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Prion Disease

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South Netherlandish, The Unicorn Is Found, from Hunt of the Unicorn (1495–1505)
Wool warp, wool, silk, silver and gilt wefts, 368 cm x 379 cm
The Metropolitan Museum of Art, Gift of John D. Rockefeller Jr., 1937

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Chronic Wasting Disease and Potential Transmission to Humans

Ermias D. Belay,* Ryan A. Maddox,* Elizabeth S. Williams,† Michael W. Miller,‡ Pierluigi Gambetti,§ and Lawrence B. Schonberger*

Chronic wasting disease (CWD) of deer and elk is endemic in a tri-corner area of Colorado, Wyoming, and Nebraska, and new foci of CWD have been detected in other parts of the United States. Although detection in some areas may be related to increased surveillance, introduction of CWD due to translocation or natural migration of animals may account for some new foci of infection. Increasing spread of CWD has raised concerns about the potential for increasing human exposure to the CWD agent. The foodborne transmission of bovine spongiform encephalopathy to humans indicates that the species barrier may not completely protect humans from animal prion diseases. Conversion of human prion protein by CWD-associated prions has been demonstrated in an in vitro cell-free experiment, but limited investigations have not identified strong evidence for CWD transmission to humans. More epidemiologic and laboratory studies are needed to monitor the possibility of such transmissions.

Chronic wasting disease (CWD) is classified as a transmissible spongiform encephalopathy (TSE), or prion disease, along with other animal diseases, such as scrapie and bovine spongiform encephalopathy. The only known natural hosts for CWD are deer (*Odocoileus* species) and Rocky Mountain elk (*Cervus elaphus nelsoni*) (1,2). CWD and other TSEs are believed to be caused by a pathogenic effect on neurons of an abnormal isoform of a host-encoded glycoprotein, the prion protein. The pathogenic form of this protein appears to be devoid of nucleic acids and supports its own amplification in the host. TSEs in animals primarily occur by transmitting the etiologic agent within a species, either naturally or through domestic husbandry practices. In contrast, most such encephalopathies in humans occur as a sporadic disease with no identifiable source of infection or as a familial disease linked with mutations of the prion protein gene (3). A notable exception among the human TSEs is the variant form of Creutzfeldt-Jakob disease (vCJD), which is believed to have resulted from the foodborne transmission of bovine spongiform encephalopathy (BSE) to humans (4,5).

CWD was first identified as a fatal wasting syndrome of captive mule deer in the late 1960s in research facilities in Colorado and was recognized as a TSE in 1978 (6,7). Subsequently, this wasting disease was identified in mule deer in a research facility in Wyoming and in captive elk in both the Colorado and Wyoming facilities (6–8). The disease was first recognized in the wild in 1981, when it was diagnosed in a free-ranging elk in Colorado (1,9). By the mid-1990s, CWD had been diagnosed among free-ranging deer and elk in a contiguous area in northeastern Colorado and southeastern Wyoming, where subsequent surveillance studies confirmed it to be endemic (10). Epidemic modeling suggested that this wasting disease might have been present among free-ranging animals in some portions of the disease-endemic area several decades before it was initially recognized (10). On the basis of hunter-harvested animal surveillance, the overall prevalence of the disease in this area from 1996 through 1999 was estimated at approximately 5% in mule deer, 2% in white-tailed deer, and <1% in elk (10). In 2000, surveillance data indicated that the disease-endemic focus extended eastward into adjacent areas of Nebraska (1,11), and ongoing surveillance continues to redefine the limits of this focus.

Clinical manifestations of CWD include weight loss over weeks or months, behavioral changes, excessive salivation, difficulty swallowing, polydipsia, and polyuria (1,6–8). In some animals, ataxia and head tremors may occur. Most animals with the disease die within several months of illness onset, sometimes from aspiration pneumonia. In rare cases, illness may last for ≥1 year. In captive cervids, most cases occur in animals 2–7 years of age; however, the disease has been reported in cervids as young as 17 months and as old as >15 years of age (1). This disease can be highly transmissible within captive deer and elk populations. A prevalence of >90% was reported among mule deer in facilities where the disease has been endemic for >2 years (2,6,7,12). The mode of transmission among deer and elk is not fully understood; however,

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †University of Wyoming, Laramie, Wyoming, USA; ‡Colorado Division of Wildlife, Fort Collins, Colorado, USA; and §Case Western Reserve University, Cleveland, Ohio, USA

evidence supports lateral transmission through direct animal-to-animal contact or as a result of indirect exposure to the causative agent in the environment, including contaminated feed and water sources (12).

