, 26, 37, 38) and the vole-type (only 2 spacers, 37 and 38) have been well described; both types are involved in human infections (*5*,*7*). The international spoligotyping database (SpolDB4) (*14*) includes 40 *M. microti* strains, 37 of which are from the United Kingdom and Western Europe. Although there are no published reports of *M. microti* infections from the United States, 3 of the strains in SpolDB4 are from this country. *M. microti* strains yield broadly similar, high–copy number fingerprints by the insertion sequence *6110*–based restriction fragment length polymorphism method (IS*6110* RFLP) (*7*).

In the 12-year period from 1994 through 2005, we isolated *M. microti* from 4 humans and from 5 animals (2 cats, a llama, a badger, and a ferret). No clinical details were available for the animal cases. The animal and human cases were from different locations in Scotland. No epidemiologic links were apparent.

The Patients

Patient 1 was a 41-year-old woman in whom sputum smear–positive tuberculosis was diagnosed in 2001. She was treated with isoniazid, rifampin, ethambutol, and pyrazinamide for 2 months and for 4 months more with rifampin and isoniazid. She made good clinical progress, but sputum samples remained positive for acid-fast bacilli (AFB), although cultures were negative. She was re-treated with isoniazid, rifampin, ethambutol, and pyrazinamide for 6 months. She became sputum negative and remained clinically well at her 6-month follow-up visit. She was not immunocompromised. No other patients with tuberculosis were identified in contacts, and no relevant animal contact had occurred.

Patient 2 was a 39-year-old man for whom HIV was diagnosed in 2003, who had bilateral pulmonary consolidation. The patient lived on a farm. He was initially treated with co-trimoxazole for suspected *Pneumocystis carinii* infection, and rifampin, isoniazid, and pyrazinamide were added when AFB were seen in the sputum sample. The patient's condition deteriorated, and he died despite this drug treatment and intensive therapy unit support. No other patients with tuberculosis were identified in connection with this case.

Patient 3 was a 76-year-old woman who had received a diagnosis of pulmonary tuberculosis in 2005. She made an uneventful recovery following standard therapy with isoniazid, rifampin, and ethambutol for 2 months, followed by rifampin and isoniazid for a further 4 months. She was not immunocompromised, and she reported no major animal contact. No cases of tuberculosis were identified in connection with this patient.

Patient 4 was a 45-year-old woman who was seen in 2005 for hemoptysis; a diagnosis of cavitating pulmonary tuberculosis was made. She received treatment with isoniazid, rifampin, ethambutol, and pyrazinamide for 2 months and rifampin and isoniazid for 4 months more. She remained unwell, with further hemoptysis, and a residual cavity was shown on chest x-ray. Chemotherapy was rein-

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Table 1. Summary of all reported cases of human infections with *Mycobacterium microti* *

Case-patient no.	Ref	Age, sex, country	Immune status	Infection site	Animal contact	Laboratory findings	Outcome
1	(*8*)	48 y, M, Germany	HIV positive	Lung	None	Llama type. Good growth in liquid medium. Poor on pyruvate. Drug susceptible. Curved bacilli.	Cured
2	(*7*,*10*)	39 y, M, the Netherlands	HIV positive	Lung; lymph nodes	House mice	Cultures negative; curved bacilli in sputum. Vole type on direct spoligotyping.	Cured after prolonged therapy†
3	(*7*)	12 y, M, the Netherlands	Renal transplant	Lung meninges	None	Vole type. Other details unavailable.	Cured
4	(*7*)	41 y, M, the Netherlands	Renal transplant	Peritoneal	Wild small rodents	Vole type. Other details unavailable.	Died despite therapy
5	(*7*)	34 y, M, the Netherlands	Normal	Lung	Lived in mobile home	Vole type. Other details unavailable.	Cured
6	(*9*)	53 y, M, Germany	Normal	Lung	None	Llama type. AFB film negative. Liquid culture better than pyruvate agar. No growth on normal egg media. Fully drug susceptible. Noncurved bacilli.	Cured
7	(*9*)	58 y, M, Germany	Diabetic	Lung	None	Vole type. Growth on liquid culture only (poor). Susceptibility not done. Noncurved bacilli.	Cured
8	(*5*)	Not known; England or Wales	Not known	Not known	Not known	Llama type.	Not known
9–12	(*5*)	Not known; England or Wales	Not known	Not known	Not known	Vole type.	Not known
13	(*11*)	69 y, sex not known, Germany	Normal	Abdominal/ miliary	Not known	Vole type. Primary culture in liquid. Subculture in solid agar.	Died, despite appropriate therapy

*Ref, reference; M, male; AFB, acid-fast bacilli.
†Three household contacts were found to be tuberculin positive.

troduced. She was not known to be immunocompromised. She had a pet cat and a dog, both in good health. No cases of tuberculosis were identified in contacts.

The laboratory characteristics of the isolates are shown in Table 2. Biochemical tests were not possible because of sparse growth. Isolates were identified as *M. tuberculosis* complex by using the Accuprobe culture confirmation assay (GenProbe, San Diego, CA, USA), and species identification as *M. microti* was confirmed by spoligotyping. Since we do not perform drug susceptibility testing using solid media, only the 3 strains that grew well in liquid subculture were tested. Genotyping data on our isolates are summarized in Table 2 and the Figure.

Conclusions

M. microti infection is widespread in wild small rodent populations in the United Kingdom (*2*). There are sporadic reports, all from the United Kingdom and Western Europe, of *M. microti* infection in other mammals. Certain animals, such as cats (*4*,*5*) and New World camelids domesticated in Europe (*6*), seem to be particularly susceptible. The reported animal cases have all been detected in clinical veterinary practice and are unlikely to reflect the true field incidence. Difficulties with laboratory diagnosis probably further contribute to the underestimation of the incidence. *M. microti* grows poorly on traditional solid egg media, and modern automated liquid culture techniques do not seem to yield better results. Moreover, even when a mycobacterial infection is diagnosed, routine veterinary diagnostic procedures often do not identify the mycobacterium to species level. It is likely also that known animal cases are not all formally reported in the literature.

The transmission of *M. microti* to pets, particularly cats, is of particular concern. Cats are assumed to acquire the *M. microti* infection from infected wild rodents, but this assumption is not supported by the genotyping evidence. Most of the strains isolated from cats are genotypically very distinct from wild rodent strains, as shown in our cases and in the literature (*5*). Very little is known about the incidence and ecology of *M. microti* infection in farm and domestic animals.

Many of the human patients with *M. microti* infection appear to have no immunologic deficits (3 of our 4 patients and 3 of the 8 published cases for which relevant clinical details were available). However, inherited defects of interleukin receptor function are known to specifically predispose to intracellular infections, particularly mycobacterial infection (*15*). Therefore, some persons with apparently normal immunity infected with *M. microti* may in fact have undetected specific immune defects.

Table 2. Laboratory features of *Mycobacterium microti* isolates from Scotland*†

Source	Specimen	Direct AFB	Growth on primary isolation: Solid culture IUT	Solid culture PYR	Liquid culture MB/MGIT	Drug susceptibility	Genotype
Human 1	Sputum	Positive	–	+	–	Failed to grow in liquid cultures	Llama type SIT641 (spacers 4–7, 23,24, 37, and 38)
Human 2	Sputum	Positive (many)	–	+	–	Failed to grow in liquid cultures	Llama type SIT641
Human 3	Sputum	Positive (many)	–	–	+	Susceptible to R, I, E; resistant to P	Llama type SIT641
Human 4	Sputum	Positive (few)	+	+	–	Failed to grow in liquid culture	Llama type (spacers 4–7 and 23,24 only)
Cat 1	Tissue/lymph node	Negative	–	+	–	Failed to grow in liquid cultures	Llama type SIT641
Badger	Tissue/lung	Strongly positive	+	+	±	Inadequate growth	Vole type SIT 539 (spacers 37 and 38)
Cat 2	Tissue/lymph node	Negative	–	+	–	Susceptible to R,I,E,P (grew on liquid subculture)	Vole type SIT539
Llama	Tissue/lung	Positive	+	+	–	Failed to grow in liquid culture	Llama type SIT641
Ferret	Tissue	Positive	±	±	±	Susceptible to R,I,E,P	Not tested

*AFB, acid-fast bacilli; IUT, International Union Against Tuberculosis formulation of solid egg medium; PYR, IUT medium with pyruvate supplementation; MB, MBBact, Biomerieux, Basingstoke, United Kingdom; MGIT, Mycobacteria Growth Indicator Tube, Becton Dickinson; R,I,E,P, rifampin, isoniazid, ethambutol, pyrazinamide; SIT, Spoligo-International-Type.
†SIT numbers: designations in International spoligotyping database (Spol DB4) (*14*).

Human-to-human transmission of *M. microti* infection seems rare. In the single instance in which this possibility is moot, the secondary cases all occurred in the same mice-infested household (*10*).

Extensive trials of *M. microti* as a vaccine suggest that it lacks virulence for humans with normal immunity. However, it remains a potential threat to the substantial pool of persons with compromised immunity, including the unknown number who may have genetic defects specifically predisposing to mycobacterial infections.

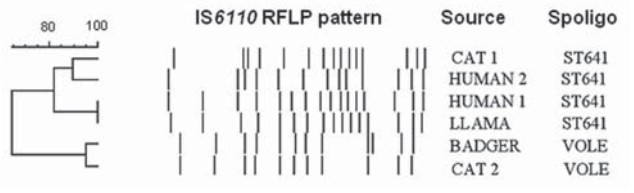

Figure. Comparison of the restriction fragment length polymorphism patterns of *Mycobacterium microti* strains from Scotland. Spoligo, spoligotyping.

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Dr Emmanuel retired recently from his post as consultant medical microbiologist at the Department of Laboratory Medicine at the Royal Infirmary of Edinburgh. The department incorporates the Scottish Mycobacteria Reference Laboratory, which he directed during the period that this research was carried out. His research interests include the molecular epidemiology of *Mycobacterium tuberculosis* complex.

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African Swine Fever Virus DNA in Soft Ticks, Senegal

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African swine fever is a highly contagious disease of pigs in Africa. Although its persistence in Senegal may be caused by asymptomatic carriers involved in the domestic transmission cycle, we demonstrated that the soft tick *Ornithodoros sonrai* can be naturally infected with the causative agent.

African swine fever (ASF) is one of the most severe diseases of pigs in Africa. It is caused by African swine fever virus (ASFV), an *Asfaviridae* virus, and usually results in acute hemorrhagic fever in susceptible animals with mortality rates up to 100% in some herds (*1*). ASF is defined by the World Organization for Animal Health as a highly contagious disease that can spread rapidly and have serious socioeconomic effects in international trade of pigs or pig products and food security. No treatment or vaccine is currently available, and control is essentially based on sanitary measures (*1*).

ASF is endemic in eastern and southern Africa, where ASFV is maintained either in a sylvatic cycle between warthogs (*Phacochoerus aethiopicus*) or bushpigs (*Potamochoerus* spp.) and soft tick vectors of the *Ornithodoros moubata* complex or in a domestic cycle that involves pigs of local breeds with or without tick involvement (*2*–*4*). Long-term persistence of ASFV caused by the presence of the soft tick vector *O. erraticus* (*5*) has also been reported in the Iberian Peninsula.

In west Africa, ASFV has been introduced several times since the 1970s in different countries by importing infected pigs or meat. These imports resulted in massive sporadic outbreaks that have been eradicated (*6*). Senegal has had several outbreaks caused by regular reemergence of ASFV since its first description in 1959, which suggests a unique epidemiology that has not been reported in most west African countries infected with ASFV (*6*). The presence of warthogs (*7*) and the soft tick *O. sonrai* (*8*) in Senegal suggest a sylvatic cycle of ASF. *O. sonrai* is closely related to *O. erraticus* and the *O. moubata* complex and shares similar vector competence for some pathogens, such as *Borrelia*, which causes human relapsing fever in Africa (*9*). This article reports preliminary results on potential involvement of *O. sonrai* in persistence and transmission of ASFV and discusses the role of reservoirs or vectors in control measures.

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The Study

Tick investigations were conducted in January 2006 in the Fatick region of Sine-Saloum in west-central Senegal (Figure 1). This region is a major area of pig production and a center for trade with Dakar and Casamance in Sengal and Bissau-Guinea (*10*). Despite no national reporting, ASF outbreaks occur almost every year in Sine-Saloum (*6*,*10*). *O. sonrai* has also been found in the Fatick region of Senegal in previous investigations on human relapsing fever (*11*).

Three criteria were selected to assess the role of *O. sonrai* in ASF (*12*): presence of this tick in domestic pig buildings and warthog habitats, its probability of contact with domestic pigs and warthogs, and its natural infection with ASFV. We searched for *O. sonrai* in pigpens in 5 villages or groups of villages, 20–30 km apart per sampling site, along a north-south transect, as well as in warthog burrows in wild areas from 3 different forests (Figure 1). For tick collection, we used a portable gasoline-powered vacuum cleaner adapted for burrow-dwelling ticks (*13*) (Figure 2, panel B). Specimens were stored in liquid nitrogen. Pig pens and warthog burrows were systematically described to determine ecologic preferences of *O. sonrai*. Rodent or insectivore burrows, which are known to be favorable natural habitats for *O. sonrai*, were also examined at each sampling site to determine the presence or absence of the tick. Collected ticks were tested for ASFV infection by nested PCR amplification of the VP72 gene, a method considered most sensitive for detection of viral DNA in ticks (*14*).

Figure 1. Sampling sites in the Fatick region of Sine-Saloum, Senegal.

Table. Tick collections in villages and detection of African swine fever virus in ticks, Senegal*

Sampling site	Pigpen no.	Burrows	Presence *of Ornithodoros sonrai*†			Infection with *O. sonrai*‡	Last ASF outbreak
			Reference	Within buildings	Near buildings		
Sassa-Niakhar-Diohine	1	11	+	–	+ (4)	0/4	S 2005
	2	6		–	+ (5)	1/6	S 2005
	3	1		–	–		
	4	1		–	–		S 2005
	5	0		–	–		S 2005
Fatick	6	7	+	+ (3)	+ (2)	1/2	
	7	4		+ (2)	+ (2)	0/6	
	8	0		–	–		
	9	1		–	+ (1)	0/1	
	10	2		–	+ (1)		
Foundiougne	11	7	+	–	+ (1)	1/2	S 2005
	12	3		–	–		S 2005
	13	2		–	–		S 2005
	14	1		–	–		Sp 2005
	15	2		–	–		S 2005
Sokone	16	6	+	–	+ (1)	1/7	S 2004
	17	3		–	–		S 2004
	18	1		–	–		
	19	3		–	+ (3)	0/6	
	20	4		–	-		
Karang	21	6	–	–	–		
	22	10		–	–		
	23	2		–	–		S 2005
	24	4		–	–		
	25	2		–	–		

*ASF, African swine fever; +, positive; –, negative; S, summer; Sp, spring. Two other ticks from wild areas in Fatalah and Sipo forests were tested for AFSV and found negative.
†Values in parentheses are no. infested burrows or cracks.
‡Values are no. infected ticks/no. tested.

O. sonrai was found in 11 of the 25 examined pigpens in villages in the 4 most northern sampling sites (Table). Specimens were always found in rodent and insectivore burrows, or in deep hollows, in openings inside pig buildings, or near sleeping or foraging areas around pig buildings, as described for the closely related Iberian soft tick *O. erraticus* during investigations of ASF (*5*) (Figure 2, panel A). *O. sonrai* was not found in litter or buildings, except at 1 farm in Fatick, where nearby burrows were highly infested. The village of Karang showed negative results, even in suitable microhabitats, a finding that confirmed the southern distribution limit of *O. sonrai* proposed by Morel (*8*). In wild areas, *O. sonrai* was not found in 10 warthog burrows examined (Figure 2, panel B), although its presence was confirmed in contiguous rodent or insectivore burrows. Of 36 ticks tested for ASFV infection, 4 from the 4 most northern sampling sites were positive for ASFV (Table). The farms where ASFV was detected in ticks had reported recent outbreaks in the summers of 2004 and 2005, except for the farm in Fatick. This farm, which belonged to a fattener/collector, had a high turnvover rate of pigs that may have caused difficulties in monitoring their health. Sequencing and BLAST analysis (www.ncbi.nlm.nih.gov) of PCR products confirmed a 100% relationship with ASFV. One sample was positive by repeated analysis. Three samples showed doubtful results when retested by PCR, which indicated low virus titers.

Conclusions

To our knowledge, this study demonstrated for the first time that *O. sonrai* is naturally infected with ASFV. Although these preliminary results suggest a role for *O. sonrai* in persistence of ASFV within a sylvatic cycle, only experimental infections will enable formal testing of *O. sonrai* as a reservoir and competent vector for AFSV. Additional tick sampling and virus detection analyses are also being conducted to estimate its natural prevalence of infection. If one considers that the ability of pathogens to infect a wide range of hosts is a risk factor for disease reemergence (*15*), our findings are useful for the design of control measures for ASF in Senegal, which currently focus only on pig slaughtering and environment disinfection.

Although contact between soft ticks and domestic pigs has been confirmed in villages in this study, this contact is considered limited. *O. sonrai* colonizes mainly rodent or insectivore burrows with high humidity and a cool temperature favorable for its development and survival. It is rare that such favorable microhabitats are near pigpens and enable ticks to feed on pigs instead of small mammals inhabiting burrows. In addition, heterogeneous and relatively low

Figure 2. Favorable and unfavorable habitats examined for *Ornithodoros sonrai*. A) Favorable rodent or insectivore burrows infested with *O. sonrai* inside pig buildings. B) Unfavorable warthog burrows negative for *O. sonrai* dug under a termite mound. The portable gasoline-powered vacuum cleaner used for tick collection is also shown.

infestation rates for such microhabitats have been shown in a previous study (*11*). Conversely, in wild areas, contact between ticks and warthogs was unlikely, which is contrary to the situation in eastern and southern Africa. In Senegal, warthogs inhabit mainly dry forests and dig superficial burrows under termite mounds, which are not optimal conditions for *O. sonrai*. To more clearly quantify contacts between ticks and domestic pigs or warthogs and assess their effect on ASF transmission, analyses of mammalian cytochrome B in tick blood meals and detection of antibodies to tick saliva in serum samples of pigs and warthogs are being conducted.

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Enhanced Subtyping Scheme for *Salmonella* Enteritidis

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To improve pulsed-field gel electrophoresis–based strain discrimination of 76 *Salmonella* Enteritidis strains, we evaluated 6 macro-restriction endonucleases, separately and in various combinations. One 3-enzyme subset, *Sfi*I/*Pac*I/*Not*I, was highly discriminatory. Five different indices, including the Simpson diversity index, supported this 3-enzyme combination for improved differentiation of *S.* Enteritidis.

Since 1987, *Salmonella* Enteritidis has been one of the most frequently isolated salmonellae associated with foodborne outbreaks (*1*). Illness from *S.* Enteritidis is linked to consumption of chickens, eggs, and foods that contain eggs (*2*). *S.* Enteritidis presents an interesting challenge from an epidemiologic perspective. Several reports documented a limited number of genotypes among ecologically diverse *S.* Enteritidis, reinforcing the notion that most *S.* Enteritidis strains are derived from a few endemic clones (*3*,*4*).

Pulsed-field gel electrophoresis (PFGE) is an integral subtyping tool used by several national public health networks (e.g., PulseNet, FoodNet, and VetNet) to differentiate outbreak strain clusters (*5*). The genetic homogeneity of *S.* Enteritidis, however, confounds many subtyping approaches, including PFGE (*6*,*7*). Conventional PFGE protocols lack discriminatory power to cull the subtle genotypic differences that distinguish *S.* Enteritidis strains. A more discriminatory scheme that incorporates combinations of potentially more informative enzymes may be attainable. We explored the discriminatory power of 6 enzymes, individually and in combination, to identify a more informative PFGE-based subtyping scheme for this important foodborne pathogen.

The Study

We examined 76 strains of *S.* Enteritidis and 74 strains of *S.* Typhimurium. Strains were isolated from poultry and poultry-related sources and were obtained from the Center for Veterinary Medicine and Center for Food Safety and Applied Nutrition of the US Food and Drug Administration and from the University of Georgia. After screening numerous restriction enzymes, the 6 selected were *Xba*I, *Bln*I, and *Spe*I, all used in PulseNet protocols (*5*); *Sfi*I and *Pac*I, previously used to improve resolution in PFGE studies involving *Escherichia coli* O157:H7 (*8*,*9*); and *Not*I, found to yield an optimal number of cut sites (*10*). The standard PulseNet PFGE protocol for non-typhoidal *Salmonella* was performed as described (*11*,*12*). Individual run conditions are listed in Table 1.

Five diversity indices were used to assess discriminatory potential among enzymes. First, unique PFGE patterns or pattern combinations, when analyzing ≥2 enzymes, were tallied. Second, the mean number of strains per polytomy (an unresolved strain cluster) was calculated as the number of polytomous strains divided by the number of polytomies in the tree. Third, the percentage of polytomous strains (of 76 *S.* Enteritidis strains) was calculated. Fourth, the node:strain ratio was calculated as the number of nodes (bifurcating tree forks) divided by 76 *S.* Enteritidis strains. A node:strain value closer to 1 indicated a more resolved tree. Finally, the Simpson diversity index was calculated as a numerical assessment of the relative discriminatory potential of each enzyme and enzyme combination (*13*).