Geographic Distribution of Chronic Wasting Disease

The geographic extent of CWD has changed dramatically since 1996 (2). Two largely independent and simultaneous epidemics, one in free-ranging deer and elk and another in the captive elk and deer industry, appear to represent the main framework for explaining the disease's current distribution (2). More extensive and coordinated surveillance has provided a clearer picture of its distribution over the last few years. Since 2000, the disease in free-ranging cervids has been increasingly identified outside of the original CWD-endemic areas of Colorado and Wyoming (Figure). The observed distribution seems to be related in part to natural movement of deer and elk and to commercial movement of infected animals to areas far from the disease-endemic zone. Considerable attention has been given to recent increases in the geographic spread of the disease, which in some areas is likely a result of increased surveillance rather than evidence of explosive geographic spread.

No single original event or source links all wasting disease foci documented to date. Given the disease's insidious nature and the apparent duration (at least several decades) of epidemics among captive and free-ranging cervids, gaps in knowledge about its spread and distribution are not surprising, particularly within the captive deer and elk industry. However, our current knowledge cannot explain some of the distinct foci of CWD among free-ranging animals (e.g., in New Mexico and Utah). Thus, unidentified risk factors may be contributing to the occurrence of CWD among free-ranging and captive cervid populations in some areas.

Chronic Wasting Disease in Free-ranging Deer and Elk

In 2000, surveillance of hunter-harvested deer first detected the occurrence of CWD in counties in southwestern Nebraska, adjacent to the previously recognized areas of Colorado and Wyoming that are endemic for this disease (Figure) (1,11). It was reported subsequently in other Nebraska counties, including among deer and elk in a commercial, large enclosure surrounded by a fence in northwestern Nebraska, where the prevalence of CWD was >50% (11). Free-ranging deer from areas surrounding the enclosure also tested positive for the disease but at substantially lower rates. In 2001, CWD in a free-ranging deer was identified in the southwestern part of South Dakota along the Nebraska border close to an area where the disease had been reported among captive elk (13). Since then, additional CWD-positive free-ranging deer and elk have been identified in southwestern South Dakota.

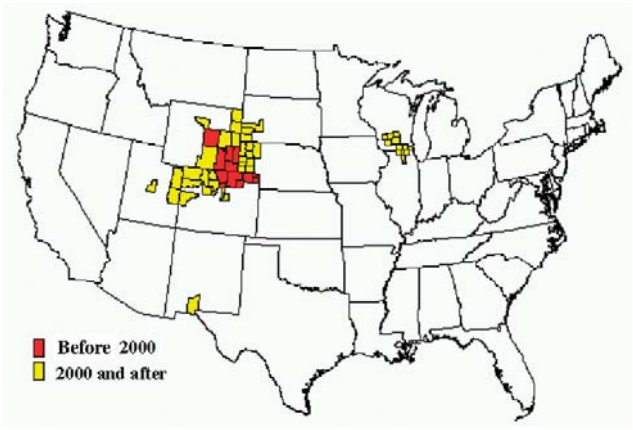

Figure. Chronic wasting disease among free-ranging deer and elk by county, United States.

CWD in free-ranging cervids was first reported east of the Mississippi River in Wisconsin among white-tailed deer harvested in the 2001 hunting season (14). Subsequent surveillance indicated that this CWD epidemic focus was limited to several counties in the south-central region of Wisconsin, although a second focus spanning the Illinois border was also detected (15). The absence of evidence for a widespread occurrence of CWD and its low prevalence, despite a highly dense deer population, indicate that the disease probably was recently introduced into Wisconsin. Because the distance from the CWD-endemic area of Colorado-Wyoming effectively precludes eastward migration of animals as a logical source of infection, CWD in Wisconsin was more likely introduced by an imported infected cervid or some other unidentified source (14). The proximity of the Wisconsin-Illinois focus to a white-tailed deer farm with infected animals appears to support this explanation, as highlighted by the report of CWD in a previously captive white-tailed deer approximately 7 months after it escaped into the wild in southern Wisconsin (14). The disease among the captive deer herd from which the white-tailed deer escaped was demonstrated earlier, when a still-captive deer tested positive for the disease. The captive source herd was held in a facility 30–50 km from the Illinois location where CWD was recently identified in a free-ranging deer (16). In 2002, the Wisconsin Department of Natural Resources launched an ambitious culling program by providing special hunting permits to eliminate the disease in a designated "eradication zone" around the areas where it was detected (15,17). Whether such aggressive management will succeed in eliminating free-ranging foci of CWD remains to be determined.