*Xba*I-*Bln*I patterns from 76 *S.* Enteritidis strains and 74 *S.* Typhimurium strains were analyzed simultaneously for a direct comparison of PFGE diversity. The resultant dendrogram yielded striking topologic differences between the 2 serovars (Figure 1). *S. Typhimurium* strains were almost entirely resolved; nearly every strain possessed its own branch on the dendrogram. In contrast, *S.* Enteritidis strain discrimination was sharply weaker, affirming extensive genetic homogeneity among strains. For example, 6 polytomies were evident in the *S.* Enteritidis portion of the tree, 5 of which comprised 4 or more strains and 1 of which comprised 24 strains. In total, 76% of *S.* Enteritidis strains occupied unresolved clusters with an average of ≈10 strains per cluster. Moreover, the *S.* Typhimurium dendrogram retained a nearly 1:1 ratio of nodes to strains, indicating that almost every strain retained a unique *Xba*I/*Bln*I pattern combination. *S.* Enteritidis, however, yielded a node:strain ratio of 1:3, indicating a relatively poorly bifurcated tree. Together, these observations highlighted the difficulty in differentiating *S.* Enteritidis with conventional PFGE approaches.

To develop a more discriminatory scheme for *S.* Enteritidis, we examined pattern diversity for 4 additional restriction endonucleases (*Spe*I, *Sfi*I, *Pac*I, and *Not*I). Diversity indices associated with each of the 6 enzymes are listed in Table 2. Many of the indices designated *Not*I as being effective for discriminating *S.* Enteritidis. Among the 6 enzymes, *Not*I yielded the highest number of unique patterns (n = 26), the fewest average number of strains per polytomy (5.2), the lowest percentage of strains captured

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Table 1. Pulsed-field gel electrophoresis run conditions for 6 restriction enzymes used to subtype *Salmonella* Enteritidis*

Enzyme	Digestion temperature, °C	Enzyme units, per plug	Run time, h†	Initial switch time, s	Final switch time, s
*Xba*I	37	50	19	2.16	63.8
*Bln*I	37	30	19	2.16	63.8
*Spe*I	37	30	20.5	5	25
*Sfi*I	50	30	20.5	5	25
*Pac*I	37	30	20.5	0.1	15
*Not*I	37	30	20.5	0.1	15

*All digestion times were 2–3 h.
†Digestions were separated in 1% agarose gels (Cambrex, Baltimore, MD, USA) at 6V/cm by using a CHEF-Mapper (Bio-Rad, Hercules, CA, USA).

by polytomies (82%), the highest node:strain ratio (0.47), and the highest Simpson diversity value (0.92). *Not*I was followed closely by *Pac*I for most indices, which suggests that *Pac*I was also useful for *S.* Enteritidis strain discrimination.

Previous studies that used PFGE noted the combining of restriction enzyme data into a single analysis as an approach for improving strain differentiation (*8*,*9*). In our study, a dendrogram of the combined 6-enzyme *S.* Enteritidis data was highly resolved and yielded 57 unique pattern combinations. The tree contained, on average, 3.6 strains per polytomy (n = 8), and only 38% of the strains in the 6-enzyme tree were associated with unresolved clusters (Table 2). The node:strain ratio was 0.78, and the Simpson index was 0.98, surpassing the accepted threshold (0.95) for a useful subtyping scheme (*14*).

Although highly effective for *S.* Enteritidis discrimination, 6-enzyme simultaneous analysis would increase

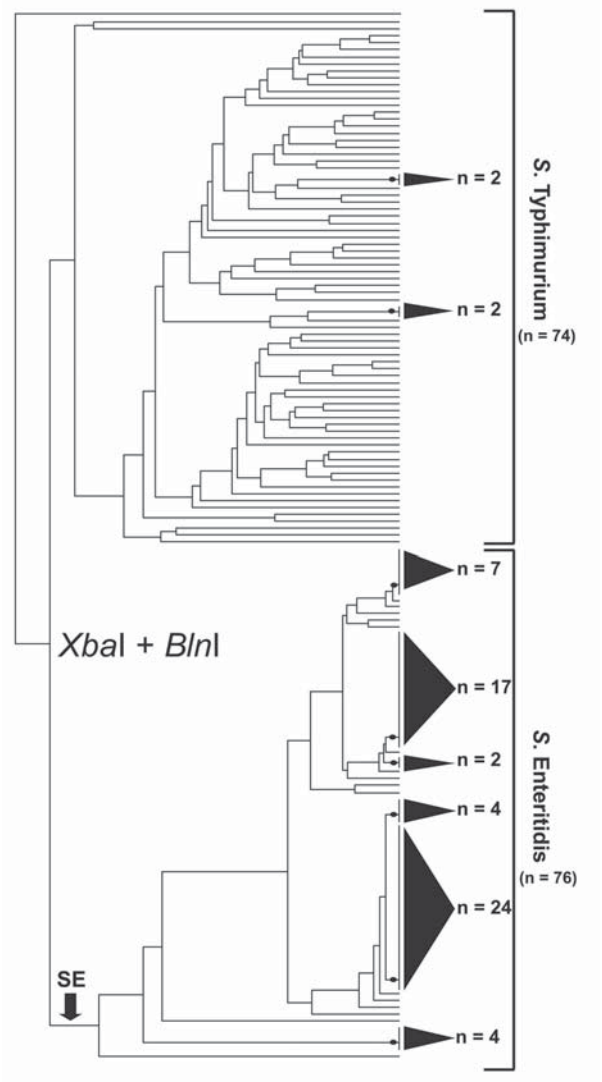

Figure 1. Simultaneous cluster analysis of *Salmonella* Enteritidis and *S.* Typhimurium that used a standard *Xba*I/*Bln*I combined PFGE protocol. The dendrogram incorporates 76 *S.* Enteritidis strains and 74 *S. Typhimurium* strains and depicts the contrasting ability of pulsed-field gel electrophoresis (PFGE) to genetically differentiate these 2 *Salmonella* subspecies I serovars. The dendrogram was generated in BioNumerics v.4.061 (Applied Maths, Sint-Martens-Latem, Belgium) by using band-matched *Xba*I/*Bln*I PFGE data in conjunction with an unweighted pair group method with arithmetic mean clustering algorithm and a Dice similarity coefficient. Shaded cones to the right of terminal tree branches denote polytomies within the dendrogram; adjacent numbers (n) show the strain totals composing that polytomy. An arrow near the bottom of the tree denotes the basal branch of the *S.* Enteritidis cluster. The *S.* Enteritidis portion of the dendrogram comprises strains isolated from Georgia (n = 31), Maryland (n = 8), Pennsylvania (n = 3), Connecticut (n = 3), North Carolina (n = 2), Iowa (n = 2), Tennessee (n = 2), Minnesota (n = 1), Mexico (n = 11), and the People's Republic of China (n = 6).

Table 2. PFGE diversity indices for various combinations of restriction enzymes in *Salmonella* Enteritidis

Enzyme/combination	PFGE patterns*	Mean no. strains/ polytomy†	% polytomous strains‡	Node:strain ratio§	Simpson diversity index
*Xba*I	19	9.1	84	0.30	0.83
*Bln*I	15	16.3	85	0.22	0.76
*Spe*I	16	9.6	88	0.28	0.80
*Sfi*I	15	13.2	87	0.24	0.67
*Pac*I	20	9.0	83	0.32	0.74
*Not*I	26	5.2	82	0.47	0.92
*Xba*I/*Bln*I	24	9.7	76	0.37	0.84
*Xba*I/*Spe*I	26	6.6	78	0.43	0.88
*Xba*I/*Sfi*I	30	5.2	75	0.51	0.92
*Xba*I/*Pac*I	30	5.6	74	0.50	0.91
*Xba*I/*Not*I	38	4.2	66	0.63	0.95
*Bln*I/*Spe*I	22	8.6	79	0.36	0.84
*Bln*I/*Sfi*I	26	6.6	78	0.43	0.89
*Bln*I/*Pac*I	28	6.3	75	0.46	0.88
*Bln*I/*Not*I	36	4.3	68	0.61	0.94
*Spe*I/*Sfi*I	27	5.9	78	0.46	0.90
*Spe*I/*Pac*I	30	6.8	71	0.46	0.89
*Spe*I/*Not*I	35	3.9	72	0.61	0.96
*Sfi*I/*Pac*I	30	5.2	75	0.51	0.87
*Sfi*I/*Not*I	43	4.0	58	0.67	0.96
*Pac*I/*Not*I	42	3.6	62	0.70	0.96
*Spe*I/*Sfi*I/*Not*I	48	3.8	50	0.64	0.97
*Spe*I/*Pac*I/*Not*I	47	3.6	53	0.72	0.97
*Sfi*I/*Pac*I/*Not*I	51	3.3	47	0.79	0.98
6 enzymes	57	3.6	38	0.78	0.98

*PFGE, pulsed-field gel electrophoresis. Absolute tally of unique PFGE fingerprints or fingerprint combinations derived from the corresponding enzyme or group of enzymes.
†Total no. polytomous strains/no. polytomies in the dendrogram.
‡No. polytomous strains/76 (total *S.* Enteritidis strains in the analysis).
§Total no. nodes in the dendrogram/76 *S.*Enteritidis strains.
¶Derived from the formula for assessing discriminatory capability of a subtyping method (*13*).

the time and resources needed to complete investigations. Thus, a streamlined PFGE scheme with enhanced discrimination was desirable. First, 2-enzyme combinations were assessed to ascertain a minimal enzyme set for useful discrimination of *S.* Enteritidis. Combinations *Sfi*I/*Not*I and *Pac*I/*Not*I yielded optimal diversity measures, compared with the others (Table 2). However, because no 2-enzyme pair rivaled the discriminatory potential of the 6-enzyme analysis, 3-enzyme combinations were explored. A *Sfi*I/*Pac*I/*Not*I combination was found to be superior in all diversity categories (Table 2). It yielded 51 unique pattern combinations, an average of 3.3 strains per unresolved cluster, a node:strain ratio of 0.79, and a Simpson index of 0.98. The latter 3 measures matched or exceeded corresponding values in the 6-enzyme analysis, which indicates that most of the diversity in the 6-enzyme analysis was captured by this 3-enzyme combination.

The *Sfi*I/*Pac*I/*Not*I dendrogram showed a highly resolved tree topology and earmarked this 3-enzyme combination as being particularly effective for differentiating *S.* Enteritidis strains (Figure 2, panel A). Of 11 clusters, 9 comprised 3 or fewer strains, and only 47% of *S.* Enteritidis strains composed these 11 clusters, which, aside from the 6-enzyme analysis, represented the lowest portion of polytomous strains in the study.

The robust discrimination of *S.* Enteritidis achieved from combining *Sfi*I, *Pac*I, and *Not*I was attributed to polymorphic band classes that were lacking in other enzymes. As an example, the *Sfi*I, *Pac*I, and *Not*I PFGE patterns for 4 strains (*S.* Enteritidis 9, *S.* Enteritidis 12, 22704, and 22705) that were identical using *Xba*I and *Bln*I were examined for band variation. In contrast to *Xba*I, *Sfi*I, *Pac*I, and *Not*I, all showed some level of polymorphism among DNA fragments (Figure 2, panel B). Band differences for these 3 enzymes partitioned the 4 *S.* Enteritidis strains into disparate dendrogram positions.

Conclusions

On the basis of common geography and *Xba*I/*Bln*I pattern homogeneity, several clusters of *S.* Enteritidis strains appeared to exhibit clonal relatedness. However, the combined analysis of *Sfi*I/*Pac*I/*Not*I PFGE patterns was able to differentiate not only geographically disparate *S.* Enteritidis strains but also *S.* Enteritidis isolates from within a specific geographic locale (e.g., Georgia *S.* Enteritidis strains 415–421 and 434–436). Although several parameters, such

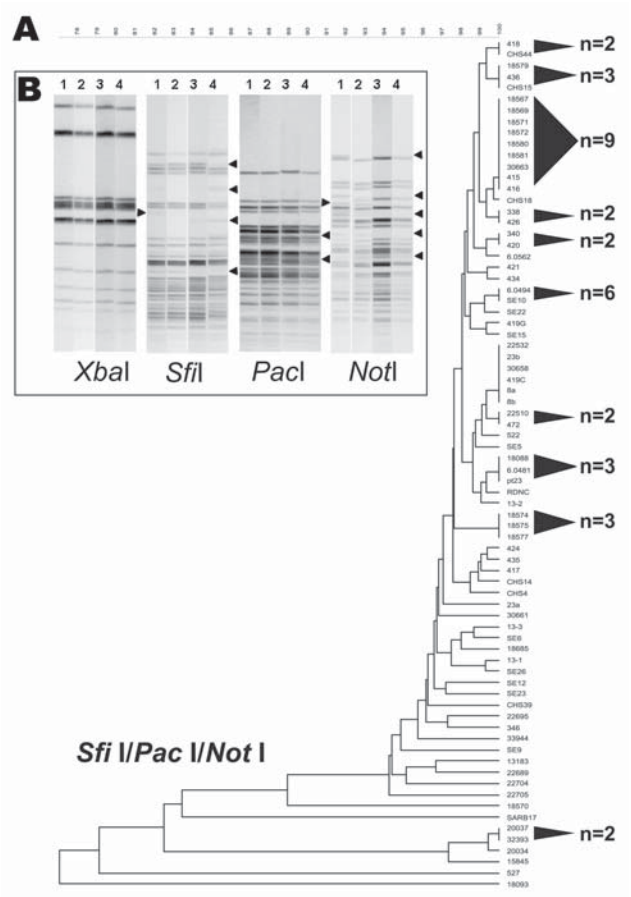

Figure 2. A 3-enzyme pulsed-field gel electrophoresis (PFGE)–based discriminatory scheme of *Salmonella* Enteritidis. A) Dendrogram derived from the combined analysis of PFGE data from *Sfi*I, *Pac*I, and *Not*I. Shaded cones to the right of the terminal branches denote polytomies within each dendrogram; adjacent numbers (n) show the strain totals composing their respective polytomies. A scale depicting percent divergence is presented above the dendrogram. B) Examples of *S.* Enteritidis strain differentiation that used *Sfi*I, *Pac*I, and *Not*I PFGE patterns. The 4 strains are numbered above the gel lanes as follows: 1, *S.* Enteritidis 9; 2, *S.* Enteritidis 12; 3, 22,704; and 4, 22,705. These strains yielded identical PFGE patterns for *Xba*I and *Bln*I. *Xba*I patterns shown here retain no variation among fragments. *Sfi*I, *Pac*I, and *Not*I showed examples of band polymorphism among DNA fragments.

as variable run conditions and times, likely preclude application to outbreak events in real time, the revised scheme presented here may be useful for retrospective epidemiologic investigations in which a more specific focus is often placed on a limited number of epidemiologically related isolates (*15*). Specifically, when *S.* Enteritidis strains are tightly linked geographically and temporally by clonal expansion, this 3-enzyme approach should provide an effective differentiation process.

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Clinical and Epidemiologic Characterization of WU Polyomavirus Infection, St. Louis, Missouri

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WU polyomavirus is a recently described polyomavirus found in patients with respiratory infections. Of 2,637 respiratory samples tested in St. Louis, Missouri, 2.7% were positive for WU polyomavirus by PCR, and 71% were coinfected with other respiratory viruses. Persistent human infection with WU polyomavirus is described.

An initial report described the identification of WU polyomavirus in 6 (0.7%) of 890 respiratory tract samples collected in St. Louis, Missouri, USA, and in 37 (3.0%) of 1,245 respiratory tract specimens tested from Brisbane, Queensland, Australia (*1*). The goal of our study was to extend these initial findings by determining the prevalence of WU polyomavirus in a larger patient cohort in St. Louis.

The Study

We tested 2,637 nasopharyngeal swabs or nasal washes (from patients 1 day to 88 years of age) submitted to the virology laboratory at St. Louis Children's Hospital for routine respiratory virus detection from July 2003 through June 2004. Of these samples, 2,263 were from children ≤4 years of age (including 419 newborns) and 374 were from children >4 years of age. The specimens were extracted with the automated Roche MagNA Pure LC extractor and MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Indianapolis, IN, USA).

For real-time PCR, amplification primers WU-TAB02-F 5′- TGTTGCATCCATTTGTTACATTCAT-3′ and WU-TAB03-R 5′-GAAAGAACTGTTAGACAAATATATAG-GCCTTA-3′ and the minor groove binder probe WU-TAB04-pro 5′-6FAMATGTCAGCAAATTCMGB-NFQ-3′ were used with a commercially available universal TaqMan real-time PCR master mix and ABI 7500 Real-Time Thermocycler (Applied Biosystems, Foster City, CA, USA). All WU polyomavirus–positive specimens were screened for 17 additional viruses (influenza A and B; RSV A and B; PIV 1–4; human metapneumovirus; adenovirus subgroups B, C, and E; rhinovirus; and coronaviruses OC43, 229E, and NL63) by using the EraGen MultiCode-PLx respiratory virus panel as described previously (*1*).

Each clinical specimen was assigned a code. Collection of clinical data was approved by the Washington University Human Research Protection Office. Pertinent demographic, historical, and clinical information, when available, was collected by using a standard collection form. Statistical significance was determined by using 2-tailed Fisher exact χ^2 tests with Epi Info software version 3.4 (Centers for Disease Control and Prevention, Atlanta, GA, USA)

Seventy (2.7%) of the 2,637 tested specimens were positive for WU polyomavirus; 71% of the positive samples were also positive for ≥1 other respiratory virus. Of the 70 positive samples, 5 were omitted from analysis because of chart unavailability. The remaining 65 samples were collected from 60 individual patients (5 specimens were serial samples associated with distinct clinical syndromes in 2 immunocompromised patients).

Of the 60 WU-positive patients, 31 (52%) were female. The ethnic breakdown was as follows: 50% African-American, 47% Caucasian, 3% other. Positive specimens were noted for patients 1 day to 15 years of age (online Appendix Table, available from www.cdc.gov/EID/content/13/12/1936-appT.htm). The highest and lowest rates of infection are displayed in the Figure, panel A.

Patients positive for WU polyomavirus were detected throughout the year. A small peak was observed in July 2003, and a second small peak was observed in April and May 2004 (Figure, panel B). WU polyomavirus was the only virus detected in a 1-day-old full-term infant delivered by cesarean section who had been transferred to St. Louis Children's Hospital with respiratory distress requiring intubation. He was afebrile with lung opacities on chest radiograph. Patent ductus arteriosus and pulmonary hypertension were eventually diagnosed.

The 3 oldest patients positive for WU polyomavirus in this cohort were immunosuppressed. They included a 12-year-old with Evans syndrome and a 15-year-old with severe combined immunodeficiency syndrome (both post–bone marrow transplant) and a 14-year-old with end-stage renal disease and asthma.

The most common clinical findings in the patients with WU polyomavirus are listed in the Table. The most frequent diagnoses were pneumonia (31%) (although 40% had positive bacterial cultures), bronchiolitis (25%), and upper respiratory tract infections (15%). We also compared all the measured parameters from the patients who were infected with WU alone to the patients who were co-infected with other viruses. In most cases, no statistically signifi-

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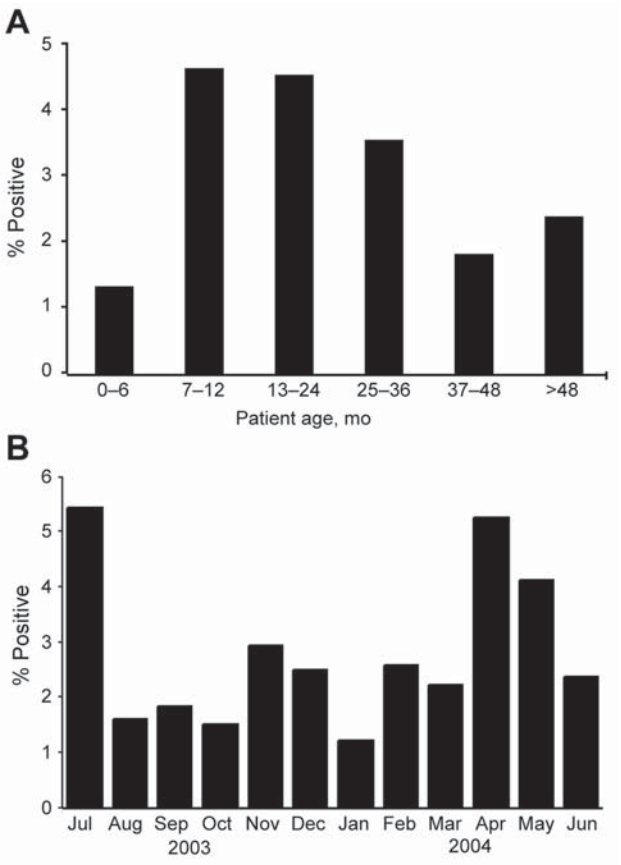

Figure. A) Percentage of samples positive for WU polyomavirus by age group. B) Percentage of samples positive for WU polyomavirus by month.

cant differences occurred between the co-infected and WU polyomavirus–only patients, except that more co-infected patients than WU polyomavirus–only patients had rhinorrhea (23/47 vs. 2/18; p = 0.005) and upper respiratory tract symptoms (30/47 vs. 6/18; p = 0.049). In addition, significantly more children with co-infection than with only WU polyomavirus had prior daycare exposure (18/45 vs. 1/15; p = 0.02).