In Colorado, the Continental Divide initially appeared to have prevented natural expansion of CWD into the

western part of the state. However, in 2002, the disease was confirmed for the first time in several free-ranging deer harvested in western Colorado in an area surrounding a commercial enclosure, where entrapped mule deer tested positive for CWD. Aggressive culling of deer and elk surrounding the enclosure was initiated to prevent further spread of the disease in the western slope of Colorado. Through the 2002 hunting season, CWD-positive deer and elk continued to be identified outside of the previously defined disease-endemic area, primarily in northwestern Colorado (18). This northwestern focus appears to be discontinuous from the previously identified CWD-endemic area, although surveys conducted in 2002 demonstrated that the western and southern boundaries of that area were wider than previously believed. The ultimate source of this wasting disease in northwestern Colorado remains unidentified.

In 2002, samples from an emaciated, free-ranging mule deer found in White Sands, New Mexico, tested positive for CWD (1,19). No cervids have been held in captivity close to the area where the New Mexico deer was found, and the origin of the disease in this deer remains unknown. In addition, CWD-positive, free-ranging deer have been identified in Wyoming to the west over the Continental Divide from the known CWD-endemic zone (20). In 2003, a mature buck deer harvested in the fall of 2002 in northeastern Utah tested positive for the disease (21); additional cases have since been found in central and eastern Utah (Figure). These cases provide additional evidence for the potential spread of this wasting disease in the wild.

In Canada, CWD was first detected in free-ranging cervids (two mule deer) in 2001 in Saskatchewan; a few additional deer tested positive in 2002 and 2003 (22). Saskatchewan Environment has implemented a herd-reduction program using "control permits" to prevent further spread of the disease among free-ranging cervids.

Chronic Wasting Disease in Captive Deer and Elk

CWD was first recognized in the captive elk industry in Saskatchewan in 1996, but subsequent investigations indicated that the most likely source of Canadian cases was captive elk imported from South Dakota prior to 1989 (2,22). Since 1996, surveillance has detected infected animals on more than 25 elk farms in Colorado, Kansas, Minnesota, Montana, Nebraska, Oklahoma, South Dakota, and Alberta, Canada, and the Republic of Korea (1,14,23,24). CWD in most of these farms was identified in the past 5 years. In 2002, the disease was detected in white-tailed deer on farms in Alberta and Wisconsin (23,25). More extensive and uniform surveillance in captive white-tailed deer is needed to determine the full extent of the disease in this industry.

Captive herds with a CWD-infected cervid are often depopulated both in Canada and the United States. Carcasses of depopulated animals are incinerated or buried in accordance with local regulations. Meat from depopulated animals has not been allowed to enter the human food and animal feed supply.

Transmission to Other Animals

Concerns have been raised about the possible transmission of the CWD agent to domestic animals, such as cattle and sheep, which may come in contact with infected deer and elk or CWD-contaminated environments. If such transmissions were to occur, they would potentially increase the extent and frequency of human exposure to the CWD agent. In addition, passage of the agent through a secondary host could alter its infectious properties, increasing its potential for becoming more pathogenic to humans. This phenomenon may have occurred with BSE when a strain of scrapie, a possible original source of the BSE outbreak, changed its pathogenic properties for humans after infecting cattle. However, the exact origin of BSE remains unknown.

Although CWD does not appear to occur naturally outside the cervid family, it has been transmitted experimentally by intracerebral injection to a number of animals, including laboratory mice, ferrets, mink, squirrel monkeys, and goats (1,26). In an experimental study, the CWD agent was transmitted to 3 of 13 intracerebrally injected cattle after an incubation period of 22 to 27 months (27). The susceptibility of cattle intracerebrally challenged with the agent of this disease was substantially less than that observed after intracerebral scrapie challenge: nine of nine cattle succumbed to scrapie challenge after intracerebral injection (28). In ongoing experimental studies, after >6 years of observation, no prion disease has developed in 11 cattle orally challenged with the CWD agent or 24 cattle living with infected deer herds (E.S. Williams and M.W. Miller, unpub. data) (1). In addition, domestic cattle, sheep, and goat residing in research facilities in close contact with infected cervids did not develop a prion disease.

Analysis by immunohistochemical studies of the tissue distribution of prions in CWD-infected cervids identified the agent in the brain, spinal cord, eyes, peripheral nerves, and lymphoreticular tissues (Table 1) (29,30). Distribution of the CWD agent outside of the brain seems to be less widespread in elk than in deer (2). Involvement of the tonsils and peripheral nerves early in the course of experimental and natural prion infection suggests the possible involvement of the lymphoreticular and peripheral nervous systems in the pathogenesis and transmission of the disease (2,12,30,31).

Table 1. Deer tissues tested for the CWD agent by animal bioassay or immunohistochemical studies[a]

Tissues positive for CWD agent
Brain
Pituitary gland
Spinal cord
Eyes (optic nerve, ganglion cells, retina)
Tonsils
Lymphoid tissues (e.g., gut-associated, retropharyngeal, posterior nasal septum)
Spleen
Pancreas
Peripheral nerves (e.g., brachial plexus, sciatic nerve, vagosympathetic trunk)
Tissues negative for CWD agent
Dorsal root ganglia
Parotid and mandibular salivary glands, tongue, esophagus, small intestine, colon
Thymus
Liver
Kidneys, urinary bladder, ovary, uterus, testis, epididymis, placentomes
Myocardium, Purkinje fibers, arteries, veins
Trachea, bronchi, bronchioles, aleveolar parenchyma
Bone marrow
Thyroid gland, adrenal gland
Skeletal muscle
Skin

[a]CWD, chronic wasting disease.

Risk for Transmission to Humans

Epidemiologic Studies

The increasing detection of CWD in a wider geographic area and the presumed foodborne transmission of BSE to humans, resulting in cases of vCJD, have raised concerns about the possible zoonotic transmission of CWD (32). In the late 1990s, such concerns were heightened by the occurrence of CJD among three patients ≤30 years of age who were deer hunters or ate deer and elk meat harvested by family members (Table 2). However, epidemiologic and laboratory investigations of these case-patients indicated no strong evidence for a causal link between CWD and their CJD illness (33). None of the patients were reported to have hunted deer or eaten deer meat harvested in the CWD-endemic areas of Colorado and Wyoming. Such a history in unusually young CJD patients, if present, would have supported a causal link with CWD. Moreover, the testing of brain tissues from >1,000 deer and elk harvested from areas where the patients hunted or their venison originated did not show any evidence of CWD (33). In addition, the lack of homogeneity in the clinicopathologic manifestation and codon 129 of the prion protein gene among the three patients suggested that their illnesses could not be explained by exposure to the same prion strain. In vCJD, homogeneity of the genotype at codon 129 and the clinical and pathologic phenotype were attributed to the patients' exposure to the same prion strain, the agent of BSE.

In 2001, the case of a 25-year-old man who reportedly died of a prion disease after an illness lasting ≈22 months was investigated (Table 2). Although this man had hunted deer only rarely, his grandfather hunted deer and elk throughout much of the 1980s and 1990s and regularly shared the venison with the case-patient's family. The grandfather primarily hunted in southeastern Wyoming, around the known CWD-endemic area. The case-patient's illness began with a seizure and progressed to fatigue, poor concentration, and depression. Memory loss, ataxia, speech abnormalities, combative behavior, and recurrent seizures also developed. Histopathologic, immunohistochemical, and Western blot testing of brain autopsy samples confirmed a prion disease diagnosis. Analysis of the prion protein gene indicated a P102L mutation coupled with valine at the polymorphic codon 129 in the mutant allele, confirming a diagnosis of Gerstmann-Sträussler-Scheinker syndrome (GSS). This case-patient was unusually young even for a person with a GSS P102L mutation. It remains unknown whether the possible exposure of the case-patient to CWD-infected venison potentially contributed to the early onset of his prion disease.

In 2001, two additional CJD patients 26 and 28 years of age were reported from a single state (Table 2) (34). The patients grew up in adjacent counties and had illness onset within several months of each other. As a result of this fact and their unusually young age, a possible environmental source of infection, including exposure to CWD-infected venison, was considered. One of the patients died after an illness lasting 5–6 months that was characterized by progressive aphasia, memory loss, social withdrawal, vision disturbances, and seizure activity leading to status epilepticus and induced coma. Histopathologic, immunohistochemical, and Western blot testing of brain biopsy and autopsy samples confirmed a CJD diagnosis. The patient's disease phenotype corresponded to the MM2 sporadic CJD subtype reported by Parchi et al. (35). This patient did not hunt, and family members provided no history of regularly eating venison. The patient may have occasionally eaten venison originating from the Upper Peninsula of Michigan while away from home during his college years. However, ongoing surveillance has not detected CWD in Michigan deer (36).