The cohort of 2,637 samples included several sets of sequential samples taken from the same patient during the course of prolonged illness. In 2 patients, sequential samples obtained over a span of 6–8 weeks were positive for WU polyomavirus. The first patient was a 4-year-old girl with hemophagocytic lymphohistiocytosis, who had 4 distinct respiratory specimens that tested positive during a 2-month period. Her first positive specimen was obtained while she was asymptomatic during admission for a bone marrow transplant in September 2003. A second sample was obtained during a clinic visit for nasal congestion and cough in November 2003. Her third sample (also positive for coronavirus OC43) was obtained during an admission 5 days later for pneumonia. Finally, a fourth sample (negative for the previously detected coronavirus OC43) was taken 7 days later during the same admission after an episode of apnea.

The second patient was a 16-month-old child with biliary atresia admitted for a liver transplant in September 2003. He had 3 positive samples during a 6-week period. An initial sample taken on admission was negative for WU polyomavirus. Six weeks after transplant, fever and shortness of breath requiring intubation developed. A sample taken then was positive for both WU polyomavirus and adenovirus. Four weeks later, worsening shortness of breath and fever developed. His blood cultures were now also positive for

Table. Clinical parameters in episodes of WU polyomavirus infection

Symptoms and physical examination findings (n = 65)	%
Symptoms	
Cough	57
Upper respiratory tract symptoms	55
Rhonchi/crackles/coarse breath sounds	46
Shortness of breath or increased work of breathing	42
Wheezing	40
Rhinorrhea	38
Retractions	37
Decreased oral intake	34
Vomiting	32
Diarrhea	18
Stridor	6
Rash	6
Apnea	5
Signs	
Tachypnea* (n = 57)	79
Hypoxia† (n = 55)	47
Fever‡ (n = 63)	41
Bandemia (n = 42)	40
Leukocytosis§ (n = 42)	31
Leukopenia¶ (n = 42)	10
Radiographic or computed tomographic findings (n = 50)	
Infiltrate or consolidation	72
Hyperinflation	14
Peribronchial cuffing	12
Effusions	6
Treatment	
Antimicrobial agents (n = 65)	58
Bronchodilators (n = 65)	38
Steroids (n = 65)	37
Oxygen (n = 65)	25
Intubation (n = 63)	11
Intensive care (n = 63)	19
Risk factors	
Daycare	32
Sick contacts	25
Medical history	
Asthma (n = 60)	37

*Defined per National Institutes of Health clinical center guidelines.
†$SaO_2 \leq 95\%$ or $PaO_2 > 80\%$.
‡Temperature $\geq 38°C$.
§Leukocyte count >15,000/mm^3.
¶Leukocyte count <4,000/mm^3.

Klebsiella and *Enterobacter* spp., and his third respiratory sample demonstrated both WU polyomavirus and rhinovirus. Two weeks later, fever, hypoxia, and increasing secretions developed, with his fourth sample positive only for WU polyomavirus. Followup samples obtained 3 months later for each patient indicated clearance of WU virus.

Conclusions

Patients infected with WU polyomavirus in this cohort were primarily hospitalized with pneumonia, bronchiolitis, and upper respiratory tract infections. One new observation is that multiple respiratory specimens sampled from the same patient over 6–8 weeks were positive for WU polyomavirus, which suggests that WU polyomavirus may persistently infect humans. Both patients were immunocompromised, although they were able to clear infections with other viruses (coronavirus OC43, rhinovirus, adenovirus), which suggests that the continued detection of WU polyomavirus was not due to a completely incapacitated immune system. Sequence analysis of a 250-bp fragment of the VP2 gene of WU polyomavirus amplified from multiple samples from the 2 patients showed no sequence polymorphisms between the initial and later samples (data not shown). As some sequence variation has previously been reported in this locus (*1*), these data are consistent with the model of persistent infection. The detection of WU polyomavirus in the respiratory secretions of a 1-day-old infant suggests that vertical transmission of WU polyomavirus from mother to fetus may occur, although further studies are needed to verify this suggestion.

In conclusion, WU polyomavirus was detected in 2.7% of patients with respiratory tract infections. A high percentage of coinfection with other respiratory viruses was detected, complicating interpretation of the clinical findings. However, WU polyomavirus was the sole virus detected in 20 specimens from patients with respiratory illness, which suggests that it may be a respiratory pathogen. Finally, the observed persistence of this virus suggests analogy to BK and JC viruses in this regard (*2*).

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All experiments described in this manuscript were performed at Washington University, St. Louis, MO, USA.

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WU Polyomavirus in Children, Canada

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WU polyomavirus was detected in nasopharyngeal aspirates in 2 (2.5%) of 79 children with respiratory infections (both infected with respiratory syncytial virus) and in 5 (6.4%) of 78 asymptomatic children during the same winter season in Canada. The strains were closely related to Australian and American viruses based on analysis of large T antigen (TAg) and VP2 genes. The pathogenic role of WU virus is still uncertain.

Polyomaviruses are nonenveloped viruses that have an icosahedral capsid containing a small, circular, double-stranded DNA genome (*1*). These viruses have been identified in a variety of mammals and birds worldwide, and the most studied polyomavirus species infecting animals are the mouse polyomavirus (*2*) and the simian vacuolating (SV40) virus (*3*). In 1971, 2 human polyomavirus species named BK and JC viruses, respectively, were first isolated from the urine of a kidney allograft recipient with chronic pyelonephritis and advanced renal failure (*4*) and from the brain of a patient with progressive multifocal leukoencephalopathy (*5*). Recently, 2 new human polyomavirus members were described. The KI virus was identified in nasopharyngeal aspirates (NPA) and feces from patients with respiratory tract infections in Sweden (*6*). Also, Gaynor and colleagues (*7*) reported the detection and molecular characterization of the WU virus in clinical respiratory samples from patients with acute respiratory tract infections (ARTI). Although the pathogenesis of BK and JC viruses has been clearly established, the role of the KI and WU viruses as respiratory pathogens has yet to be demonstrated. In this article, we report on the molecular detection and characterization of WU viruses in NPA from hospitalized children with or without respiratory tract infections.

The Study

We tested 157 NPA specimens obtained from a case-control study on the incidence of respiratory viral agents, the results of which have been partly reported by our group (*8*). Participants were children ≤3 years of age who were hospitalized from December 2002 through April 2003 at Laval University Hospital Center in Quebec City, Quebec, Canada. Case-patients were children admitted for ARTI (mostly bronchiolitis, pneumonitis, and laryngotracheobronchitis) who had an NPA collected as part of the investigation of their illness. A research nurse administered a specific questionnaire at admission in the presence of the parents. At the end of the hospitalization, the children's charts were reviewed to collect clinical and laboratory data. Eligible controls were children hospitalized during the same period for any elective surgery. These children had no concomitant respiratory symptoms or fever at admission, although they might have had an ARTI in the weeks before hospitalization. The study nurse obtained a signed consent from parents and a NPA was obtained during surgery. The original study was approved by the ethics committee of the Center Hospitalier Universitaire de Quebec.

NPA samples (200 μL) were used for nucleic acid extraction using the QIAamp viral RNA Mini Kit (QIAGEN, Inc., Mississauga, Ontario, Canada), which has been shown to recover both RNA and DNA. These specimens were previously analyzed by using a multiplex real-time RT-PCR assay for influenza A and B viruses, human respiratory syncytial virus (hRSV), and human metapneumovirus (hMPV) (*8*). For symptomatic children, viral cultures and antigen detection assays were performed at the treating physician's request. The specimens were frozen at –80°C during the 4 years before PCR studies began for WU polyomavirus. All specimens were first tested for WU virus DNA by using primers AG0048 and AG0049, which allowed the amplification of a 244-bp product in the 3′ end of the large T antigen (TAg) region (*7*). A plasmid containing the partial WU genomic DNA from the original Australian virus (B0 strain) served as the positive control in each PCR batch (*7*). For WU-positive samples with the TAg primers, a confirmatory PCR assay was performed with primers AG0044 and AG0045 to amplify a 250-bp fragment from the VP2 region (*7*). PCR products were analyzed by agarose gel electrophoresis. Positive amplicons were subsequently purified and sequenced by using the respective PCR primers.

The Canadian WU VP2 sequences were compared with those of 18 WU viruses that originated in Brisbane, Queensland, Australia, and St Louis, Missouri, USA (*7*). For this purpose, multiple nucleotide sequence alignments were performed by using the ClustalW program followed by phylogenetic analyses, which were conducted with the MEGA version 3.1 software using the neighbor-joining algorithm with Kimura-2 parameters (*9*).

By using the PCR assay with primers targeting the large TAg, WU sequences were detected in 2 (2.53%) of 79 symptomatic children (the 2 children were 13 months old) and in 5 (6.41%) of 78 asymptomatic children, 13–24 months of age (mean age 20 months) (Table). Symptomatic children had a diagnosis of bronchiolitis (patient 1) and pneumonitis (patient 2) and were both coinfected with hRSV. In contrast, no other viruses were detected in the asymptomatic children who underwent elective surgery. The duration of hospitalization for the 2 symptomatic children

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Table. Clinical data from 7 WU polyomavirus–infected children, Canada, 2003*

Patient no.	Sex	Age, mo	Date sample collected	Sample type	PCR for WU virus LTAg/VP2	Diagnosis	Copathogen
1	F	13	Feb 13	NPA	+/+	Bronchiolitis	hRSV
2	M	13	Mar 5	NPA	+/–	Pneumonitis	hRSV
3	F	13	Feb 24	NPA	+/+	None†	None
4	M	20	Mar 13	NPA	+/–	None†	None
5	F	24	Mar 17	NPA	+/+	None†	None
6	M	19	Mar 31	NPA	+/+	None†	None
7	F	24	Apr 1	NPA	+/–	None†	None

*NPA, nasopharyngeal aspirates; hRSV, human respiratory syncytial virus.
†No fever or respiratory symptoms at the time of elective surgery.

with dual WU/hRSV infection (3 and 4 days) was similar to that of 120 children with single hRSV infection (median: 4 days). By using the PCR assay with primers targeting the VP2 region, WU sequences were detected in 4/7 previously positive children, including 1 symptomatic (patient 1) and 3 asymptomatic (patients 3, 5 and 6) patients (Table). The 7 large TAg nt sequences of Canadian WU viruses were 100% identical and had 99.5% identity to the WU sequence contained in the control plasmid from Australia (data not shown). In addition, 100% identity was found between the VP2 nt sequences of the 4 Canadian WU viruses (data not shown). The latter also shared 100% identity with the most frequently observed WU genotypes (represented by previously-reported WU strains B9, S6, B28, B37, B22, B24, B35, B10, B1, and B17 [GenBank accession numbers: EF444592, EF444593, EF444590, EF444589, EF444588, EF444587, EF444586, EF444584, EF444583 and EF444582, respectively]) and, obviously, clustered together in the phylogenetic tree (Figure).

Conclusions

In this study, we report for the first time, to our knowledge, the presence of the newly described WU polyomavirus in Canadian children. We found that more asymptomatic (6.4%) than symptomatic (2.5%) children shed viral DNA in their respiratory tract. The WU polyomavirus was previously identified in respiratory tract samples from Australian and American patients, which suggests its worldwide distribution. Efforts to culture this new virus by using PCR-positive respiratory specimens have thus far been unsuccessful (D. Wang, unpub. data).

The 7 sequences of the large TAg region and the 4 sequences of the VP2 region from the Canadian WU strains displayed no sequence variations. This could be due to the short size of these PCR products (244 and 250 bp, respectively) and the stability of this double-stranded DNA genome. A similar finding of limited sequence variation was reported in the previous molecular study performed with Australian and American WU strains (*7*). Indeed, 4 Canadian WU strains had 100% nucleotide identity with 10 strains selected from these cohorts when VP2 sequences were compared (Figure).

Since we aimed at evaluating the possible contribution of the WU polyomavirus in respiratory tract infections of children, we tested NPA samples from symptomatic and asymptomatic subjects of the same age (≤3 years) that were collected during the same winter period at the same institution. The WU virus was detected in 5 asymptomatic children at the time of an elective surgery and in 2 symptomatic children (bronchiolitis and pneumonitis) who were also

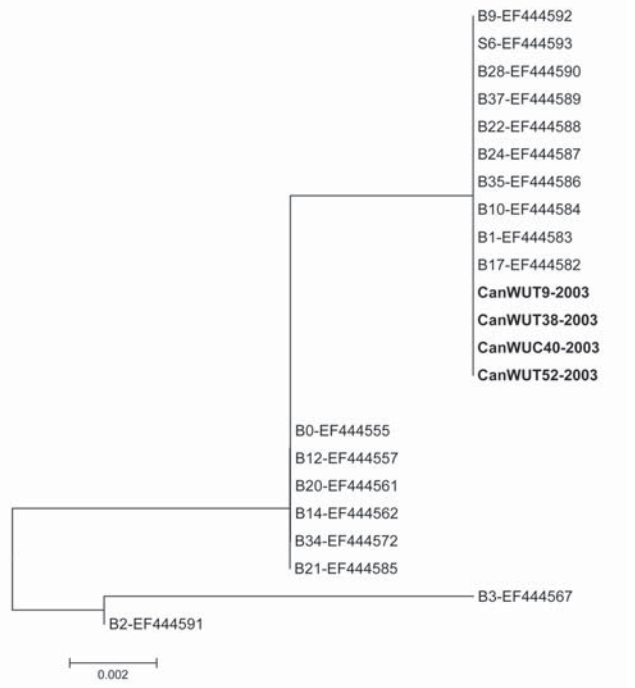

Figure. Phylogenetic analysis of Canadian WU polyomavirus strains CanWUT9–2003, CanWUT38–2003, CanWUC40–2003 and CanWUT52–2003 (shown in **boldface**), based on nucleotide sequences of the VP2 region. Multiple nucleotide sequence alignments were performed by using the ClustalW program and a phylogenetic tree was constructed with the MEGA 3.1 software using the neighbor-joining algorithm with Kimura-2 parameters (*9*). The analysis included WU strains previously identified from Australian and American cohorts (*7*) i.e., B9, S6, B28, B37, B22, B24, B35, B10, B1, B17, B0, B12, B20, B14, B34, B21, B3, and B2 (GenBank accession nos.: EF444592, EF444593, EF444590, EF444589, EF444588, EF444587, EF444586, EF444584, EF444583, EF444582, EF-444555, EF444557, EF444561, EF444562, EF444572, EF444585, EF444567, and EF444591, respectively).

infected with hRSV. A high rate of coinfection was also noted in the Australian cohort (68%) and in the American cohort (100%) (*7*). Notably, similar findings were obtained in a study on the related KI polyomavirus in which another viral pathogen was found in 5/6 KI-positive samples (*6*). By analogy with other human polyomaviruses (BK and JC), WU and KI possibly could establish a latent infection with subsequent asymptomatic reactivation; further studies are needed for confirmation. The presence of WU virus in the control children could also represent prolonged shedding from a prior respiratory tract infection. In conclusion, although this study confirmed the presence of the WU polyomavirus in NPA samples from Canadian children and suggests that its distribution is worldwide, its role in respiratory tract diseases of children remains undetermined.

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Rhodococcus equi Infection after Alemtuzumab Therapy for T-cell Prolymphocytic Leukemia

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Rhodococcus equi, mainly known from veterinary medicine as a pathogen in domestic animals, can also cause infections in immunocompromised humans, especially in those with defects in cellular immunity. Alemtuzumab, an anti-CD52 monoclonal antibody, causes lymphocytopenia by eliminating CD52-positive cells. We report a patient in whom *Rhodococcus equi* infection developed after alemtuzumab therapy.

Rhodococcus equi is a soil-borne, asporogenous, nonmotile, obligate aerobe; it is also a facultative, intracellular, gram-positive microorganism that can survive inside macrophages, the characteristic considered the basis for its pathogenicity (*1*). In foals and other domestic animals, it is an important respiratory and intestinal pathogen (*2*). Human infection with *R. equi* is rare but can occur in immunocompromised patients, especially those who have HIV infection and a $CD4^+$ cell count $<100 \times 10^6$/L (*3*). The clinical manifestations are diverse, although 80% of patients have some pulmonary involvement (*3*). In recent decades, an increased incidence of *R. equi* infections in humans has been reported. This increase may be due to the rising number of immunocompromised patients as a result of increasing numbers of organ transplantations and intensified antitumor chemotherapy. We describe a patient with T-prolymphocytic leukemia (T-PLL) in whom a febrile disease with lung abscess due to *R. equi* developed 10 weeks after the complete remission of leukemia was induced by chemotherapy combined with alemtuzumab.

Case Report

A 68-year-old man with T-PLL (leukocyte count 174.5 $\times 10^9$/L, 96% lymphoid cells) was treated with chemotherapy consisting of cyclophosphamide, doxorubicin, vincristine, and prednisolone every 2 weeks (CHOP14), in combination with alemtuzumab 30 mg subcutaneously on days 1, 5, and 9 of each cycle. This combined therapy was well tolerated. Complete cytologic and immunohistochemical remission was confirmed by blood and bone marrow examination 2 weeks after the latest chemotherapy treatment. Ten weeks later, the patient experienced flu-like symptoms and had a fever of 38.9°C. One week earlier, the antimicrobial prophylaxis, which consisted of valacyclovir, 500 mg 2 times/day, and trimethoprim-sulfamethoxazole, 960 mg 3 times/week, had been stopped, although the alemtuzumab-induced lymphocytopenia was still present (leukocytes 7.2 $\times 10^9$/L, 84% neutrophils, 0.6% lymphocytes). Outpatient evaluation showed 2 lung abscesses. From 3 consecutive blood cultures and from the bronchoalveolar lavage fluid, a gram-positive bacillus with mucoid growth was isolated and identified as *R. equi* (API Coryne, bioMérieux, Marcy l'Etoile, France). The isolated strain was resistant to β-lactam antimicrobial drugs and trimethoprim-sulfamethoxazole and susceptible to aminoglycosides, tetracyclines, fluoroquinolones, glycopeptides, erythromycin, and rifampin. Treatment with moxifloxacin and rifampin was begun. After 3 weeks of treatment, fever developed in the patient again. Blood cultures grew *R. equi*. The patient was admitted to the hospital for intravenous treatment with imipenem/cilastatin, 500 mg/500 mg 3 times/day, and vancomycin, 1.5 g once a day. A computed tomographic scan of the chest showed progression of the pulmonary abscesses and mediastinal lymphadenopathy. Clarithromycin, 500 mg 2 times/day, was added, and the vancomycin was increased to 2 g once a day, which resulted in clinical improvement. Purple, subcutaneous, oval lesions, 2–3 cm in diameter and not painful to palpation, were seen on the upper portion of both legs. Pathologic examination of these lesions after biopsy showed suspected localization of T-PLL. *R. equi* could not be demonstrated in these skin lesions by either pathologic or microbiologic examination. After 2 weeks of receiving intravenous antimicrobial drugs, the patient was discharged with oral rifampicin, 600 mg once a day; ciprofloxacin, 750 mg twice/day; and azithromycin, 500 mg once a day.

He was readmitted to our hospital 9 weeks later because he had become dyspneic and febrile. Evaluation showed pleural effusion on the right side. Progression of the T-PLL was also diagnosed. After 1 week's incubation of the pleural fluid, mucoid nonpigmented colonies were growing, consisting of gram-positive coccoid rods, which were catalase positive. *Rhodococcus* infection was suspected and confirmed by 16S rDNA sequencing without further conventional identification. The isolate showed intermediate susceptibility to ciprofloxacin (MIC 0.75 mg/L), moxifloxacin (MIC 0.5 mg/L), and erythromycin (MIC 1.5 mg/L). Drainage of the pleural

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fluid resulted in a trapped lung due to pleural thickening. A pleurectomy was considered but was refused by the patient, considering his poor overall prognosis based on the relapse of T-PLL. On his request, the antimicrobial drugs were stopped, and he went home with palliative treatment consisting of morphine and prednisone. He died 3 months later. Overall, he had been treated with antimicrobial agents for 19 weeks.

Conclusions

The described patient acquired a *R. equi* infection during alemtuzumab-induced lymphocytopenia. *R. equi* infection is predominantly airborne, acquired through the respiratory tract. Exposure to domestic animals, such as horses and pigs, may play a role in acquisition of this organism. The patient denied any such contact, as do two thirds of all patients infected with *R. equi* (*3*).

Alemtuzumab is approved as a second-line treatment in chronic lymphatic leukemia and is increasingly used in therapeutic trials for T-cell malignancies. It is a recombinant DNA-derived, humanized monoclonal antibody directed against CD52 (*4*). CD52 is a membrane glycoprotein expressed mainly by lymphocytes, especially T cells. Alemtuzumab causes lysis of these cells by binding to CD52, resulting in lymphocytopenia, which can persist for up to 320 days after treatment (*5*). While the patient is experiencing lymphocytopenia, prophylaxis with an antiviral agent and trimethoprim-sulfamethoxazole are mandatory to prevent the most frequent opportunistic infections (*6*). Reduced cellular immunity is known to predispose to infection with *R. equi* (*7*). Primary prophylaxis is not routinely recommended because no data are available to support its efficacy and because the infection is rare (*3*). Due to variable susceptibility to trimethoprim-sulfamethoxazole, the prophylaxis regimen used after alemtuzumab therapy will not prevent *R. equi* infection in all patients, as this case illustrates.