The second patient died from an illness lasting ≈16 months. The patient's illness began with behavioral changes, including unusual outbursts of anger and depression. Confusion, memory loss, gait disturbances, incontinence, headaches, and photophobia also developed. Western blot analysis of frozen brain biopsy tissue confirmed a prion disease diagnosis. Immunohistochemical analysis of brain tissue obtained after the patient's death

Table 2. Creutzfeldt-Jakob disease investigated for possible causal link with chronic wasting disease of deer and elk, United States[a]

Case no.	Age at death (y)	Year of death	Codon 129	Western blot	Final diagnosis	Eating of venison from CWD-endemic area
1	25	2001	M/V	Type 1	GSS 102	Yes
2	26	2001	M/M	Type 2	CJD	No
3[b]	28	2002	nd	nd	GSS 102	No
4	28	1997	M/M	nd	CJD	No
5	28	2000	M/V	Type 1	CJD	No
6	30	1999	V/V	Type 1	CJD	No
7	54	2002	V/V	Type 2	CJD	No
8[c]	55	1999	M/M	Type 1	CJD	No
9[d]	61	2000	M/M	Type 1	CJD	Yes
10	63	2002	V/V	Type 1	CJD	No
11[e]	64	2002	M/M	Type 1	CJD	Yes
12	66	2001	M/M	Type 1	CJD	No

[a]CWD, chronic wasting disease; GSS, Gerstmann-Sträussler-Scheinker syndrome; CJD, Creutzfeldt-Jakob disease; nd, not done.
[b]Immunohistochemical analysis of postmortem brain tissue was consistent with GSS, and a GSS 102 mutation was confirmed in the family.
[c]Investigated as part of a cluster of three case-patients who participated in "wild game feasts" in a cabin owned by one of the decedents.
[d]Patient grew up in an area known to be endemic for CWD and ate venison harvested locally; however, the CJD phenotype fits the most common form of sporadic CJD.
[e]Patient may have been successful in harvesting two deer since 1996 from a CWD-endemic area, but both deer tested negative for CWD.

showed prion deposition consistent with GSS. A prion protein gene analysis could not be performed because appropriate samples were lacking. However, prion protein gene analysis of a blood sample from one of the patient's parents indicated a GSS P102L mutation. The patient did not hunt but may have eaten venison from Michigan once when he was 1–2 years old. The GSS diagnosis greatly reduced the likelihood that the two patients reported from adjacent counties had disease with a common origin.

Recently, rare neurologic disorders resulting in the deaths of three men who participated in "wild game feasts" in a cabin owned by one of the decedents created concern about the possible relationship of their illnesses with CWD (Table 2) (37). Two of the patients reportedly died of CJD, and the third died from Pick's disease. More than 50 persons were identified as possibly participating in these feasts; the three patients were the only participants reported to have died of a degenerative neurologic disorder. Reanalysis of autopsy brain tissues from the three patients at the National Prion Disease Pathology Surveillance Center indicated that two of them had no evidence of a prion disease by immunohistochemical analysis. CJD was confirmed in the third patient, who had clinicopathologic, codon 129, and prion characteristics similar to the most common sporadic CJD subtype (MM1/MV1) (35). This patient participated in the feasts only once, perhaps in the mid-1980s. In addition, the investigation found no evidence that the deer and elk meat served during the feasts originated from the known CWD-endemic areas of Colorado and Wyoming.

In 2003, CJD in two deer and elk hunters (54 and 66 years of age) was reported (38). The report implied that the patients had striking neuropathologic similarities and that their illness may represent a new entity in the spectrum of prion diseases. A third patient (63 years of age), who was also purported to have been a big game hunter, was subsequently reported from the same area. However, none of the three patients were reported to have eaten venison from the CWD-endemic areas of the western United States. The 66-year-old patient hunted most of his life in Washington State. Although information about the 54-year-old patient was limited, there was no evidence that he hunted in CWD-endemic areas. The third patient was not a hunter but ate venison harvested from Pennsylvania and Washington. The neuropathologic changes, Western blot profile, and genotype at codon 129 of the three patients each fit the MM1, VV1, or VV2 sporadic CJD subtype, indicating absence of phenotypic similarity among the cases or atypical neuropathologic features (35).

To date, only two nonfamilial CJD cases with a positive history of exposure to venison obtained from the known CWD-endemic areas have been reported. One of the patients was a 61-year-old woman who grew up in an area where this disease is known to be endemic, and she ate venison harvested locally. She died in 2000, and analysis of autopsy brain specimens confirmed that the patient's CJD phenotype fit the MM1 subtype, with no atypical neuropathologic features. The second patient was a 66-year-old man who was reported to have eaten venison from two deer harvested in a CWD-endemic area. Both deer tested negative for CWD, and the patient's illness was consistent with the MM1 CJD phenotype.

Despite the decades-long endemicity of CWD in Colorado and Wyoming, the incidence of CJD and the age distribution of CJD case-patients in these two states are similar to those seen in other parts of the United States.

From 1979 to 2000, 67 CJD cases from Colorado and 7 from Wyoming were reported to the national multiple cause-of-death database. The average annual age-adjusted CJD death rate was 1.2 per million persons in Colorado and 0.8 in Wyoming. The proportion of CJD patients who died before age 55 in Colorado (13.4%) was similar to that of the national (10.2%). The only CJD case-patient <30 years of age in Colorado had iatrogenic CJD linked to receipt of human growth hormone injections. CJD was not reported in persons <55 years of age in Wyoming during the 22-year surveillance period.

Laboratory Studies

The possible interspecies transmission of prions can be assessed with laboratory methods. In BSE and vCJD, several laboratory studies provided crucial evidence that helped establish a causal link between the two diseases (39–41). These studies characterized the molecular similarities of the agents causing BSE and vCJD and determined the lesion profile and incubation period patterns of different panels of mice inoculated by the two agents. Limited laboratory studies have been performed to molecularly characterize CWD-associated prions and to compare them with prions from human case-patients and other species. Strain typing studies involving wild-type inbred mice indicated that the CWD agent from a mule deer produced incubation-period and brain-lesion profiles different from those produced by the agents causing BSE and scrapie (39,42). These same strain-typing techniques had identified the similarities of the etiologic agents of BSE and vCJD, providing strong laboratory evidence for a link between the two diseases.

In human prion diseases, two major types of the proteinase-K–resistant prion protein fragment have been identified on the basis of their molecular size by one-dimensional immunoblot analysis: type 1 migrating at 21 kDa and type 2 at 19 kDa (35). N-terminal protein sequencing indicated that the cleavage site of the type 1 fragment is generally at residue 82 and that of type 2 is at residue 97 (43). Prion strain diversity is believed to be encoded in the three-dimensional conformation of the protein, which determines the cleavage site and molecular size of proteinase-K–treated prion fragment, indicating that the difference in molecular size may correlate with strain differences. However, one-dimensional immunoblot analysis may not identify more subtle differences that may influence the conformation of different prion strains. Analysis of the glycoform ratios of prion fragments and application of a two-dimensional immunoblot may help further identify these subtle differences. On one-dimensional immunoblot analysis, the prion fragment from several CWD-infected deer and elk migrated to 21 kDa, corresponding to the type 1 pattern. This specific type has been identified in most cases of sporadic CJD in the United States. However, the deer and elk prion fragment differs from that in sporadic CJD cases in the glycoform ratio. In the CWD-associated prion fragment, the diglycosylated form was predominant, but in the CJD-associated prions, the monoglycosylated form was predominant. Preliminary analysis using two-dimensional immunoblot indicated that the CWD-associated prion fragment exhibited patterns different from that of the CJD-associated prion fragment from a human patient with the type 1 pattern (S. Chen, pers. comm.). Although analysis of more samples from cervids and humans is needed before meaningful conclusions can be made, these molecular techniques could potentially be used to study the similarities or differences in prion strains from cervids and humans with possible exposure to CWD.

The likelihood of successful interspecies transmission of prion diseases is influenced by the degree of homology of the infecting prion with that of the host endogenous prion protein. Such observations have given rise to the concept of a "species barrier," which would need to be overcome before an infecting prion strain caused disease in a recipient host. In vitro cell-free conversion reaction experiments have been developed to assess the degree of molecular compatibility of disease-associated prions from one species with normal prion protein obtained from a different species (44,45). Such experiments specifically assess the likelihood that an infecting prion would potentially initiate the formation and propagation of pathogenic prions if it came in contact with normal prion protein. A cell-free conversion experiment indicated that CWD-associated prions can convert human prion protein into its abnormal conformer, albeit at a very low rate (44). The efficiency of this conversion was >14-fold weaker than the homologous conversion of cervid prion protein and >5-fold weaker than the homologous conversion induced by CJD-associated prions. A similar low efficiency conversion of human prion protein by bovine- and scrapie-associated prions was also reported (44,45). Although a high level of compatibility of prions in in vitro conversion reactions is believed to correlate with in vivo transmissibility of the agents, the threshold of compatibility efficiency below which no natural transmission should be anticipated is unknown. A low level of compatibility of infecting prions and host prion protein does not necessarily rule in or out natural interspecies transmission of prion diseases. However, the comparably low-level in vitro conversion of bovine prion protein by CWD-associated prions is consistent with the relative in vivo resistance of cattle to CWD under all but the most extreme experimental challenges. In addition, several other factors may determine the in vivo transmission of disease-associated prions, including dose, strain of the agent, route of infection, stability of the agent inside and outside the host, and the efficiency of agent delivery to the nervous system (44,46).