Standard treatment regimens for *R. equi* infections have not been established. Weinstock and Brown advised intravenous therapy with 2 or 3 drug regimens that include vancomycin, imipenem, aminoglycosides, ciprofloxacin, rifampin, or erythromycin (*3*). This recommendation was based on in vitro susceptibility data and published case reports. Treatment should preferably be guided by susceptibility testing. After clinical improvement (usually after 2–4 weeks), oral antimicrobial agents can then be substituted and continued until all culture results are negative and the patient's symptoms and signs have resolved. A minimum of 6 months of antimicrobial drug therapy is typically required for immunocompromised patients with pulmonary, bone and joint, or central nervous system infections (*3*).

Our patient started treatment with oral antibiotics, guided by susceptibility tests. Although moxifloxacin and rifampin are known for their good oral resorption, and despite initial clinical improvement, progression of the infection was apparent by the clinical course. Susceptibility testing was not performed at this time, but testing later in the clinical course suggested a decrease in susceptibility by the *R. equi* strain to the antimicrobial agents given.

After this regimen failed, intravenous therapy with 3 antimicrobial drugs was instituted. However, also this strategy ultimately failed. Apart from persistence of bacilli due to poor penetration at the site of infection, and the possible development of resistance, this lack of response is likely due to persistent lymphocytopenia resulting from previous treatment with alemtuzumab and progression of T-PLL.

In summary, longstanding alemtuzumab-induced lymphocytopenia is the most likely cause of the uncontrollable opportunistic *R. equi* infection in the described patient. This case illustrates the therapeutic challenges of this kind of infection in severely immunocompromised patients.

Dr Meeuse is a completing a residency in internal medicine at the University Medical Center Groningen. He is also a PhD candidate in the field of palliative medicine, focusing on measurement and treatment of pain of malignant origin.

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Antimicrobial Drug Resistance in Singapore Hospitals

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A new national antimicrobial resistance surveillance program in Singapore public hospitals that uses WHONET detected high levels of methicillin resistance among *Staphylococcus aureus* (35.3%), carbapenem resistance among *Acinetobacter* spp. (49.6%), and third-generation cephalosporin resistance among *Klebsiella pneumoniae* (35.9%) hospital isolates in 2006. Antimicrobial drug resistance is a major problem in Singapore.

Bacterial antimicrobial drug resistance is a worldwide problem that is exacerbated by the diminishing number of new antimicrobial drugs in the pharmaceutical pipeline (*1*,*2*). This is an emerging public health problem, especially in hospitals of the newly industrialized countries of Asia and the Pacific. In 2001, the World Health Organization (WHO) launched the first global strategy to counter this phenomenon (*3*), a key component of which is the development of surveillance programs to monitor trends in antimicrobial drug resistance and use (*3*).

Overarching surveillance programs monitoring antimicrobial drug–resistance trends on a national or regional level are present in Australia (*4*) and Europe (*5*). Such is not the case in Singapore, where surveillance efforts have generally been conducted only at the institutional level, with limited sharing and analysis of data. As a result, the actual scale of local antimicrobial drug resistance is not well defined. The Network for Antimicrobial Resistance Surveillance (Singapore), a voluntary group of healthcare professionals, was established in December 2005 to fill this gap.

The Study

A laboratory-based surveillance program was established in 2006 to monitor the antimicrobial drug–resistance trends of 6 common nosocomial pathogens: *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus* spp., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. Excluding coagulase-negative staphylococci, these organisms collectively account for >90% of positive bacterial cultures from nosocomial infections locally.

All 6 public sector acute-care hospitals in Singapore—2 tertiary-care hospitals, 3 secondary-care hospitals, and 1 institution dedicated to pediatrics and obstetrics/gynecologic services only—participated in the program. These hospitals constitute ≈76.5% of the 8,205 acute-care hospital beds available in the country (*6*).

All clinical isolates submitted to the externally accredited microbiology laboratories of these hospitals in calendar year 2006 were recorded for this study. Four laboratories performed antimicrobial drug–susceptibility testing predominantly through disk-susceptibility testing, supplemented by VITEK 2 system (bioMérieux, Marcy l'Etoile, France), following guidelines of the Clinical Laboratory Standards Institute (CSLI) (*7*). One laboratory used the VITEK 2 system exclusively, following CLSI guidelines (*7*), and the sixth laboratory used disk-susceptibility testing, following guidelines for the calibrated dichotomous sensitivity method (*8*).

Microbiologic and demographic data were extracted every quarter from the laboratory information system of each participating institution and converted into a standard format by using WHONET 5 (WHO, Geneva, Switzerland). Data were collated and analyzed centrally, with duplicates eliminated according to CLSI guidelines (*9*). Hospital bed occupancy data were obtained from the published records of each institution.

Statistical analysis was performed by using Excel 2003 (Microsoft, Redmond, WA, USA). Clinical microbiologists of the respective hospitals verified the analyzed data. Combined antimicrobial drug–susceptibility data were analyzed for the target organisms in 3 ways: for all isolates, for blood culture isolates only, and for isolates from intensive care unit (ICU) settings. The same analysis was also separately performed for data from each institution.

The distribution of resistant organisms isolated in 2006 is shown in the Table. The incidence density of resistant organisms from clinical samples for 2006 is shown in the Figure. Antimicrobial drug resistance was generally more prevalent in ICUs, but there was marked interhospital variation in resistance percentages. The tertiary hospitals had high rates of antimicrobial drug resistance, whereas the pediatric and women's hospital had much lower rates.

Antimicrobial drug resistance in the Enterobacteriaceae was prevalent for amoxicillin-clavulanate (*K. pneumoniae* 36.0%, *E. coli* 26.7%), ciprofloxacin, and third-generation cephalosporins (Table). Imipenem resistance was present in 0.2% (14 isolates) of *K. pneumoniae*. Ertapenem resistance was reported in 0.2% of all *E. coli* isolates and 0.9%

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Table. Drug-resistant clinical bacterial isolates cultured at public sector hospitals, Singapore, 2006*

Isolates	All resistant isolates		Resistant blood isolates			Resistant ICU isolates		
	No. (%) of all isolates†	% Range for single hospitals‡	No. (%) of all blood isolates†	% Range for single hospitals‡	p value§	No. (%) of all ICU isolates†	% Range for single hospitals†	p value¶
Methicillin-resistant *S. aureus*	3,517 (35.3)	18.0–44.3	497 (39.8)	23.8–44.4	<0.01	261 (46.7)	26.8–70.5	<0.01
Vancomycin-resistant enterococci (*E. faecium* or *E. faecalis*)	31 (0.8)	0–1.3	5 (1.3)	0–2.4	0.25	3 (1.2)	0–3.2	0.46
3rd-generation cephalosporin-resistant *E. coli*	2,257 (17.5)	6.1–22.8	284 (17.9)	7.4–19.0	0.66	123 (33.4)	12.7–41.4	<0.01
Quinolone-resistant *E. coli*	4,227 (34.4)	15.2–40.1	453 (28.6)	15.4–40.5	<0.01	150 (41.6)	12.0–54.6	<0.01
Cephalosporin and quinolone-resistant *E. coli*	1,080 (8.4)	0.8–19.9	181 (11.4)	5.7–15.3	<0.01	79 (21.4)	2.9–40.5	<0.01
3rd-generation cephalosporin-resistant *K. pneumoniae*	2,651 (35.9)	9.6–49.7	294 (30.6)	13.8–34.5	<0.01	187 (37.2)	8.8–46.6	0.54
Quinolone-resistant *K. pneumoniae*	3,074 (42.5)	11.5–58.3	321 (33.6)	11.1–39.6	<0.01	183 (36.7)	6.2–47.6	<0.01
Cephalosporin- and quinolone-resistant *K. pneumoniae*	1,839 (24.9)	2.0–46.1	214 (22.3)	6.9–35.2	0.05	135 (26.2)	0.0–41.2	0.47
Carbapenem-resistant *P. aeruginosa*	477 (9.6)	2.4–12.2	45 (16.5)	9.1–23.1	<0.01	74 (18.3)	3.3–27.2	<0.01
Carbapenem-resistant *Acinetobacter* spp.	929 (49.6)	16.9–65.5	86 (48.1)	18.2–66.7	0.66	164 (59.7)	31.6–68.8	<0.01
Multidrug-resistant *Acinetobacter* spp.*	354 (18.2)	3.6–26.1	34 (17.8)	0.0–29.8	0.88	64 (23.4)	0.0–30.2	0.02

*ICU, represents all intensive care units, including surgical, medical, pediatric, and neonatal; *S. aureus, Staphylococcus aureus; E. faecium* or *E. faecalis, Enterococcus faecium or Enterococcus faecalis; E. coli, Escherichia coli; K. pneumoniae, Klebsiella pneumoniae; P. aeruginosa, Pseudomonas aeruginosa*. Multidrug resistant is defined by resistance to ampicillin/sulbactam, carbapenems, all cephalosporins, aminoglycosides (gentamicin and amikacin), and ciprofloxacin.
†No. resistant isolates (e.g., methicillin-resistant *S. aureus*, carbapenem-resistant *P. aeruginosa*) from all clinical specimens from all hospitals. The percentage in parenthesis refers to the proportion of resistant isolates over all isolates of the same species (resistant plus susceptible).
‡Range of proportions of resistant isolates over all isolates of the same species obtained from individual hospitals, expressed as percentages.
§p value for χ^2 test comparing proportion of resistant isolates in blood culture and non–blood culture isolates.
¶p value for χ^2 test comparing proportion of resistant isolates in ICU vs. non–ICU culture isolates.

of all *K. pneumoniae* isolates at the institutions that routinely test for this agent.

Despite the relatively small numbers of *Acinetobacter* spp. isolates compared with the other organisms, carbapenem-resistant *Acinetobacter* spp. were found in all ICUs at a high incidence density; as many as 69% of all isolates at 1 ICU were carbapenem resistant. Fully 18.2% of all *Acinetobacter* spp. were resistant to ampicillin/sulbactam, cephalosporins, carbapenems, ciprofloxacin, and aminoglycosides; these particular isolates were susceptible to only the polymyxins. Carbapenem resistance was also found in 9.6% of all *P. aeruginosa* isolates and in up to 27.2% of ICU isolates.

Methicillin resistance occurred in 35.3% of all *S. aureus* isolates. Methicillin-resistant *S. aureus* (MRSA) strains showed correspondingly high resistance levels to macrolides (90.2%), ciprofloxacin (93.9%), and trimethoprim-sulfamethoxazole (49.9%). Vancomycin resistance was reported in 0.8% of all enterococci.

Based on incidence density calculations, MRSA was the predominant drug-resistant pathogen at all hospitals. It had the highest incidence density for blood and ICU cultures (0.31/1,000 inpatient-days and 4.48/1,000 ICU inpatient-days, respectively) among all organisms surveyed. Third-generation cephalosporin-resistant *K. pneumoniae* was the predominant gram-negative resistant pathogen, with an incidence density of 0.19/1,000 and 3.21/1,000 inpatient days for blood and ICU cultures, respectively.

A comparison between organisms isolated from blood cultures and other cultures demonstrated statistically significant differences with regard to percentage resistance for *S. aureus*, *P. aeruginosa*, and the Enterobacteriaceae. The reason for these finding is not evident. In general, ≈10% of all resistant organisms were isolated from blood cultures.

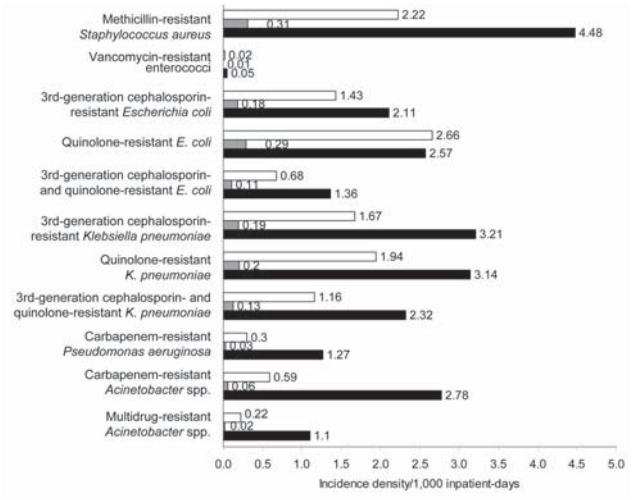

Figure. Incidence density of various antimicrobial drug–resistant bacteria isolated in public sector hospitals, Singapore, 2006. White bars, incidence density, all isolates (per 1,000 inpatient-days); gray bars, incidence density, blood isolates (per 1,000 inpatient-days); black bars, incidence density, intensive-care unit (ICU) isolates (per 1,000 ICU inpatient-days). *S. aureus, Staphylococcus aureus; E. coli, Escherichia coli; P. aeruginosa, Pseudomonas aeruginosa*.

Conclusions

This is the first comprehensive national survey of antimicrobial drug resistance in Singapore public hospitals. We believe that our findings represent the endemic antimicrobial drug resistance situation in our hospitals; quarterly data analysis did not show any overt outbreak. These results, although new, are not surprising. Previous regional surveys and local studies had already hinted at the extent of the problem in Singapore (*10*–*12*). Similar data have also been reported from other countries in the Asia Pacific region (*10*,*11*).

Use of both incidence density and percentage resistance enabled a more nuanced analysis of the scale of the problem. Although almost half of all *Acinetobacter* spp. clinical isolates were resistant to imipenem, the relative rarity of isolating this organism from clinical specimens renders it a smaller problem compared with MRSA or quinolone-resistant Enterobacteriaceae outside the ICU setting.

In comparison with similar data from Europe (*5*) and Australia (*4*), prevalence of resistance in gram-negative organisms is much higher but prevalence of vancomycin-resistant enterococci is lower. MRSA rates are comparable to those in some countries in southern Europe (*5*) but higher than those in Australia. The reasons for the differences in antimicrobial drug–resistant patterns might be related to infection control practices or to timing of the introduction of resistant organisms. However, more research is needed to clarify these differences.

There are several limitations of this work. First, the inability to segregate nosocomial and community infections prevented a more detailed analysis of antimicrobial drug–resistance issues pertaining to community and hospital settings. Second, the use of different laboratory standards and methods potentially adds a degree of inaccuracy in the analyses. Third, routine laboratory data did not enable us to distinguish the different mechanisms of resistance, particularly among gram-negative bacteria, or to determine the presence of any predominant clone responsible for the high endemic levels of antimicrobial resistance.

Nevertheless, the results can serve to direct any national effort aimed toward reducing the antimicrobial resistance problems of local hospitals. The issues of MRSA in general and carbapenem-resistant *Acinetobacter* spp. and *P. aeruginosa* in local ICUs are particularly pressing. Continued surveillance will also serve as an impartial feedback on the efforts of infection control programs for the future. For a small city-state, comprehensive national surveillance is relatively easier for Singapore than for larger countries. Such surveillance of clinical microbiology isolates is a critical first step toward controlling the growing worldwide threat of antimicrobial drug resistance, and WHONET is a useful tool in this respect.

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Bartonella DNA in Dog Saliva

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Bartonella species, transmitted by arthropods or animal bites and scratches, are emerging pathogens in human and veterinary medicine. PCR and DNA sequencing were used to test oral swabs collected from dogs. Results indicated the presence of 4 *Bartonella* species: *B. bovis, B. henselae, B. quintana*, and *B. vinsonii* subspecies *berkhoffii*.

Bartonella species are being recognized as increasingly important bacterial pathogens in veterinary and human medicine. These organisms can be transmitted by an arthropod vector or alternatively by animal scratches or bites (*1*). Among the 11 species or subspecies known or suspected to be pathogenic in humans, 8 have been detected in or isolated from pet dogs or cats, thereby highlighting the zoonotic potential of these bacteria (*2*). In general, cats are implicated in the transmission of *Bartonella henselae*, typically resulting in cat-scratch disease; however, there have also been sporadic reports of *Bartonella* transmission by dogs (*3*–*5*). When *B. henselae* prevalence was evaluated in a population of 52 dogs, 4 dogs were seroreactive at reciprocal titers of 64 or 128, and *Bartonella*-positive PCR results were found in 3 of 52 blood samples, 5 of 9 oral swabs, and 5 of 9 nail clippings (*5*). Based on these reports and the recent recognition of *B. henselae* and *B. vinsonii* subspecies *berkhoffii* bacteremia in veterinarians and veterinary technicians who experience frequent cat and dog scratches and bites (*6*), we speculated that *Bartonella* species may be present in the saliva of dogs. The purpose of this study was to determine whether *Bartonella* DNA could be detected in oral swabs collected from dogs.

The Study

As part of an ongoing study from November 2004 to December 2006 to investigate the prevalence of *Anaplasma, Bartonella*, and *Ehrlichia* infections in healthy golden retrievers and golden retrievers with lymphoma, a buccal swab was collected using a sterile cotton applicator. The swab was placed against the inside surface of the dog's cheek. Saliva and tissue were collected by rolling the swab firmly against the cheek. Subsequently, the swab was placed into a sterile, no additive, Vacutainer (Becton Dickinson**,** Franklin Lakes, NJ, USA) serum tube and allowed to air dry for 10 to 15 minutes at room temperature before the tube was recapped.

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Cells on the air-dried swab were resuspended in 500 μL of QuickExtract DNA Extraction Solution (EPICENTRE Biotechnologies, Madison, WI, USA), according to the manufacturer's instructions. Total DNA was isolated using 200 μL of the QuickExtract resuspension, which was extracted through a QIAamp DNA Blood Mini-Kit (QIAGEN, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Similarly, total DNA was extracted from 200 μL of EDTA-anticoagulated whole blood using the QIAamp DNA Blood Mini-Kit.

Oral swabs and blood samples (n = 44 each) were screened for the presence of *Bartonella* by 2 previously described PCR methods (*7*). The first PCR targeted a fragment of the 16S-23S intergenic transcribed spacer (ITS) region; samples that were PCR positive for *Bartonella* DNA by the ITS primers were subsequently analyzed by a second PCR targeting the heme-binding protein gene, Pap31. Positive and negative controls were used in all processing steps, including DNA extraction. PCR amplicons were sequenced to identify species (Davis Sequencing, Davis, CA, USA). Sequence analysis and alignment with GenBank sequences were performed (AlignX, Vector NTI Suite 6.0, InforMax, Inc., Frederick, MD, USA). Additionally, serum samples were analyzed for IgG antibodies to *B. henselae* and *B. vinsonii* (*berkhoffii*) using an indirect immunofluorescence assay (IFA), as described previously (*8*). Reciprocal titers ≥64 were considered seroreactive.

Of the 44 dogs surveyed, oral swabs collected from 5 (11.4%) dogs were PCR-positive for *Bartonella* DNA. Sequencing indicated that 5 different *Bartonella* species or subtypes were present: *B. bovis*, *B. henselae*, *B. quintana*, and *B. vinsonii* subsp. *berkhoffii* types I and II (Table). PCR amplification and sequencing of blood samples from these 5 dogs showed *B. henselae* and *B. vinsonii* (*berkhoffii*) DNA in 2 dogs (Table). None of these 5 dogs was seroreactive to *B. henselae* or *B. vinsonii* (*berkhoffii*) antigens. Contamination was not detected in any of the negative control samples at any stage of processing or at any time during the study. As this work was part of an ongoing study of golden retrievers with and without lymphoma, dogs 1 and 2 had lymphoma; the remaining 3 dogs were clinically healthy (Table).

Conclusions

These results demonstrate the presence of *Bartonella* DNA in oral swabs obtained from dogs. Notably, 3 *Bartonella* species and 2 *B. vinsonii* (*berkhoffii*) types were found in dog saliva. *B. bovis*, formerly referred to as *B. weissii*, was initially isolated from the blood of cats (*9*). Subsequently, this organism was isolated from the blood of cows in the United States, Europe, and Africa (*10*–*12*). To our knowledge, this is only the second known report of the detection of *B. bovis* DNA in a sample obtained from a dog

Table. PCR, DNA sequencing, and serologic results for the 5 dogs positive for *Bartonella* DNA in oral swabs*

Dog no.	*B. henselae* IFA	*B. vinsonii* (*berkhoffii*) IFA	PCR target	DNA sequence in blood	DNA sequence in oral swab
1	<16	<16	ITS, Pap31	*B. henselae* and *B. vinsonii* (*berkhoffii*) type II, *B. henselae*	*B. bovis,* negative
2	<16	<16	ITS, Pap31	*B. henselae, B. henselae*	*B. vinsonii* (*berkhoffii*) types I and II, *B. henselae* and *B. vinsonii* (*berkhoffii*) type II
3	32	<16	ITS, Pap31	Negative, negative	*B. vinsonii* (*berkhoffii*) type II, negative
4	<16	<16	ITS, Pap31	Negative, negative	*B. vinsonii* (*berkhoffii*) type II, negative
5	<16	<16	ITS, Pap31	Negative, negative	*B. quintana,* negative

*ITS, 16S–23S intergenic transcribed spacer region; Pap31, heme-binding protein gene; IFA, indirect immunofluorescence assay.

(*13*). All 5 dogs in this study lacked serologic evidence of *Bartonella* infection, a finding which has been previously reported in bacteremic dogs and humans (*6*,*13*,*14*).