Conclusions

The lack of evidence of a link between CWD transmission and unusual cases of CJD, despite several epidemiologic investigations, and the absence of an increase in CJD incidence in Colorado and Wyoming suggest that the risk, if any, of transmission of CWD to humans is low. Although the in vitro studies indicating inefficient conversion of human prion protein by CWD-associated prions raise the possibility of low-level transmission of CWD to humans, no human cases of prion disease with strong evidence of a link with CWD have been identified. However, the transmission of BSE to humans and the resulting vCJD indicate that, provided sufficient exposure, the species barrier may not completely protect humans from animal prion diseases. Because CWD has occurred in a limited geographic area for decades, an adequate number of people may not have been exposed to the CWD agent to result in a clinically recognizable human disease. The level and frequency of human exposure to the CWD agent may increase with the spread of CWD in the United States. Because the number of studies seeking evidence for CWD transmission to humans is limited, more epidemiologic and laboratory studies should be conducted to monitor the possibility of such transmissions. Studies involving transgenic mice expressing human and cervid prion protein are in progress to further assess the potential for the CWD agent to cause human disease. Epidemiologic studies have also been initiated to identify human cases of prion disease among persons with an increased risk for exposure to potentially CWD-infected deer or elk meat (47). If such cases are identified, laboratory data showing similarities of the etiologic agent to that of the CWD agent would strengthen the conclusion for a causal link. Surveillance for human prion diseases, particularly in areas where CWD has been detected, remains important to effectively monitor the possible transmission of CWD to humans. Because of the long incubation period associated with prion diseases, convincing negative results from epidemiologic and experimental laboratory studies would likely require years of follow-up. In the meantime, to minimize the risk for exposure to the CWD agent, hunters should consult with their state wildlife agencies to identify areas where CWD occurs and continue to follow advice provided by public health and wildlife agencies. Hunters should avoid eating meat from deer and elk that look sick or test positive for CWD. They should wear gloves when field-dressing carcasses, bone-out the meat from the animal, and minimize handling of brain and spinal cord tissues. As a precaution, hunters should avoid eating deer and elk tissues known to harbor the CWD agent (e.g., brain, spinal cord, eyes, spleen, tonsils, lymph nodes) from areas where CWD has been identified.

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Dr. Belay is a medical epidemiologist in the Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention (CDC); he coordinates the CDC prion disease surveillance and research activities. His research areas of interest include the interspecies transmission of prion diseases, Kawasaki syndrome, and Reye syndrome.

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Address for correspondence: Ermias D. Belay, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop A39, Atlanta, GA 30333, USA; fax: 404-639-3838; email: ebelay@cdc.gov

Salmonella-based Rodenticides and Public Health

John A. Painter,* Kåre Mølbak,*† Jacob Sonne-Hansen,† Tim Barrett,* Joy G. Wells,* and Robert V. Tauxe*

Several countries still permit strains of *Salmonella enterica* serotype Enteritidis, a leading cause of gastrointestinal illness in humans, to be used in rat baits. To assess the human health risk associated with such rat bait, we first reviewed historic data on health hazards associated with Ratin, a rodenticide that was used in Europe until the early 1960s. Ratin caused outbreaks of human illness, including several deaths. We then compared *S.* Enteritidis isolated from a current commercial product, Biorat, with *S.* Enteritidis from Ratin and found that the strains were both phage type 6a. Based on the similarity of the strains, currently available *Salmonella*-based rodenticides likely are as great a threat to public health as past strains were. Health officials should be aware that the continued use of *Salmonella*-based rodenticides is a risk to public health and should take appropriate measures to prevent use in their jurisdictions.

Salmonella enterica serotypes Typhimurium and Enteritidis have been used as rodenticides since the late nineteenth century. This use was explored after *S.* Typhimurium was discovered during a lethal epizootic in a research mouse colony (1,2). Researchers soon realized that the strains of *S.* Typhimurium used as rodenticide were identical to strains causing "meat poisoning" and might cause disease among humans. Use of *S.* Typhimurium rodenticides was discontinued early in the twentieth century, but *S.* Enteritidis continued to be used as a rodenticide in the United Kingdom and Denmark until the early 1960s. In 1954 (3) and again in 1967 (4), the World Health Organization (WHO) recommended that *Salmonella*-based rodenticides not be used because they posed a hazard to human health.