Previous studies have shown that targeting multiple *Bartonella* genes provides molecular evidence of coinfection with more than 1 *Bartonella* species or strain type (*6*,*7*,*13*). In the current work, the inability to confirm the ITS PCR results with a second PCR target has been previously reported by our laboratory (*6*,*13*,*14*) and likely reflects differences in PCR sensitivity, interference or inhibition of the PCR reaction by oral bacteria that are present in greater numbers than the *Bartonella*, or the lack of a known heme-binding protein gene in various *Bartonella* species, such as *B. bovis*. The limit of detection (LOD) of *Bartonella* ITS PCR is 2 copies/reaction, while the LOD of Pap31 assay is 10 copies/reaction. Further, although *B. henselae* has a detectable Pap31 protein (Table), several researchers in our laboratory have successfully isolated *B. henselae* strains that lack a PCR-detectable heme-binding protein (unpub. data). Upon recognition of the discordance between ITS and Pap31, additional genes such as 16S, *glt*A, and *rpo*B were targeted; however, these analyses were negative for *Bartonella* and resulted in nonspecific bacterial amplification. Because inhibition of ITS PCR was suspected due the presence of other oral bacteria, *Bartonella*-negative DNA extracts from oral swabs were spiked with *B. henselae* DNA at 1.5, 2.5, 5, and 10 (0.002 pg/μL) copies/reaction. Inhibition was detected at up to 5 copies/reaction, while the 10 copies/reaction sample was consistently amplified by the ITS primers.

These data, in conjunction with previous case reports (*3*–*5*), suggest that potentially viable *Bartonella* organisms may be transmitted to humans after a dog bite. The detection of DNA by PCR does not necessarily indicate the viability of *Bartonella* organisms. However, due to the extremely slow growth characteristics of *Bartonella* spp., isolation from the oral cavity does not seem feasible, because of competition with numerous other rapidly growing oral bacterial species. Recently, *Bartonella* DNA has been amplified from peripheral lymph nodes of healthy dogs (*14*). *B. henselae* was also amplified from salivary gland tissues from a dog with saladenitis (*15*). There are several plausible routes by which a *Bartonella* sp. could gain entry to the oral cavity. Future studies should determine if the tonsilar lymphoid tissues, salivary glands, or periodontal, gingival, or other oral tissues can serve as sources of *Bartonella* spp. contamination of canine saliva. As *Bartonella* infection may represent an occupational risk for veterinary professionals and others with extensive animal contact (*6*), additional studies should address the risk of transmission from dogs to humans following bite wounds.

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Use of Fly Screens to Reduce *Campylobacter* spp. Introduction in Broiler Houses

Birthe Hald,* Helle M. Sommer,† and Henrik Skovgård‡

Fly screens that prevented influx of flies in 20 broiler houses during the summer of 2006 in Denmark caused a decrease in *Campylobacter* spp.–positive flocks from 51.4% in control houses to 15.4% in case houses. A proportional reduction in the incidence of chicken-borne campylobacteriosis can be expected by comprehensive intervention against flies in broiler production houses.

Campylobacteriosis is a severe gastroenteric human disease of global significance. The incidence correlates with the prevalence of thermophilic *Campylobacter* spp., predominantly *C. jejuni* and *C. coli* (*1*), in chickens and follows a seasonal cycle in temperate climates for reasons not fully elucidated. The number of cases is lowest in winter and highest in summer (*2*). In Denmark, the prevalence of *Campylobacter* spp.–infected chicken flocks peaked at 60%–80% in recent summers (*3*). The population size of flies displays a similar cycle *(4)*. Flies, in particular the house fly, *Musca domestica*, are well-known vectors of several enteric bacterial diseases (*5*) and are known to carry *Campylobacter* spp. (*6–10).* Vector flies can transmit *Campylobacter* spp. from outside farm livestock to broiler flocks because large numbers of flies may enter broiler houses by ventilation air (*7*,*11*). Our aim was to evaluate the effect of insect screens in addition to existing biosecurity measures against *Campylobacter* spp. infection of broiler chickens in summer.

The Study

Potential study sites were identified in the Danish Poultry Council´s national surveillance database *(3*) on the basis of the number of *Campylobacter* spp.–positive flocks produced in broiler houses during 2003–2005. All farms practiced hygiene procedures such as separating clean and dirty zones, changing footwear and clothes, and washing hands with disinfecting soap before entering the broiler room. Furthermore, a 3-m zone with short-cut grass or gravel surrounded the houses. Houses were emptied, cleaned, and dried before each new flock of chickens was brought in. All farmers were instructed to maintain biosecurity and management routines as before the study. Case and control groups were assigned to match each other in *Campylobacter* spp. prevalence and were composed so that the distribution of previous *Campylobacter* spp. prevalence of flocks for each group (June to November during 2003–2005) were equal (Figure 1) and with similar distribution in the presence of other livestock in a periphery of 1.5 km around the farms. Farmers consented to participate before study groups were composed.

According to data from the national Danish *Campylobacter* surveillance program (*3*), the historical *Campylobacter* spp. prevalence at slaughter during 2003–2005 from June to November had been 51.6% (95/184) (95% confidence interval [CI] 44.3%–59.0%) in case houses and 51.7% (123/238) (95% CI 45.2%–58.2%) in control houses. Thus, before the study, the baseline prevalence for houses in the case and control groups were not significantly different from each other ($p = 0.99$ by χ^2 test).

Twenty houses on 11 farms in Jutland, Denmark, were equipped with fly screens by June 1, 2006 (photographs available from www.vet.dtu.dk/default.aspx?id=20832). Fifty-two broiler flocks stocked in the houses after June 1 constituted the cases; the last flock was slaughtered on November 6, 2006. Controls were 70 broiler flocks reared in 25 matched broiler houses on 13 other farms without fly screens; the last flock was slaughtered on November 13, 2006. All houses were ventilated through wall inlets in the long sides of the houses, air outlets through chimneys in the roofs, and gable fans. The study design was based on experience gained in a pilot study in 2004 (*11*) of 5 farms with parallel case and control houses on each farm. The pilot study showed a significant delay of *Campylobacter* spp. introduction in case houses. However, only a 37% reduction in positive broiler flocks was obtained at slaughter due

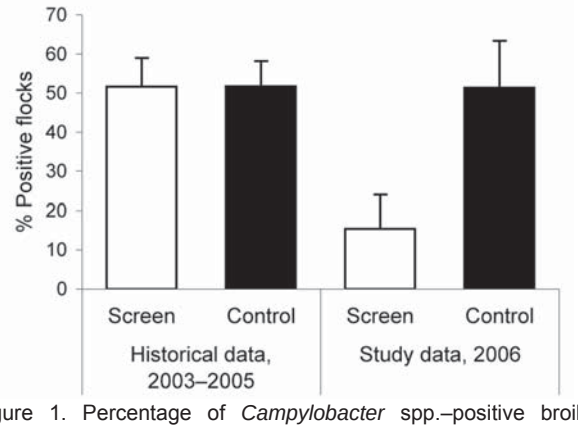

Figure 1. Percentage of *Campylobacter* spp.–positive broiler flocks produced in fly screen houses and control houses June 1 to November 13 during 2003–2005 (historical data) and in 2006 (during intervention). Error bars indicate standard deviation.

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to transmission of *Campylobacter* spp. from control houses to the corresponding case houses.

Broiler flocks were sampled at days 21, 28, and 35. Boots with over-shoe covers were used to walk through the broiler rooms. The over-shoe covers (photographs available from www.vet.dtu.dk/default.aspx?id=21756) were analyzed for *Campylobacter* spp. Results are shown in Table 1. Flocks were slaughtered between days 35 and 42 and sampled by collection of 10 cloacal swabs per flock at the abattoir. Results of the current national surveillance program of *Campylobacter* spp. in broiler production were included in the study as reference to ordinary Danish broiler production. All samples were analyzed by PCR (DANAK [The Danish Accreditation and Metrology Fund] accredited method) detecting thermophilic *Campylobacter* spp. (*12*).

In fly screen houses (case houses), 15.4% (95% CI 7.7%–27.8%) of the flocks reared during the study period were *Campylobacter* spp. positive at slaughter, whereas the prevalence in *Campylobacter* spp.–positive flocks reared in the control houses was 51.4% (95% CI 40.0%–62.7%). The prevalence in the control houses remained unchanged ($p = 0.68$ by χ^2 test) compared with the historical prevalence during June–November, 2003–2005. Figure 1 shows the *Campylobacter* spp. prevalence of flocks from the study with the historical data. The average flock *Campylobacter* spp. prevalence per month in 2006 in fly screen houses and in control houses is shown in Figure 2 with the results of the national Danish *Campylobacter* surveillance program of 1,504 broiler flocks slaughtered in Denmark in specific months.

Data were analyzed with SAS software (SAS Institute, Cary, NC, USA) in the SAS procedure proc genmod with a logit link function and a repeated statement where subject = flock. The repeated statement accounts for the intraclass correlation. In the model, the effects of the fly screen "Screen" of the time between 21 and 35 days "Time", the interaction "Screen Time" and the effect of the average monthly prevalence level at slaughter "Month" (analyzed as regressor) were analyzed. The status at day 35 was chosen in the analysis instead of the results at slaughter to avoid biases in data due to the increased risk of introducing *Campylobacter* spp. in those flocks slaughtered later and during depopulation and transportation to slaughter. Only 4 flocks were slaughtered during November and were merged with the October flocks in the analysis.

The analysis shows a clear effect of fly screens ($p = 0.0002$) by either complete prevention of infection or by a significant ($p<0.0001$) delay in onset of infection of the broiler flocks. Results of analyzed sources and estimates of flock *Campylobacter* spp. status in fly screened and in unprotected houses at days 21 and 35 predicted by the applied statistical model are shown in Table 2.

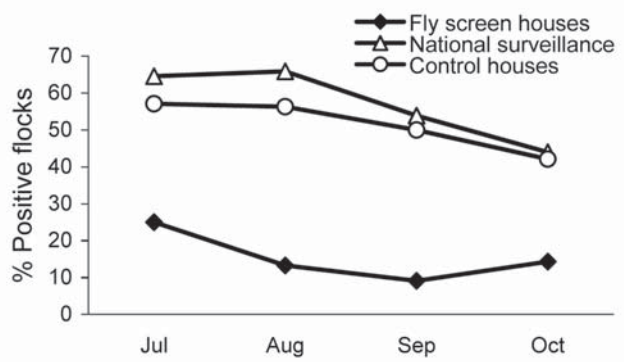

Figure 2. Prevalence per month of *Campylobacter* spp.–positive broiler flocks during the study period (June 1–November 13, 2006) in fly screen houses (52 flocks) and control houses (70 flocks), and the national flock *Campylobacter* spp. prevalence at slaughter of 1,504 flocks according to surveillance data for the same period.

Conclusions

We showed that preventing flies from entering broiler houses in the summer of 2006 caused a drop in prevalence of *Campylobacter* spp.–positive flocks at slaughter from 51.4% in control houses to 15.4% in case houses. It seems reasonable that the main results found in this study can be extrapolated to the national situation because the selected control houses had a prevalence similar to the national prevalence level for the same period (Figure 2). Installation of effective fly screens in broiler houses in Denmark would most likely decrease the average yearly *Campylobacter* spp. prevalence, and show a major decrease in the summer peak. Presumably, the risk for infection from eating chicken, the main cause of campylobacteriosis in Denmark (*13*), would be reduced. The expected effect on the incidence of chicken-borne campylobacteriosis has been calculated by Rosenquist et al. (*14*) to be proportional to the decline in flock *Campylobacter* spp. prevalence.

Our study provides evidence that flies are vectors for *Campylobacter* spp. in broilers and furthermore, probably explains the seasonal variation of *Campylobacter* spp. in

Table 1. *Campylobacter* spp. positive and negative flocks by type of house

	Day 21		Day 28		Day 35	
Type of house	No. positive (%)	No. negative	No. positive (%)	No. negative	No. positive (%)	No. negative
Fly screened (n = 52)	3 (5.8)	49	3 (5.8)	49	4 (7.7)	48
Control (n = 70)	8 (11.4)	62	20 (28.6)	50	30 (45.5)	36

Table 2. Results of analyzed sources and estimates of flock *Campylobacter* spp. status from the applied statistical model

Type of result	p value
Source of variation*	
Screen	0.0002
Time (day of rotation)	<0.0001
Screen time	0.07
Month	0.80
Predicted prevalence of *Campylobacter* spp.–positive flocks (day 21/35), %	
Fly screen houses	3/ 11
Houses without fly screens	14/ 42

*Analysis of variance, type 3 test. Significant effects if p<0.05.

chicken products. Flies may also play a role in direct transmission of *Campylobacter* spp. to humans (*14*,*15*). Certainly, the issue deserves further scientific investigation.

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Multidrug-Resistant Typhoid Fever Outbreak in Travelers Returning from Bangladesh

To the Editor: Enteric fever (typhoid and paratyphoid fever) is a systemic infection caused by several *Salmonella enterica* serotypes including *S.* Typhi and *S.* Paratyphi A. The Indian subcontinent, which has the highest incidence of the disease worldwide, is also an epicenter of enteric fever caused by multidrug-resistant (MDR; resistant to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole) and nalidixic acid–resistant (NAR) strains, i.e., strains with decreased susceptibility to ciprofloxacin (*1*–*3*). A total of 57% of *S.* Typhi strains isolated at a referral center in Dhaka, Bangladesh, in 2005 were MDR and NAR (*4*).

More than 80% of 442 enteric fever cases reported in Japan during 2001–2004 were imported (*5*). Most Japanese persons, especially the younger generation, are not immune to enteric fever as are persons living in other industrialized countries.

Although the proportion of enteric fever cases related to international travel has increased in industrialized countries, few outbreaks of enteric fever have been reported in travelers (*6*,*7*). We describe an outbreak of MDR and NAR typhoid fever in young Japanese travelers returning from Bangladesh. This outbreak highlights the need for standard treatments for MDR and NAR enteric fever.

Ten Japanese junior and senior high school students living in the Tokyo metropolitan area took part in a 9-day study tour to Dhaka in March–April, 2004. They were escorted by 2 Japanese college students and a 28-year-old Japanese instructor. The 13 participants returned to Japan on April 4, 2004. The purpose of the study tour was to acquire knowledge about street children in Dhaka. The students stayed at a guesthouse and visited orphanages in the city. The itinerary included a visit to a local home, where the family served them a meal. They shared all their meals during the tour.

Fever and diarrhea developed in 2 participants on April 3 and 5, and these symptoms were later shown to be caused by shigellosis. On April 19, the index patient became febrile. From that date until April 28, there were 6 confirmed and 2 probable typhoid fever cases reported in the 13 tour participants, resulting in an attack rate of 62%. The median age of the patients was 17 years (range 12–28 years); 5 patients were female. No other cases of typhoid fever were reported in that period in Japan.

All 6 *S.* Typhi isolates were Vi-phage type E9. These isolates were also MDR and NAR, and the MIC for ciprofloxacin for the 6 isolates was 0.38 μg/mL. It was strongly suspected that a single-point exposure to *S.* Typhi occurred in the tour participants during their stay in Bangladesh and caused this exceptional outbreak. None of the participants had received a typhoid vaccination.

The 8 patients were admitted to 5 hospitals in the Tokyo metropolitan area. Four different antimicrobial drug regimens were used on the basis of the age of the patients and the hospital in which each patient was hospitalized (Table). Four patients at 2 hospitals who received fluoroquinolone monotherapies were given other regimens on days 4–6 of treatment because of concern of treatment failure. The median fever clearance time was 6 days

Table. Characteristics of 8 case-patients with typhoid fever, Bangladesh, 2004*

Case-patient†	Age, y/ sex	Date of onset	Vi-phage type	Ciprofloxacin MIC, μg/mL	Cefotaxime MIC, μg/mL	Treatment‡	FCT, d
1C	28/F	Apr 19	E9	0.38	0.094	Ciprofloxacin 500 mg 2× a day for 3 d, cefotaxime 1 g every 12 h + tosulfoxacin 300 mg 2× a day for 11 d	4§
2C	17/F	Apr 20	E9	0.38	0.094	Levofloxacin 200 mg 2× a day for 14 d	6
3C	17/F	Apr 21	E9	0.38	0.094	Ciprofloxacin 500 mg 2× a day for 3 d, cefotaxime 1 g every 12 h + tosulfoxacin 300 mg 2× a day for 11 d	3
4P	19/F	Apr 21	NA	NA	NA	Levofloxacin 200 mg 2× a day for 3 d, cefotaxime 1 g every 12 h + tosulfoxacin 300 mg 2× a day for 13 d	12
5C	12/M	Apr 22	E9	0.38	0.094	Azithromycin 1 g for 1 d, 500 mg a day for 2 d; norfloxacin 250 mg 3× a day for 11 d	7
6C	16/F	Apr 23	E9	0.38	0.094	Levofloxacin 500 mg a day for 14 d	5
7C	19/M	Apr 23	E9	0.38	0.064	Ciprofloxacin 500 mg 2× a day for 5 d, ceftriaxone 2 g every 12 h for 16 d	6
8P	15/M	Apr 28	NA	NA	NA	Levofloxacin 200 mg 2× a day for 18 d	7

*MICs were determined by E-test (AB Biodisk, Solna, Sweden). MICs of chloramphenicol, ampicillin, trimethoprim-sulfamethoxazole, and nalidixic acid were >256 μg/mL, >256 μg/mL, >32 μg/mL, and >256 μg/mL, respectively. FCT, fever clearance time (time from the start of treatment until the body temperature reached 37.5°C and remained at 37.5°C for 48 h); NA:, not available.
†C, confirmed case, i.e., a patient with fever (>38°C) for >3 d and a laboratory-confirmed positive blood culture for *Salmonella enterica* serotype Typhi; P, probable case, i.e., a patient with fever (>38°C) for >3 d without isolation of *S.* Typhi.
‡All fluoroquinolones were given orally. Tosufloxacin is a fluoroquinolone with properties similar to those of levofloxacin.
§Fever relapsed 15 d after completion of treatment. Retreatment with tosufloxacin, 600 mg/d for 23 d, was successful.

(range 3–12 days). No complications occurred during any of the treatment regimens. Although a relapse occurred 15 days after completion of treatment in the oldest patient, who had received cefotaxime and oral tosufloxacin, retreatment cured the infection without fecal carriage.

The high attack rate may reflect the high sensitivity of adolescents to typhoid fever and the high level of bacterial contamination in food the participants had eaten during travel (*2*). Although the meal at the private home was suspected as the source of infection, we could not determine the exact cause of this outbreak.

The optimum treatment for MDR and NAR enteric fever has not yet been established. A third-generation cephalosporin or high doses of fluoroquinolones (e.g., ciprofloxacin, 20 mg/kg/day or levofloxacin, 10 mg/kg/day) for 10–14 days are the drugs of choice (*1*,*2*). Azithromycin is also a promising agent (*8*). However, for any of the regimens, the mean fever clearance times are relatively long (≈7 days), and the relapse rates are high (*1*). Although all 6 isolates showed reduced susceptibility to ciprofloxacin, a long course (14 days) of fluoroquinolones was still effective in this outbreak. However, clinicians should be aware of treatment failure in MDR and NAR enteric fever (*3*). The combination therapy of cefotaxime and a fluoroquinolone used in 3 patients has not shown greater efficacy than monotherapies. In fact, 1 patient who received this combination therapy experienced a relapse.

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Human Rabies Cluster Following Badger Bites, People's Republic of China

To the Editor: From February 2002 to April 2004, 7 rural residents of Coteau County (population 450,000) in western Zhejiang Province in eastern People's Republic of China died of rabies following badger bites (Figure). In this county, 89% of residents are farmers. The county covers 4,475 km^2, and the terrain is mountainous. No other cases of human rabies had been reported from this county since 1986. We investigated the cluster to ascertain characteristics of these exposures.

Rabies testing was not readily available. In China, the national case definition is based on clinical compatibility with appropriate animal exposure. Doctors are required to report rabies according to a general case description published by the Ministry of Health. Laboratory confirmation is not generally performed. We defined a rabies case as any person from Coteau County in whom rabies was diagnosed by a physician from February 2002 through March 31, 2007. We interviewed family members of case-patients and neighbors about the char-

acteristics of the illness and activities associated with badgers, dogs, and other animals that are potential rabies reservoirs.

From February 2002 to April 2007, a total of 8 human rabies cases were reported from Coteau County. Seven case-patients had badger exposure and 1 had cat exposure. Badger-associated rabies occurred from February 2002 to December 2004; 1- to 2-month intervals generally occurred between cases. The average yearly incidence rate for human rabies in the county was 0.52 per 100,000 compared to 0.15 per 100,000 for China for the same period. Patients ranged in age from 18 to 76 years (mean 54 years). Badger-associated rabies was confined to 7 contiguous townships in the center of the county. Signs and symptoms were typical of rabies, namely, fever, excitation, aerophobia, hydrophobia, dysphagia, and hypersalivation, leading to coma and death. Incubation periods ranged from 31 to 100 days (mean 45 days).

All 7 case-patients with badger-associated rabies had tried to catch badgers that were sluggish and could not escape. All bites occurred on the fingers, when the badger was captured or carried home. The captors killed and ate 2 badgers, 4 badgers died spontaneously, and the fate of 1 badger was not known. The cat-associated rabies case from the same area occurred in February 2004. The cat died spontaneously during the same period when some badgers died spontaneously nearby. We found no other villagers who had been bitten by these or other badgers. The case-patients and family members did not know that badgers can transmit rabies and did not seek treatment or postexposure prophylaxis. These case-patients had no other exposure to bites from other potentially rabid animals in the 10 years before onset.

The 7 case-patients lived in villages covering an area of ≈10 km^2, representing ≈0.2% of the total county area. The individual villages were 1,500–3,000 m apart. All were on the same side of a mountain ridge. Mountainous terrain and limited transportation isolate this county from nearby counties. Villagers reported seeing dead badgers before human cases occurred. During the past 20 years in this county, ≈15,000 persons received rabies postexposure prophylaxis after dog bites, but no rabies occurred. During 2002–2004, no human rabies cases followed exposure to dogs that were within 50 km of this county.

After 2004, we set up a rabies surveillance and health education system in this county. At the end of 2004, we advised the public in this and 5 neighboring counties to avoid catching and killing badgers and, if bitten, to seek postexposure prophylaxis. Since that recommendation, no human rabies has occurred in the area. In 2006, a total of 1,719 residents were treated for animal bites. The incidence of animal exposures in this county is higher than in the United States (*1*). Dog bites accounted for 86% (1,471), cat bites for 9.5% (164), and other animals for 4.9% (84) of exposures. However, no badger bites were reported.

We concluded that an epizootic of badger rabies affected a limited area of Coteau County from 2002 through 2004. Badgers can easily transmit the virus and could be an important secondary host of rabies (*2*). Research is needed on badgers as a natural reservoir of human rabies and on control of this disease in wildlife hosts (*3*,*4*). A national surveillance system for animal rabies should be set up in this region (*5*).

A major limitation of this study is the lack of laboratory support for surveillance of both human and animal rabies. Accordingly, we based our conclusion on clinical and epidemiologic histories. The lack of human cases from dogs could be attributed to effective postexposure prophylaxis of humans following dog bites. On the other hand, rabies following dog bites is the number-one cause of death from infectious diseases in China, in part because of absent or incomplete postexposure prophylaxis for poor rural residents. Thus, the complete absence of reported dog-associated rabies is unusual. China is planning increased investment in rabies surveillance and prevention that will include recommended laboratory support and should help alleviate this situation in the future (*3*).

Figure. Badger: a new natural reservoir of human rabies? (Image source: Ian Stickland)

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Diphyllobothrium latum Outbreak from Marinated Raw Perch, Lake Geneva, Switzerland

To the Editor: *Diphyllobothrium latum*, a fish tapeworm, has a complex cycle including copepods and freshwater fish as intermediate hosts. Humans are infected by eating raw or undercooked fish meat. Clinical consequences of human infection are generally absent or mild, although anemia due to vitamin B12 deficiency was described in Scandinavia (*1*). Freshwater fish host the parasite in some lakes of Switzerland, Italy, Scandinavia, northeastern Canada, and South America (*1*–*4*). Lake Geneva, in Switzerland, harbors perch, pike, and char, which are considered to be food delicacies and may act as secondary intermediate hosts. Perch are heavily infested (*5*,*6*). To date, *D. latum* has reportedly caused only sporadic cases in western Europe. One outbreak has previously been described in South Korea after 5 persons ate raw redlip mullet. Identification of the *Diphyllobothrium* species in that outbreak was uncertain (*7*).

Since 2001, medical centers in the lake region have reported an increasing number of human cases. We report, to our knowledge, the first outbreak of *D. latum* infections in this region, which occurred after a wedding party in June 2006. The menu included raw, marinated perch fillets caught the same day in Lake Geneva. After *D. latum* infection was diagnosed in 2 guests, all those who attended (n = 32) were contacted within 4 months after the wedding. Information was collected with a standardized questionnaire on personal characteristics; past infection with *D. latum*; consumption of raw perch during the wedding, raw freshwater fish in the last 5 years, or both; and symptoms or visible proglottids in stools. All participants who ate the raw perch dish during the wedding had a stool sample examined for ova and proglottids at the Laboratory of Parasitology of the Geneva University Hospitals. Species identification relied on egg and proglottid morphologic characteristics and epidemiologic factors.

A confirmed case-patient was defined as a case in a guest who ate raw perch at the wedding and had characteristic eggs or proglottids in stool. A probable case-patient was defined as a person who ate raw perch during the wedding and reported a "tagliatelle-like" worm of varying length in stools, without a history of consumption of raw beef, pork, or other raw fish in the previous 5 years and in the absence of laboratory examination of stool sample. All confirmed case-patients received a single 10-mg/kg dose of praziquantel. Stool examination was repeated after treatment.

Twenty-six wedding guests ate raw marinated perch. Seven confirmed cases and 1 probable case of *D. latum* infection occurred (attack rate 30.8%). Infected persons had a median age of 34 years (range 24–60 years) and were more likely to be female. Microscopic examination showed characteristic eggs in 7 patients' stools and both eggs and proglottids in 3 patients.

None of the patients reported symptoms within 7 days after the dinner. Two patients remained asymptomatic at interview but both were reporting visible worm segments in stools. Six patients (75%) reported symptoms that started 20–91 days after the wedding (median 56 days). Reported symptoms were diarrhea (6 patients), fatigue (5), abdominal pain (4), nausea (3), loss of weight (2), vomiting (1), or dizziness (1). No patient required urgent medical care or missed work. The mean interval between the wedding and the first observation of visible proglottids in stool was 40 days. Seven patients were treated with a single 10-mg/kg dose of praziquantel

with no adverse effects reported. One patient treated herself with albendazole (400 mg/day for 3 days) before she was seen at a hospital. All patients became asymptomatic and had negative stool examination results 2–10 weeks after treatment.

None of the patients reported previous or subsequent consumption of raw freshwater fish. Raw fish preparations such as sushi, sashimi, carpaccio, and ceviche are increasingly popular and are now also prepared with local freshwater fish. These new food habits represent a clear risk factor for human infection (*5*,*7*).

The plerocercoid larvae in the fish muscles are easily missed during food preparation. Nor are local fish systematically inspected, as imported fish are. The role of paratenic hosts (e.g., dogs, foxes) in transmission is not fully understood.

Information given to the public and professionals such as food handlers, restaurant owners, and fishermen is a key measure to promote safer food practices. Avoiding serving preparations of raw freshwater fish or selecting fish that are not intermediate hosts of *D. latum* would decrease parasite transmission. Cooking the fish at 55°C for 5 minutes efficiently kills the larvae. Freezing the fish at –20°C for 24 hours is also efficient. International regulations recommend freezing all fish that are expected to be served raw. Notable exceptions are fish from farm culture or from areas where strong evidence proves no source or cases of infection (European community rules 853/2004 annexe III, available from www.paquethygiene.com/reglement_ce_853_2004/reglements_ce_853_2004_du_parlement_europeen_et_du_conseil_annexe_3_section_8.asp#debut). However, enforcing these rules proves very difficult for food safety administrations.

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Human Papillomavirus Vaccination Strategies

To the Editor: An article by Elbasha et al. in the January 2007 issue of Emerging Infectious Diseases showed an economic evaluation of human papillomavirus (HPV) vaccination strategies (*1*). In this model, incremental cost-effectiveness ratio (ICER) calculations were based on costs measured as US dollars for 2005 and effectiveness measured as quality-adjusted life years (QALYs). Authors presented these data transparently and showed costs and QALYs of each strategy in 2 tables, where they did not show ICER of dominated options; i.e., "Strategy A is dominated if there is another strategy, B, that is more effective and less costly than strategy A" (*1*). Unfortunately, splitting data into 2 tables can be misleading.

First, ICERs of strategies for vaccination at the age of 12 (70% coverage) compared with a strategy of no vaccination showed that the strategy of vaccinating 12-year-old girls and boys is dominated by other strategies. Furthermore, vaccination of 12-year-old girls only and vaccination of 12-year-old girls only with catch-up (vaccination of girls and women 12–24 years of age) have lower ICERs, which could be interpreted as the most cost-effective approaches.

Finally, ICERs of strategies of vaccinating at 15 and 18 years of age (50% coverage) are presented without comparison strategies. Thus, one might assume that these strategies are compared with the baseline strategy (vaccination of 12-year-old girls only); however, they are compared with the no-vaccination strategy.

The transparency of the Elbasha et al. article enabled us to build a new table based on their data (Table). In our table, ICERs of the whole set of strategies showed that vaccination of

Table. Cost-effectiveness analysis of alternative human papillomavirus vaccination strategies*

Strategy	Discounted		Incremental†		
	Cost	QALY	Cost	QALY	ICER ($/QALY)‡
No vaccination	$72,659,302	2,698,711	–	–	–
12-y-old girls	$74,042,990	2,699,178	$1,383.688	467	Dominated
18-y-old women + 18–24-y-old female catch-up	$73,553,847	2,699,192	$894,545	481	$1,860
15-y-old girls + 15–24-y-old female catch-up	$73,895,046	2,699,214	$341,199	22	$15,509
12-y-old girls and boys	$78,707,825	2,699,327	$4,812,779	113	Dominated
12-y-old girls + 12–24-y-old female catch-up	$74,815,667	2,699,343	$920,621	129	$7,137
18-y-old women and men + 18–24-y-old female and male catch-up	$77,535,383	2,699,385	$2,719,716	42	$64,755
15-y-old girls and boys + 15–24-y-old female and male catch-up	$78,455,750	2,699,404	$920,367	19	$48,440
12-y-old girls and boys + 12–24-y-old female catch-up	$79,746,357	2,699,461	$1,290,607	57	$22,642
12-y-old girls and boys + 12–24-y-old female and male catch-up	$81,761,210	2,699,506	$2,014,853	45	$44,775

*QALY, quality-adjusted life year; ICER, incremental cost-effectiveness ratio; $, US dollars.
†Based on discounted costs reported by Elbasha et al. (*1*).
‡Compared with the preceding nondominated strategy. Strategy A is dominated if there exists another strategy, B, that is more effective and less costly than strategy A.

12-year-old girls only is dominated by the vaccination of 18-year-old women plus a catch-up strategy (women 18–24 years of age), although older groups have lower coverages.

In addition, I point out 2 particulars. First, epidemiology of HPV varies between countries (*2*), probably because of differences in culture and sexual habits. Thus, vaccination at older ages should be considered in countries in which prevalence of adolescent sexual activity or HPV is low. Second, higher vaccine coverage in older groups would decrease ICERs of these strategies (*1*). Both facts could reflect the real situation in some countries, e.g., Spain (*2*,*3*).

In conclusion, economic evaluations of HPV vaccination strategies should have broader sensitivity analysis to include as many country-specific realities as possible. To avoid misunderstandings that could lead policymakers to misallocate funds, these results should be evident to readers.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Distemper in a Dolphin

To the Editor: Deaths caused by new members of the genus *Morbillivirus*, family Paramyxoviridae (*1*), have occurred in recent decades among phocine and cetacean species, particularly harbor seals (*Phoca vitulina*) in 1988 (*2*) and 2002 (*3*). Endangered Mediterranean striped dolphins (*Stenella coeruleoalba*) died in 1990 and 1991 (*4*), and common dolphins (*Delphinus delphis ponticus*) from the Black Sea died in 1994 because of infection with dolphin morbillivirus (DMV) (*5*). A similar virus caused deaths in bottlenose dolphins (*Tursiops truncatus*) in the Gulf of Mexico from 1987 through 1994 (*6*). Closely related morbilliviruses caused deaths in harbor porpoises (*Phocoena phocoena*) in European waters in 1988 (*7*) (*Porpoise morbillivirus*) and endangered Mediterranean monk seals (*Monachus monachus*) in 1997 (*8*) (*Monk seal morbillivirus*). After these epidemics, the viruses disappeared and no marine or terrestrial reservoirs have been identified.

In January 2007, a moribund, subadult, white-beaked dolphin (*Lagenorhynchus albirostris*) was found stranded on the North Friesian coast of Germany. The animal was humanely

killed and a complete necropsy was performed. The main lesion was a nonsuppurative meningoencephalitis with neuronal degeneration and few eosinophilic cytoplasmic inclusion bodies characteristic of a viral disease. Lungs showed suppurative and interstitial pneumonia. Paraffin-embedded sections of brain were examined for morbillivirus antigen by using an immunoperoxidase technique. We used various monoclonal antibodies that recognize different morbilliviruses. Tissues from a seal infected with phocine distemper virus and a dog with canine distemper were used as positive controls. Tissues from a white-beaked dolphin that underwent an autopsy in 2006 were used as negative controls. In the diseased dolphin, morbillivirus antigen was found exclusively in neurons and glial cells of the brain (Figure, panel A).

Frozen tissue samples and blood were examined for morbillivirus nucleic acid by reverse transcription–PCR with a set of universal morbillivirus primers that are specific for highly conserved regions of virus nucleoprotein (N) (*9*) and phosphoprotein (P) (*10*). A 457-bp amplicon of the P gene (GenBank accession no. EF451565) and a 287-bp amplicon of the N gene (GenBank accession no. EF469546) were detected in brain tissue. Our isolate, DMV/DE/2007, showed homologies of 99% with the N gene and 98% with the P gene of DMV isolated from Mediterranean striped dolphins. Phylogenetic analysis showed that isolate DMV/DE/2007 is closely related to DMV (Figure, panel B), porpoise morbillivirus, and monk seal morbillivirus (*8*).

Histologic changes in the dolphin resembled those of distemper in seals (*3*), porpoises (*7*), and other dolphins (*4*–*6*). Identification of morbillivirus antigen in diseased tissues and isolation of genome fragments of a morbillivirus provide conclusive evidence for a primary etiologic role of this virus. Sequencing of the virus and phylogenetic comparison showed that the virus is closely related to previously described dolphin morbillivirus and porpoise and monk seal morbilliviruses (*8*). To our knowledge, this is the first report of morbillivirus infection in a white-beaked dolphin in German waters and in a marine mammal since the last epidemic among harbor seals in northern Europe in 2002. Isolation of DMV has not been reported since 1994.

Our findings indicate that DMV is still circulating in some marine mammals. Similar to infections in terrestrial hosts, morbillivirus infections may occur in marine mammals in cycles without overt clinical disease in susceptible animals, as documented for harbor seals (*2*,*3*). Serum samples collected from 1995 through 1999 from cetacean species in various regions were positive for DMV, but porpoises and striped dolphins showed a decrease in humoral immunity, making them vulnerable to new epidemics. No data exist on seroprevalence of morbillivirus-specific antibodies

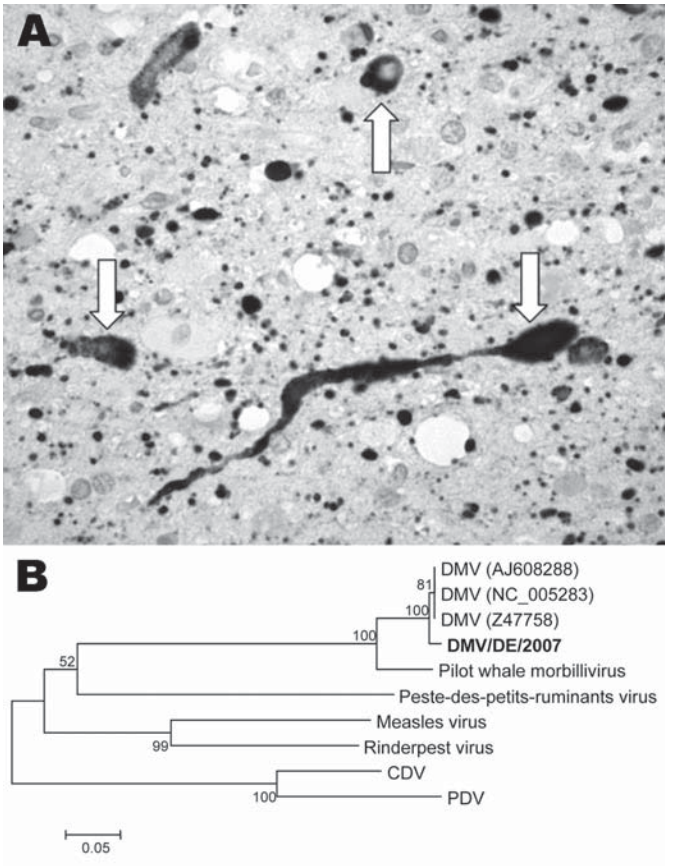

Figure. A) Immunohistologic demonstration of morbillivirus antigen in cytoplasm and nuclei of neurons (arrows) and glial cells in the brain of a white-beaked dolphin, using a monoclonal antibody (GenWay, San Diego, CA, USA) against nucleoprotein of canine distemper virus (CDV)/phocine distemper virus (PDV) visible as numerous black dots (magnification ×630). B) Unrooted neighbor-joining phylogenetic tree constructed by using 353 nt from the gene coding for the morbillivirus phosphoprotein. Alignments were calculated with ClustalX version 1.83 (http://bips.u-strasbourg.fr/fr/documentation/ClustalX). Bootstrapping (values indicated in %) was performed with 1,000 replicates using MEGA 3.1 software (www.megasoftware.net/mega.html). The new isolate from this study is shown in **boldface**. The following sequences were included: dolphin morbillivirus (DMV) (GenBank accession nos. NC_005283, Z47758, AJ608288), pilot whale morbillivirus (AF200817), Peste-des-petits-ruminants virus (NC_006383), measles virus (NC_001498), Rinderpest virus (NC_006296), CDV (NC_001921), and PDV (D10371). Scale bar shows nucleotide substitutions per site.

in white-beaked dolphins. We do not know how the dolphin contracted the infection and whether this remains an isolated case or the beginning of a new zoonosis.

White-beaked dolphins are found in moderate and subarctic waters of the Atlantic Ocean between the eastern coast of North America and northern Europe. They may migrate hundreds of kilometers within days. Therefore, these dolphins may play a role as a reservoir and vector for this morbillivirus, which is infectious for harbor porpoises, bottlenose dolphins, and other cetacean species (*10*). The reappearance of a morbillivirus represents a serious threat to susceptible marine mammals in northern European and American waters, with potentially devastating consequences and possibly the beginning of a new epidemic.

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Bartonella australis sp. nov. from Kangaroos, Australia

To the Editor: During April–May 1999, 3 *Bartonella* isolates (AUST/NH1, AUST/NH2, AUST/NH3) were cultivated and established from the blood of 5 *Macropus giganteus* gray kangaroos from central coastal Queensland, Australia. We used multigene sequencing to evaluate whether these *Bartonella* isolates fulfill the minimum requirements for classification as a new species.

DNA from each *Bartonella* isolate was extracted by using the QIAamp tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Partial PCR amplification and sequencing of the genes encoding the 16S rDNA (*rrs*), citrate synthase (*gltA*), β-subunit of the RNA polymerase (*rpoB*), and cell division protein (*ftsZ*), as well as for the 16S–23S rDNA intergenic spacer (ITS) were attempted by using previously described primers and conditions (*1*). *Bartonella* sp. isolates AUST/NH1 to AUST/NH3 exhibited identical sequences for all 4 genes and the spacer studied, and isolate AUST/NH1 was selected as type strain among kangaroo isolates. Similarity rates between strain Aust/NH1 and validated *Bartonella* species (online Appendix Table, available from www.cdc.gov/EID/content/13/12/1961-appT.htm) ranged from 84.7% to 91.6%, from 97.5% to 98.5%, from 79.6% to 87.2%, from 85.4% to 95.0%, and from 83.5% to 87.1% for the ITS and *rrs*, *gltA*, *rpoB,* and *ftsZ* genes, respectively. Therefore, for each of these 4 genes or the spacer, strain AUST/NH1 exhibited similarity rates with all other species lower than the cutoffs published to classify *Bartonella* isolates within a validated species (*1*). It may thus be regarded as a new species.

To estimate the genomic G+C content of strain AUST/NH1, we amplified and sequenced its *ftsY* gene as described (*2*) by using the BartftsyF (5′-ATGACAAAAYCYTTTATMAA-3′) and BartftsyR (5′-TCATGAGTGTCTTCCTGC-3′) primers. The *ftsY* G+C content was 37.7%; the calculated genomic G+C content was 39.51%. The *ftsY* sequence was deposited in GenBank under accession no. DQ538398.

The phylogenetic relationships among the studied bartonellae were inferred from sequence alignments of each gene and from concatenated gene sequences by using the maximum parsimony and neighbor-joining methods within the MEGA version 2.1 software package (*3*) and the maximum-likelihood method within the PHYLIP software package (*4*). Using *rrs*, *gltA*, and *rpoB* sequences, the phylogenetic position of strain AUST/NH1 was supported by bootstrap values <70%. In contrast, by using the ITS, *ftsZ,* and concatenated sequences, strain

AUST/NH1 clustered with a group of *B. tribocorum*, *B. grahamii*, and *B. elizabethae*, with elevated bootstrap values according to the 3 analysis methods (Figure).

The *Bartonella* strains we describe are the first, to our knowledge, obtained from kangaroos and, more generally, from marsupials. Before this study, the only 2 *Bartonella* species found in Australia were *B. henselae* (*5*) and *B. quintana* (*6*). We demonstrated that strain AUST/NH1 was reliably associated with a well-established cluster, including the rodent-associated *B. elizabethae*, *B. grahamii*, and *B. tribocorum* (*7*). Therefore, we are confident that the phylogenic position of the new *Bartonella*, which was similar according to 3 analysis methods and supported by high bootstrap values, is reliable. Although *B. grahamii* (*8*) and *B. elizabethae* (*9*), members of the same phylogenetic cluster as strain AUST/NH1, cause human infections, the pathogenicity of *B. tribocorum* is as yet unknown. Its pathogenicity should therefore be investigated, especially for persons who come in contact with kangaroos.

B. australis is a facultative intracellular gram-negative bacterium. It grows on Columbia agar with 5% sheep blood at 32°C to 37°C in a moist atmosphere containing 5% CO_2. A primary culture was obtained after 7 days, and subculture was obtained after 4 days under the same conditions. Colonies are homogeneous, smooth, round, and gray-white. The 3 strains tested were oxidase negative, catalase negative, and nonmotile. Pathogenicity for humans is, as yet, unknown.

The type strain is strain AUST/NH1. The new species is distinguished from other *Bartonella* species by its 16S rRNA, *gltA*, *rpoB*, *ftsZ* gene sequences, as well as its 16S–23S rRNA ITS sequence. The estimated G+C content is 38%. The type strain exhibits a specific serotype (*10*) and was susceptible to amoxicillin, ceftriaxone, imipenem, erythromycin, clarithromycin, ofloxacin, ciprofloxacin, rifampin, and tetracycline (unpub. data). The type strain AUST/NH1 has been deposited in the Collection of the World Health Organization Collaborative Center for Rickettsioses, Borrelioses and Tick-borne Infections (CSUR), Marseille, France, under reference CSUR B1; in the Collection de l'Institut Pasteur (CIP) under reference CIP 108978T; and in the Culture Collection of the University of Göteborg (CCUG), Sweden, under reference CCUG 51999. The strains AUST/NH2 and AUST/NH3 have been deposited in CSUR under references CSUR B2 and CSUR B3, in the CIP under references CIP 108980 and CIP 108979, and in CCUG under references CCUG 52000 and CCUG 52001, respectively.

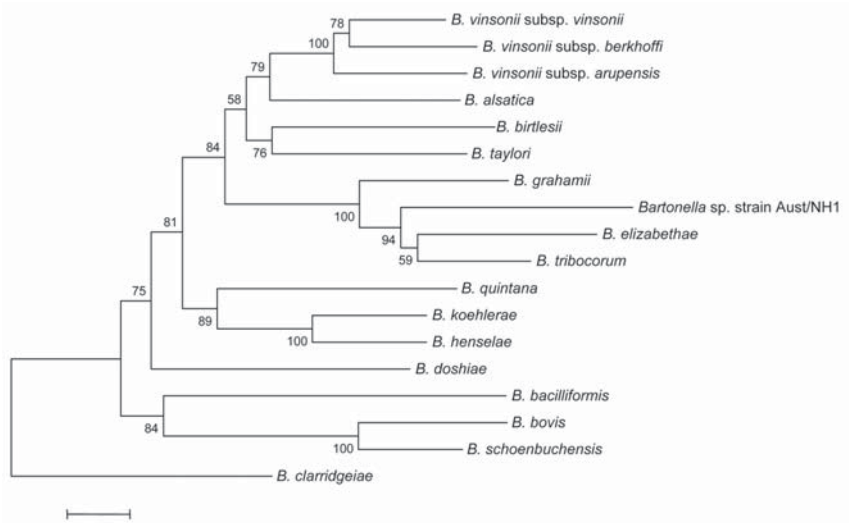

Figure. Unrooted dendrogram showing the phylogenetic position of *Bartonella* sp. strain AUST/NH1 among *Bartonella* species inferred from the comparison of concatenated sequences from the *rrs*, *gltA*, intergenic spacer, *rpoB*, and *ftsZ* genes by the neighbor-joining method. We included only species for which all 5 genes were available. Bootstrap values are indicated at the nodes. The scale bar indicates nucleotide sequence divergence of 0.5%.

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Q Fever in Migrant Workers, Scotland

To the Editor: Q fever is a zoonosis caused by infection with *Coxiella burnetii* and is most commonly associated with occupational exposure to animal-slaughtering facilities. *C. burnetii* is an obligate intracellular bacterium and causes highly variable disease, ranging from asymptomatic infection to fatal chronic infective endocarditis. In June 2006, the United Kingdom experienced its largest outbreak of Q fever with 138 cases associated with a slaughterhouse near Stirling in Scotland. The slaughterhouse had been processing post-parturition ewes in the lairage (place for keeping livestock temporarily) at the end of May. These animals were thought to be among the most likely to shed the organism (*1*). Further investigation showed that a ewe had aborted in the lairage toward the end of May. Although the sheep lairage was the most likely source of the infection, no microbiologic evidence confirmed this, as *C. burnetii* was not isolated from environmental samples.

The outbreak was neither remarkable for its putative mode of transmission nor for the industry involved, but both the number and nationalities of migrant workers infected was noteworthy. Since 2004, 12 member states have joined the European Union and this has led to an influx of immigrants to the United Kingdom. The increase in migrant numbers has partly been a result of the government's managed migration policy, expanding migration to fill vacancies in skilled and low-wage occupations. Employers have difficulty recruiting UK workers because of the jobs' physical demands, long hours that limit social activities, and low pay. They therefore recruit international workers with a good work ethic and reliability; central and Eastern European workers are compared favorably with UK nationals (*2*). Migrants from Eastern and central Europe are now more likely to be found in low-wage occupations in agriculture, construction, hospitality, and au pair employment. Of the 138 cases of Q fever, 48 were immigrants from the following countries: Slovakia (41), Poland (3), Czech Republic (2), and Lithuania (2). Unsurprisingly, epidemiologic case interviews were beset with linguistic and logistic problems.

The diagnosis of Q fever relies predominantly on its serologic legacy since asymptomatic seroconversion occurs in up to 60% of patients (*3*). Analysis of our cohort found that non-UK patients were significantly less likely than their UK counterparts to have symptoms (fever, muscle pain, joint pain, headache, and cough) and to subsequently have Q fever confirmed (Table, $p<0.001$). Twenty-two patients (15 UK, 7 non-UK) did not complete epidemiologic questionnaires and were therefore not included in this analysis.

Furthermore, analysis of cases registered with general practitioners (GPs) identified a significant difference (Table, $p<0.001$) between UK and non-UK patients with the latter group less likely to be registered with a GP. Although most UK residents were registered with a general practice, only 11 of 43 non-UK cases were registered. Information on GP registration was not known for 17 patients, and these were not included in the analysis.

Although the investigating health board took stringent steps to ensure follow-up of all patients, we believe that some asymptomatic non-UK patients may have permanently returned to their native countries with undiagnosed illness, and subsequently, cannot be traced. This unfortunate scenario has potentially catastrophic implications for these patients because proper follow-up clinical management of Q fever is necessary to prevent possible endocarditis (*4*), unnecessary surgery, and premature death.

Persons with known occupational hazards have benefited from an effective Q fever vaccine; abattoir workers and farmers are routinely vaccinated

Table. χ^2 analysis of Q fever symptoms and GP registration by nationality*

Characteristic	No. (%) UK natives		All
	Yes	No	
Symptoms			
No	19 (28.4)	25 (15.6)	44
Yes	56 (46.6)	16 (25.4)	72
All	75	41	116
GP registered			
No	1 (21.3)	32 (11.7)	33
Yes	77 (56.7)	11 (31.3)	88
All	78	43	121

*Expected nos. in parentheses. GP, general practitioner; UK, United Kingdom.

in Australia (*5*). Given the aforementioned linguistic and coordination issues with follow-up of migrant workers and the potential gravity of inappropriate clinical follow-up, it may be prudent to consider Q fever vaccination for all employees who work within UK meat-processing industries.

Public health practitioners should be aware of the continuously evolving multinational makeup of the local population and this should stimulate constant review of local translation services because census data seriously underrecognize the ethnic minority migrant worker population. Furthermore, many migrant workers are unsure of their rights to access primary and hospital care and the structure of healthcare is unfamiliar to many. GPs should consider zoonotic infections, such as Q fever, when patients with acute febrile illness report occupational livestock exposure, especially because migrant workers have become an important source of labor (sometimes preferred over domestic workers) in the agricultural workforce in the United Kingdom (*2*).

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Fatal *Streptococcus equi* subsp. *ruminatorum* Infection in a Man

To the Editor: *Streptococcus equi* belongs to the pyogenic group of streptococci and to group C of the Lancefield classification. It consists of 3 subspecies of zoonotic agents rarely reported as human pathogens (*1*,*2*): *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, and *S. equi* subsp. *ruminatorum*. We report here a case of human infection caused by *S. equi* subsp. *ruminatorum*. (*3*).

A 53-year-old man was admitted to an intensive care unit of our hospital (University Teaching Hospital, Montpellier, France) on April 28, 2006, with a high fever and in a comatose state. The day before, he had experienced headache and neck pain. He had been infected with HIV for 9 years but had not had an opportunistic infection. His ongoing HIV treatment consisted of ritonavir, lopinavir, abacavir, lamivudine, and co-trimoxazole; 3 weeks before admission, his blood CD4+ T-cell count was 133/μL, and viral load was 118,000 copies/mL. At the time of admission, his body temperature was 38.9°C, heart rate was 105 beats/min, and blood pressure was 55/35 mmHg. He exhibited a fixed pupil in 1 eye, neck stiffness, and was nonresponsive. He had bilateral pulmonary infiltrates and severe hypoxemia. Treatment consisted of mechanical ventilation, fluid therapy, and norepinephrine. Laboratory investigations found the following: leukocyte count 9,600/mm^3 with 90% neutrophils, hemoglobin level 9.0 g/dL, platelet count 32,000/mm^3, C-reactive protein value 159 mg/L, and blood lactate concentration 3.2 mmol/L. Computed tomographic scanning of the brain showed no hemorrhage or edema. Lumbar puncture produced turbid cerebrospinal fluid (CSF) with 300 leukocytes/mm^3 (95% neutrophils), protein 5.6 g/L, glucose <0.1 mmol/L, and gram-positive cocci. Three sets of aerobic-anaerobic blood cultures and bronchial aspirates were sampled, and intravenous treatment with dexamethasone (10 mg/6 h/day), cefotaxime (2 g/4 h/day), and vancomycin (30 mg/kg/day) was initiated. On day 2, the hemodynamic state was stabilized, but brain death occurred.

All sets of aero-anaerobic blood cultures, CSF, and bronchial aspirate fluid yielded the growth of a catalase-negative, β-hemolytic, gram-positive cocci belonging to the Lancefield group C of streptococci. Antimicrobial susceptibility testing showed a bacterium fully susceptible to antibiotics tested. MICs of penicillin, amoxicillin, and cefotaxime were 0.047, 0.125, and 0.125 mg/L, respectively. The isolates were identified as *S. equi* by using the Vitek2 system, rapid ID32 STREP, and API 20 STREP strips (bioMérieux, Marcy l'Etoile, France), but phenotype was inconclusive for subspecies identification. The strains were identified as *S. equi* subsp.

zooepidemicus by Vitek2, but aesculin was not hydrolyzed, and D-ribose fermentation was noted, as previously described for *S. equi* subsp. *ruminatorum.* 16S rRNA gene–based identification was performed as previously described (*4*) on strain ADV 6048.06 from blood. The 1,396-bp sequence (GenBank accession no. EF362949) was compared with databases by using the BLAST program (*5*); the sequence differed by only 1 nucleotide position (>99.9% identity) from the sequence of *S. equi* subsp. *ruminatorum* CECT 5772^T. Other primarily related sequences were from *S. equi* subsp. *ruminatorum* strains of animal origin (99.5%–99.9% identity) and from *S. equi* subsp. *zooepidemicus*, (98.7% identity). Phylogenetic trees clustered the clinical isolate with *S. equi* subsp. *ruminatorum* strains to form a robust lineage, well separated from other strains of *S. equi* and supported by a high bootstrap value (Figure).

S. equi subsp. *equi* and *S. equi* subsp. *zooepidemicus* are zoonotic agents implicated in diverse animal infections such as strangles, mastitis, abscesses, wounds, and respiratory and uterine infections. Human infections caused by *S. equi* subsp. *equi*, and *S. equi* subsp. *zooepidemicus* included outbreaks of foodborne diseases (*6*,*7*), meningitis, septicemia, arthritis, pneumonia, glomerulonephritis, and streptococcal toxic shock syndrome, in both immunocompromised and immunocompetent patients (*1*,*2*,*8*,*9*). *S. equi* subsp. *ruminatorum* was described in 2004 in domestic sheep and goats with mastitis (*3*). More recently, it was isolated during severe infections in spotted hyenas and zebras (*10*). No human isolate has been reported to date. Moreover, none of the 3 subspecies of *S. equi* has been isolated from HIV-infected patients. The current case underlines the conclusion that molecular identification of *S. equi* subsp. *ruminatorum* is essential. *S. equi* subsp. *ruminatorum* could have been underestimated due to its potential misidentification as *S. equi* subsp. *zooepidemicus* by phenotypic tools. Despite the rare occurrence of group C streptococci in human infections, a high death rate is reported for invasive infections (*7*–*9*). *S. equi* subsp. *zooepidemicus* produce superantigen exotoxin that may have been implicated in the pathogenesis of fatal infection (*2*); *S. equi* subsp. *ruminatorum* should also be investigated for potential virulence factors for humans.

Figure. Neighbor-joining tree showing the phylogenetic placement of strain ADV 6048.06 (**boldface**) among members of the *Streptococcus equi* species in the pyogenic group of streptococci. Twenty-three 16S rRNA gene sequences selected from the GenBank database were aligned with that of strain ADV 6048.06 by using ClustalX 1.83 (available from http://bips.u-strasbg.fr/fr/documentation/ClustalX). Alignment of 1,263 bp was used to reconstruct phylogenies by using PHYLIP v3.66 package (http://evolution.genetics.washington.edu/phylip.html). The neighbor-joining tree was constructed with a distance matrix calculated with F84 model. Numbers given at the nodes are bootstrap values estimated with 100 replicates. *S. pneumoniae* is used as outgroup organism. Accession numbers are indicated in brackets. The scale bar indicates 0.005 substitutions per nucleotide position. Maximum likelihood and parsimony trees were globally congruent with the distance tree and confirmed the placement of the strain ADV 6048.06 in the *S. equi* subspecies *ruminatorum* (SE*R*) lineage. SE*Z*, *S. equi* subspecies *zooepidemicus.*

Epidemiologic investigations were unsuccessful in tracing the patient's infection to an animal source. The respiratory tract, from which *S. equi* subsp. *ruminatorum* was recovered in pure culture, could be considered the most probable portal of entry.

The mode of *S. equi* subsp. *ruminatorum* transmission to humans remains unknown. More information is needed on its reservoirs, but they likely resemble those of *S. equi* subsp. *equi*, and *S. equi* subsp. *zooepidemicus* (*2*,*6*,*7*). Prevention of human infections due to *S. equi* should include frequent microbiologic sampling of lactating animals and control measures for unpasteurized dairy products (*7*). Better characterization of underlying conditions that increase risk of invasive *S. equi* infections is also needed. This knowledge could help define high-risk

groups of persons and could lead to generation of specific preventive recommendations.

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Rabies Prophylaxis for Pregnant Women

To the Editor: Rabies poses a 100% risk for death to pregnant women and an indeterminate risk to the fetus (*1*,*2*). Although a theoretical risk exists for adverse effects from rabies immune globulin and killed rabies virus vaccines, several studies assessing the safety of this treatment have failed to identify these risks (*3*–*6*). Indeed, the consensus is that pregnancy is not a contraindication to rabies postexposure prophylaxis (PEP) (*7*). Despite this concensus, healthcare providers resist treating pregnant women with rabies PEP. We describe a case of a pregnant woman with uncertain rabies exposure.

A 35-year-old pregnant woman (at 34 weeks gestation) sought treatment 3 weeks after being exposed to a bat. The patient reported awakening at 3:00 AM to find a bat flying in her bedroom. She attempted to confine the bat to 1 section of the home and then called for help. A relative trapped and retrieved the bat, then disposed of the animal without further incident. The patient denied being bitten by the bat, and she had no obvious bite marks after the event. Initially, the patient sought information from online resources, her primary care physician, and her obstetrician. She was uncertain whether rabies PEP was warranted, given what she believed to be the low probability of the bat being rabid and the low likelihood of her having had direct exposure to the bat. The patient did express concern about the safety of rabies PEP in pregnant women. Because no unequivocal recommendations were made by either her primary care physician or obstetrician, she sought further advice from the Infectious Diseases Department at the University of Michigan on how best to proceed.

The 1999 recommendations of Centers for Disease Control and Prevention Advisory Committee on Immunization Practices state, "... postexposure prophylaxis can be considered for persons who were in the same room as the bat and who might be unaware that a bite or direct contact had occurred ..." (*8*). Bat bites may not be apparent when they occur, even with careful examination. In fact, most of the recent human rabies patients have no known history of exposure to a rabid animal (*9*,*10*). Of the 21 cases of bat-associated rabies in the United States during 1980–1999, 12 (57%) occurred in persons with apparent bat contact but no detectable bites (*8*). Our patient woke up with a bat flying in her room and did not know how long it had been there. The best course of action would have been to test the bat for rabies. However, because the animal had already been disposed of, laboratory testing for rabies was not possible. Furthermore, given that 5%–9% of bats tested in Washtenaw County, Michigan, are positive for rabies (www.mdch.state.mi.us/pha/epi/cded/cd/batcoframe.htm), the exposure risk was not insignificant. Therefore, it

was our opinion that this patient qualified for rabies PEP.

Several studies of the safety of rabies PEP for pregnant patients demonstrated no association between treatment and adverse outcomes (*3*–*6*). In 1 study, tissue culture-derived vaccines and human immune globulin did not lead to an increased risk for congenital anomalies; no effects were observed on intrauterine or infant growth or development with a follow-up period of 1 year postpartum (*6*). Although these studies are not comprehensive in their assessment of all reproductive outcomes, they do suggest that PEP is generally safe.

On the basis of the exposure and our literature review, we recommended that the patient receive rabies PEP. After discussing options with her husband, the patient chose not to receive treatment, citing continued concern about the effect of rabies PEP on the fetus. There must be a greater public health effort to educate clinicians and the public about proper response to bat exposures, particularly undetectable bite exposures such as this case. Had public health authorities been contacted to collect and test the captured bat for rabies, there would have been no ambiguity as to the appropriate course of action.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Novel Orthoreovirus from Diseased Crow, Finland

To the Editor: Corvids, especially American crows (*Corvus brachyrhynchos*), are reported to be highly susceptible to lineage 1 of West Nile virus (WNV), which causes them to show symptoms of encephalitis. They are regarded as indicator species in the surveillance of WNV in the United States (*1*). In parts of Europe, WNV is endemic and studies are ongoing to detect WNV in wild birds. Thus far, no evidence of WNV in birds has been found in northern Europe.

In August 2002, in southern Finland, a diseased wild hooded crow (*Corvus corone cornix*) was found flying abnormally with coordination problems, abnormal postures, cramps, and paralysis. Because WNV infection was suspected, virologic tests were performed, which resulted in the isolation of a novel orthoreovirus, which was likely the causative agent of the disease.

Avian orthoreoviruses (ARVs) belong to the family *Reoviridae*, genus *Orthoreovirus*. They infect wild and farm-raised birds and are important fowl pathogens associated with various disease conditions such as gastrointestinal malabsorption syndrome, tenosynovitis (arthritis), growth retardation, and sudden death. They have also been isolated from asymptomatic birds. The reovirus virion is icosahedral, nonenveloped, and has a double-capsid structure that shelters the segmented double-stranded RNA genome (*2*).

Heart, lung, liver, kidney, and brain tissues of the diseased crow tested negative for WNV RNA. Virus isolation from brain homogenate was carried out in BHK (baby hamster kidney)–21 cells. On day 2 after infection, a strong cytopathic effect was observed, including syncytium formation. Spherical, spiked virus particles,

consistent with those of members of the family *Reoviridae,* were observed by electron microscopy. The diameter of the particles was slightly smaller (≈70 nm) than that reported for ARV (85 nm) (*3*). Members of the genus *Orthoreovirus* differ in their host reservoir and capability of syncytium formation; most avian orthoreoviruses are fusogenic and fail to agglutinate erythrocytes, unlike the mammalian reoviruses (*4*). The isolate, designated as Tvärminne avian virus (TVAV), failed to hemagglutinate chicken, goose, or human O erythrocytes.

Members of the genus *Orthoreovirus* have a genome consisting of 10 dsRNA segments in 3 size classes, large (L1–3), medium (M1–3), and small (S1–4). The RNA was extracted from TVAV-infected BHK-21 cells with TriPure isolation reagent (Roche Diagnostics, GmbH, Mannheim, Germany). Ten double-stranded RNA genome segments were separated by electrophoresis, showing a pattern typical of ARV with the S1 segment migrating between S- and M-segment classes (*5*). The S1 segment encodes the orthoreovirus type-specific antigen, σC protein, which is the minor outer-capsid protein, a spiked structure mediating cell attachment.

For phylogenetic analyses, the partial σC gene was amplified by reverse transcription–PCR with avian reovirus–specific primers (*6*). The obtained sequence (GenBank accession no. DQ470139) was aligned with 25 published orthoreovirus sequences. The phylogenetic tree was constructed by using the maximum likelihood method, with general-time reversible model of substitution determined by Modeltest using PAUP* (*7*). The analyses showed that TVAV did not group with avian or mammalian orthoreoviruses but formed a separate clade (Figure). In further analysis, no evidence for recombination events was found. The nucleotide sequence homology of the σC gene was <50%, and amino acid homology was <40%, when compared with previously described orthoreovirus strains. Additionally, a partial M3 segment was sequenced (GenBank accession no. EU053426) that also showed low (<40%) amino acid homology and genetic relation to other orthoreoviruses, which supports the result obtained from the σC gene.

To our knowledge, no sequences of ARV isolates have been previously available from northern Europe. The TVAV isolate described differs clearly from other known ARV strains and could be considered a candidate for a new species in the genus *Orthoreovirus*. ARVs are not generally associated with encephalitic disease, in contrast to reoviruses that infect mice, baboons, and snakes (*8*,*9*). Systemic infection with ARV could cause viremia also in the brain, but since other tissues were not studied, whether they were infected remains unclear. In Finland, a bird-pathogenic orthoreovirus was isolated in the same geographic region 6 years earlier from the bursa of Fabricius from common eider (*Somateria mollissima*) carcasses and was suspected to be the cause of their death (*10*). The eider reovirus induced syncytium formation, lacked hemagglutination activity, and had an RNA genome segment migration pattern similar to that of TVAV. However, instead of showing symptoms that appeared to affect the central nervous system, experimentally infected mallards (*Anas platyrhynchos*) showed hemorrhages in liver, spleen, and bursa of Fabricius tissues. Unfortunately, no sequence data are available from the eider virus isolate that can be compared with TVAV. Be-

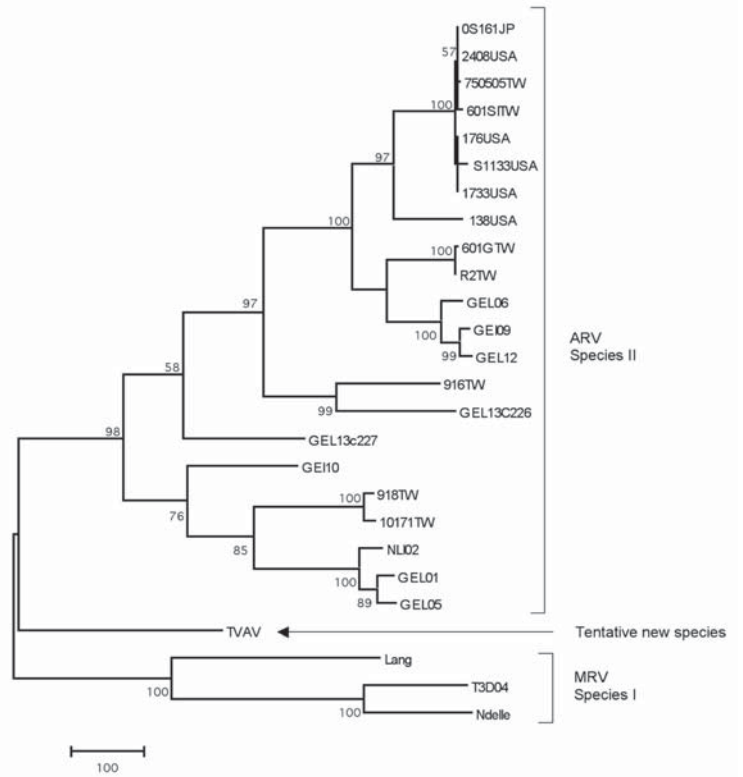

Figure. Maximum parsimony tree based on a 916-bp nucleotide sequence of the σC gene. The scale bar indicates a branch length corresponding to 100 character-state changes. Bootstrap support values <50 are not shown. The tentative species is shown together with the closest relatives within the *Orthoreovirus* genus; avian orthoreovirus (ARV), mammalian orthoreovirus (MRV). GenBank accession nos.: AF204946, AF204945, AF204950, AF204947, AF18358, L39002, AF004857, AF218359, AF297217, AF297213, AF354224, AF354220, AF354225, AF297214, AF354226, AF354227, AF354219, AF297215, AF297216, AF354229, AF354221, AF354223, DQ470139, M10260, AY785910, AF368035.

cause many ARVs are poultry pathogens of economic importance, more studies are needed to determine the taxonomic classification of the TVAV isolate and its pathogenicity for avian hosts. In addition, the recognition of potential avian pathogens in wild birds is important due to the possible threat for farm-raised birds and also for the surveillance of zoonotic viruses transmissible to humans.

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Detecting Human-to-Human Transmission of Avian Influenza A (H5N1)

To the Editor: This letter is in response to a recently published article about statistical modeling to assess human-to-human transmission of avian influenza A (H5N1) viruses in 2 case clusters (*1*). Sporadic cases and clusters of human infection with highly pathogenic avian influenza A (H5N1) viruses have occurred after direct contact with diseased or dead poultry (*2*,*3*). Limited, nonsustained human-to-human transmission of avian influenza (H5N1) viruses is believed to have occurred in some clusters (*4*). Every human infection with a novel influenza A virus should be investigated, and suspected clusters should be investigated immediately to assess exposures and transmission patterns.

Yang et al. applied a statistical model to evaluate publicly available data from 2 case clusters of human infection with avian influenza A (H5N1) viruses (*1*). These clusters were investigated in detail during 2006 by field epidemiologic investigation teams. Yang et al. suggest that statistical methods can prove or confirm human-to-human transmission, but this suggestion is misleading. Modeling approaches can suggest transmission modalities to account for case patterns, but determination of human-to-human transmission requires detailed field epidemiologic investigations in which human, animal, and environmental exposures as well as clinical and laboratory data are assessed and interpreted.

Indication that a novel influenza A virus has acquired the ability to spread among humans could be reflected by a change in the epidemiology of clusters, such as increases in 1) size and frequency of clusters, 2) cases among nonrelated persons, and 3) clinically mild cases. This ability could also be reflected in accompanying changes in viruses isolated from case-patients. When facing emerging infectious disease threats such as those posed by highly pathogenic avian influenza A (H5N1) viruses, surveillance should rapidly detect human cases and case clusters and facilitate accurate identification of the agent. Field epidemiologic investigations, initiation of evidence-based clinical management of case-patients, and epidemiologic disease-control methods (including ap-

propriate infection control measures) should be implemented immediately. Statistical modeling can provide useful and supportive insights but should not be viewed as an alternative to a detailed field epidemiologic investigation combined with laboratory data. Timely and comprehensive field investigations remain most critical to guiding decisions about containment efforts for pandemic influenza and other emerging infectious diseases (*5*).

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In Response: We thank Drs Uyeki and Bresee for their thoughtful commentary (*1*) on our article about assessing the possibility of human-to-human transmission of avian influenza A (H5N1) in observed clusters (*2*). We agree with them that statistical models are not substitutes for careful epidemiologic investigations combined with laboratory data. We believe that the statistical model that we advance can be an important tool to use in conjunction with sound and rapid epidemiologic investigation and intervention.

We do not claim, however, that our statistical model can prove or confirm human-to-human transmission, as Uyeki and Bresee state. In our article, we were careful to point out that we found statistical evidence of human-to-human transmission ($p = 0.009$) in Sumatra. We did not claim to have proven or confirmed human-to-human transmission on the basis of our statistical analysis. A strict interpretation of what we found is that the data from the cluster in northern Sumatra provided evidence to reject the null hypothesis of no human-to-human transmission. Given this, we then weighed all the epidemiologic and laboratory information available to make a scientific judgment about the likelihood of human-to-human transmission. We are not aware of any logical system analogous to Koch's postulates (*3*) (i.e., proving the causal link between an infectious agent and clinical disease) for actually proving that an infectious agent is transmitted from person to person. However, we believe that the statistical, epidemiologic, and laboratory evidence combined weigh heavily toward the presence of limited human-to-human transmission in the case of the Sumatra cluster.

This statement can be contrasted with our analysis of the cluster in eastern Turkey, where we did not find statistical evidence of human-to-human transmission of avian influenza A (H5N1). This analysis must be evaluated with the epidemiologic and laboratory data. We simply state that we do not have enough statistical evidence to reject the null hypothesis of no hu-

man-to-human spread. Whether human-to-human transmission occurred is a scientific judgment.

We agree with Uyeki and Bresee that statistical modeling can provide useful and supportive insights, and we hope that epidemiologic teams who investigate and control potential infectious disease outbreaks will carry the TranStat software (*2*) into the field with them. We are grateful that Uyeki and Bresee point out that immediate implementation of containment measures is critical, even while we sort out the cause of an observed cluster of cases. Actually containing possible spread of a potential pandemic strain of influenza (*4*,*5*) is more important than scientific arguments about the transmissibility of the infectious agent in question.

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Swimming With A Hundred Year Old Snapping Turtle

Freya Manfred

I spy his head above the waves,
big as a man's fist, black eyes peering at me,
until he dives into darker, deeper water.
Yesterday I saw him a foot from my outstretched hand,
already tilting his great domed shell away.
Ribbons of green moss rippled behind him,
growing along the ridge of his back
and down his long reptilian tail.
He swims in everything he knows,
and what he knows is never forgotten.
Wisely, he fears me as if I were the Plague,
which I am, sick unto death, swimming
to heal myself in his primeval sea.

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Silent Victories: The History and Practice of Public Health in Twentieth-Century America

John W. Ward and Christian Warren, editors

Oxford University Press, New York, USA, 2007
ISBN: 9780195150698
Pages: 484; Price: US $49.95

The 20th century witnessed some notable public health triumphs in America: improvements in the water supply, further control of several infectious diseases through vaccines and antimicrobial drugs, and increases in life expectancy with enormous improvements in survival rates of mothers and their infants. What made these improvements possible? For anyone who has ever wondered, this book is an excellent place to start looking for answers.

The stated purpose of the book is not to provide a comprehensive history of public health in America but to discuss 10 key public health advances of the 20th century. This is a broad objective in itself, which this volume richly achieves. The advances, originally chosen for MMWR (Morbidity and Mortality Weekly Report) in 1999, are each expanded into a section of the book: Control of Infectious Diseases, Control of Disease through Vaccination, Maternal and Infant Health, Nutrition, Occupational Health, Family Planning, Fluoridation, Vehicular Safety, Cardiovascular Disease, and Tobacco and Disease Prevention.

The facts and figures are all there, of course, and they are generally very well presented and referenced. Infectious diseases are well represented; their respective chapters are excellent and informative. But it would be a pity if the reader stopped there. A unique strength of the book is the pairing of these expository chapters with essays by social scientists and historians who explore aspects of the social or political context. This combination makes it a book to savor. Experienced practitioners having a hard day may be encouraged to learn that many public health triumphs we take for granted today (the apt title Silent Victories is from a 1923 lecture by C.-E.A. Winslow) were made possible only by heroic and sustained effort.

One theme that emerges is the importance of coalitions, often including not only the medical community and health departments (and sometimes industry), but also activists, reformers, and even ordinary citizens who became passionate about a cause. Getting recognition and consensus within the medical community was essential, and not always easy, as in the development of occupational health, or even pasteurization at first. Wolf's article, for example, notes that ensuring clean pasteurized milk required 30 years of effort, during which time many infants died. In traffic safety, discussed by Albert, the activists were often the ones who pushed government into taking action. With regard to the more recent efforts toward tobacco cessation, Brandt argues that the 1964 Surgeon General's Report was a watershed comparable to John Snow's work on cholera, as it developed the foundations not only for tobacco cessation but also for chronic disease epidemiology.

But, of course, public health cannot rest on these laurels. As Koplan and Thacker note in the Epilogue, public health in the coming century will face many challenges. Some are a continuation of 20th-century trends, such as emerging infectious diseases, healthy lifestyle choices, and ensuring that basic public health measures are available globally. Others will be new, including the aging of large segments of the population. As this book demonstrates, one of the best ways to meet the new challenges may well be to fully appreciate how these past successes were achieved.

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Emerging Viruses in Human Populations

Edward Tabor, editor

Elsevier, Amsterdam, the Netherlands, 2007
ISBN: 978-0-444-52074-6
Pages: 374; Price: US $94.95

With increasing international travel and globalization of the world's economies, changing climates, and altered human behavior and demographics, multiple viruses have emerged to occupy expanded ecologic niches, producing disease syndromes in parts of the world where they had never before existed. Because most emerging viral diseases in humans in the 21st century have been zoonotic, Emerging Viruses in Human Populations focuses on this group of viruses. The resulting overview is a book useful for anyone interested in a diverse group of viral agents that have recently elicited novel disease syndromes in human populations around the world. This text does an excellent job of encompassing a wide variety of contact-transmitted enzootic viruses including severe acute respiratory syndrome–associated coronavirus, Nipah and Hendra viruses, influenza virus, hantaviruses, monkeypox viruses, and vector-transmitted agents including Crimean-Congo hemorrhagic fever, dengue, West Nile, and Japanese encephalitis viruses.

Two especially informative chapters, the first and last, introduce several emerging viral disease agents that affect humans. The authors provide a synthesis of factors that could be associated with the emergence of novel viral agents, such as environmental change, altered human demographics, and human behavior. They also discuss the defining mechanisms through which emerging viral disease can be identified and monitored.

The text outlines basic virologic characterization such as replication strategy and the role of known viral proteins in viral pathogenesis, diagnostics, treatment, and vaccine availability. Additionally, it covers epidemiology of agents, relative disease manifestation, and disease patterns identified in human populations. My only criticism regarding this fine resource is the lack of a consistent level of information presented for each viral agent. In some cases, for example, extensive information was presented on the role of all known viral proteins in replication of the virus and how these proteins contribute to disease manifestations. For other agents, the epidemiology was highlighted with relatively no coverage of viral pathogenesis.

Many of the chapters are easily readable by the general public, yet the level of detail within most of the sections makes this also an excellent reference text for research and public health professionals. I recommend this book for anyone interested in obtaining a broad perspective on the emergence of viral diseases that affect humans.

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Francisella tularensis: Biology, Pathogenicity, Epidemiology, and Biodefense

Yusef Abu Kwaik, Dennis W. Metzger, Francis Nano, Anders Sjöstedt, and Richard Titball, editors

Blackwell Publishing Limited, New York, New York, USA 2007
ISBN-10: 1573316911
Pages: 352; Price: US $145.00

I am pleased to recommend *Francisella tularensis*: Biology, Pathogenicity, Epidemiology, and Biodefense, published by Blackwell Publishing Limited on behalf of the New York Academy of Sciences. This book is a much-needed comprehensive overview of recent research on the causative agent of tularemia, a potentially serious illness that occurs naturally in the United States. *F. tularensis* is a marvel among vector-borne agents of infectious disease. It has a wide geographic distribution (covering most of the Northern Hemisphere) and can be transmitted through a variety of routes including 1) tick or insect bites; 2) handling of infected animals; 3) contact with or ingestion of water, food, or soil; and 4) inhalation of contaminated aerosols. Indeed, *F. tularensis* is notorious for infecting laboratory workers and is a potential bioterrorism agent. The bacterium includes 4 biovars, with the pathogenic type A recently shown to consist of at least 2 subtypes in North America. Natural transmission cycles of *F. tularensis* are complex and poorly understood.

Research on a broad variety of topics was carried out between the 1914 recognition of *F. tularensis* as a disease agent in humans and the 1970s, but few studies focused on this pathogen during the 1980s and 1990s. The recent designations of *F. tularensis* by the National Institute of Allergy and Infectious Diseases as a priority A pathogen and a potential bioterrorism agent has resulted in an explosion of new studies on this intriguing pathogen. Primary focal points of these studies have included vaccine development, improved pathogen detection methods, evaluation of the genetic variability of *F. tularensis* biovars commonly associated with human disease, description of the *F. tularensis* genome, and determination of virulence factors. The wealth of information gained from recent studies is elegantly outlined by an impressive group of world leaders in the field of tularemia research. Chapter topics vary from molecular epidemiology, evolution, and ecology of *Francisella* to genetics, genomics, and proteomics of *F. tularensis,* molecular and genetic basis of pathogenesis of *F. tularensis*, animal models, immunity and immunopathogenesis, diagnosis and therapy, vaccine development, and biosafety issues.

Reflecting a disturbing paucity of epidemiologic and field-oriented studies in the past 20 years, especially in North America, only a few chapters include some information on epidemiology, natural transmission cycles of *F. tularensis*, and the role of different transmission routes to humans. As the field of *F. tularensis* and tularemia research moves forward in the 21st century, the explosion of knowledge related to genetics, immunology, and pathogenesis of *F. tularensis* needs to be complemented by renewed studies on natural transmission cycles, transmission routes to humans, and epidemiology.

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Census Proceedings on the Campus Martius. Altar of Domitius Ahenobarbus. Decoration from the base of a statuary group. Rome. End of second century BCE. Marble (78 cm × 559 cm). Louvre, Paris, France/Lauros/Giraudon/The Bridgeman Art Library Nationality/copyright status: out of copyright

Uncommon Denominators

Polyxeni Potter*

"Others will have greater skill for getting the breath of life to spring from bronze more fluidly .... But as for you, Roman, remember to impose your power upon nations. Your art is to decree the rules of peace, to spare the vanquished and subdue the vainglorious," advised Virgil in the Aeneid, placing himself in the service of imperial ideology (*1*). This was the reign of Augustus (27 BCE–14 CE), the first and among the most influential of Roman emperors, who enlisted literature and the arts in support of the new order.

Art of the Roman Empire, from Romulus to Constantine the Great, a period of more than 1,000 years, was expansive and diverse like the Empire itself but left few records of artists or patrons (*2*). Influenced by the Etruscans, the preceding dominant culture in Italy, and the Hellenistic world through colonies in southern Italy and Sicily, its growth awaited the evolution of political institutions during the latest period of Republican history (*3*). Before the conquest of Syracuse, wrote Plutarch, a leading thinker of the Empire's golden age, "Rome neither had nor even knew" of these refined things, "nor was there in the city any love of what was charming and elegant; rather it was full of barbaric weapons and bloody spoils" (*4*).

Hellenistic influences continued as artists were brought to Rome to repair crumbling monuments and design new ones. Hellenic bronze statues were widely copied, usually in marble. Classicism gave way to a more realistic style, particularly in portrait busts, which were very popular. Art became secular and utilitarian. Architecture flourished on a grand scale, and the vault and dome were invented. Augustus is said to have boasted that he "found Rome of brick and left it of marble" (*5*). The discovery of concrete made possible such monumental buildings as the Pantheon in Rome, which still stands. The triumphant arch, also an invention of the period, exemplified Roman civic and commemorative architecture.

In the Augustan era, sculpture still showed the idealism of Hellenic models, even relief sculpture: shallow three-dimensional carvings on arches, friezes, altars, and other flat areas of temples and public buildings. But the content of reliefs favored the historical and commemorative, intending to narrate in detail triumphant military campaigns and promote the goals of the Empire. In his Ars Poetica, Horace supported this philosophy, as he argued the superiority of painting over any other form of communication to affect

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and manipulate: "Less vividly is the mind stirred by what finds entrance through the ears than by what is brought before the trusty eyes, and what the spectator can see for himself" (*6*). Public art of the Empire aimed to "write conquerors and conquered in one community" (*7*).

The remains of an altar believed to have been set up in the Campus Martius by Domitius Ahenobarbus, father of Emperor Nero, provide a glimpse into civic commemorative art of the Empire. The Campus Martius (Field of Mars) was a public area of Rome used for military activities; as such, it was dedicated to Mars, god of war and father of Romulus and Remus, legendary founders of the city. The month March (Martius) was named after him, and the Romans called themselves "sons of Mars." The Campus later became the site of triumphant parades and celebrations and was filled with temples and public buildings.

Contiguous panels of the relief on this month's cover have the feel of narrative stream. During the census proceedings, a collection of citizens, among them military men serving as guards, are taking part in a religious rite, the *suovetaurilia* (from *sus* [pig], *ovis* [ram], *taurus* [bull]): a ceremony during which livestock were sacrificed to the gods. "Father Mars, I pray and beseech thee that thou be gracious and merciful to me, my house, and my household," read the Latin prayer (*8*). The sacrifice, whose purpose was purification, was performed at state ceremonies; during agricultural festivals to drive out evil from the fields and purify new crops; as atonement for ritual errors; before military campaigns; and at the conclusion of the census.

The census was the first and principal duty of the Roman censors, high magistrates in charge of this 5-yearly activity. To carry out the census and the purifications that concluded it, they had the power of summoning the people to the Campus Martius, each tribe separately, by public crier. Each paterfamilias appeared in person to account for himself, his family, and his property upon oath, "declared from the heart" (*9*). A person voluntarily absent from the census was considered *incensus* and risked imprisonment and death.

"It is so hard to find out the truth of anything by looking at the record of the past," wrote Plutarch; "The process of time obscures the truth of former times, and even contemporaneous writers disguise and twist the truth out of malice or flattery" (*4*). Even art can be used for promotion and persuasion. Yet this census-taking relief, a glimpse of Roman life, did more than serve the purposes of the state. It witnessed one of the foundation stones of Roman civilization; a ritual special to the Romans for it symbolized their status as a *populus*, a people, capable of collective action (*10*).

"We are all, so far as we inherit the civilizations of Europe, still citizens of the Roman Empire," wrote T.S. Eliot, poet and critic of modern European culture. And while his words may not have universal application, they do call attention to Roman legacy in some of our practices. Certainly we relate to the census. In ancient Rome, the practice served to count citizens and assess military strength and tax revenue. In public health, it helps calculate population density. The number of humans, animals, plants, wildlife, and vectors per unit area influences the spread of communicable diseases and their impact, a tax of its own. And "census numbers" of domestic and wild animals, the denominators used to calculate attack, birth, and death rates, can be strong predictors of zoonotic disease. Once again in the words of T.S. Eliot, "… withered stumps of time … told upon the walls," uncover uncommon denominators.

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EMERGING INFECTIOUS DISEASES

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Hynes Convention Center
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http://www.retroconference.org

March 16–19, 2008
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Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

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Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

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