In spite of these recommendations, *Salmonella*-based rodenticides are still produced and used in Central America, South America, and Asia (2,5). Biorat (Labiofam, Cuba), one *Salmonella*-based rodenticide that is currently used in several countries (6,7), is made by coating rice grains with a combination of *S.* Enteritidis and warfarin. Currently, the Biorat product label offers no warning regarding the risk for human salmonellosis. Indeed, product information indicates that this product contains a strain of *Salmonella* that is pathogenic to animals but not to humans (7).

In 1995, the Centers for Disease Control and Prevention (CDC) received a sample of Biorat that had been distributed in Nicaragua (2), and in July 2001, U.S. custom authorities seized a shipment of Biorat destined for distribution in the United States. These incidents prompted us to compare Biorat with Ratin, one of the major *Salmonella*-based rodenticides used before the early 1960s; in addition, we summarize the public health hazards of *Salmonella*-based rodenticides.

Microbiologic Findings

We compared three isolates of *S.* Enteritidis recovered from *Salmonella*-based rodenticides. Two isolates (of the Biorat strain) were from Biorat samples collected in 1995 and 2001. The label from the Biorat product obtained in 2001 states that Biorat contains 1.25% "monopathogenic" *Salmonella* and 0.02% hydroxycoumarin. Pooled samples of the 1995 Biorat product yielded $1x10^8$ CFU *S.* Enteritidis per gram of Biorat granules, and the 2001 product yielded 200,000 CFU per gram. A third isolate, *S.* Enteritidis var. Danysz (of the Ratin strain) was recovered from Ratin by the Danish Veterinary Laboratory in the 1920s or early 1930s.

Isolates were serotyped and biochemically characterized (8), subtyped by pulsed-field gel electrophoresis (PFGE) with the restriction enzymes *Xba*I and *Bln*I (9), and phage-typed. Phage-typing was performed at the National Laboratory for Enteric Pathogens, National Microbiology Laboratory, Canadian Science Centre for Human and Animal Health.

Both the Biorat strains and the Ratin strain were identified as *S.* Enteritidis, phage type (PT) 6a. The two Biorat strains were indistinguishable from each other by PFGE with the restriction enzymes *Xba*I and *Bln*I. PFGE patterns of the Ratin strain differed from those of the Biorat strain by three bands with *Xba*I and by five bands with *Bln*I (Figure 1).

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and †Statens Serum Institut, Copenhagen, Denmark

Neither the Biorat strains nor the Ratin strain was shown to decarboxylate lysine. By contrast, four *S.* Enteritidis PT 6a isolates in the CDC culture collection from sources unrelated to Biorat or Ratin were positive for lysine decarboxylase. Threlfall et al. reported that the strain of *S.* Enteritidis PT 6a from Biorat is indistinguishable from those of the Ratin and Liverpool rodenticide strains by plasmid profile typing (5), as both strains contained plasmids of approximately 59, 4.0, and 3.0 MDa.

Public Health Hazard

Since the mid 1980s, *S.* Enteritidis has caused a global pandemic of foodborne illness associated with eggs and poultry as a result of infection of the internal organs of chicken (10). Because of this pandemic, *S.* Enteritidis has become the most common serotype of *Salmonella* isolated from humans worldwide (11).

The *S.* Enteritidis strain found in Biorat is similar to the strain found in Ratin, a discontinued European product that caused human illness. Both strains were the same phage type, were indistinguishable by plasmid profile typing, and were different from 97% of salmonellae (8) in that they did not decarboxylate lysine. Although differences were noted between PFGE patterns of the Biorat strain and the Ratin strain, the similarities suggest that they may have originated from a common strain. Because the strains are similar and no evidence shows that the Biorat strain has decreased virulence, the Biorat strain is likely as pathogenic to humans as the Ratin strain.

In a retrospective study in Denmark from 1926 through 1956, Martin Kristensen identified 122 patients infected with the Ratin strain (12 and unpub. data), including 5 (4%) who died, 3 of whom were children. Twenty-two (18%) of 122 patients were reported to have eaten food items contaminated with Ratin, while 43 (35%) had handled the rodenticide. In 1956, Taylor (13) also reported several outbreaks of food poisoning associated with *S.* Enteritidis rodenticides and concluded that the use of bacterial rodenticides should be stopped.

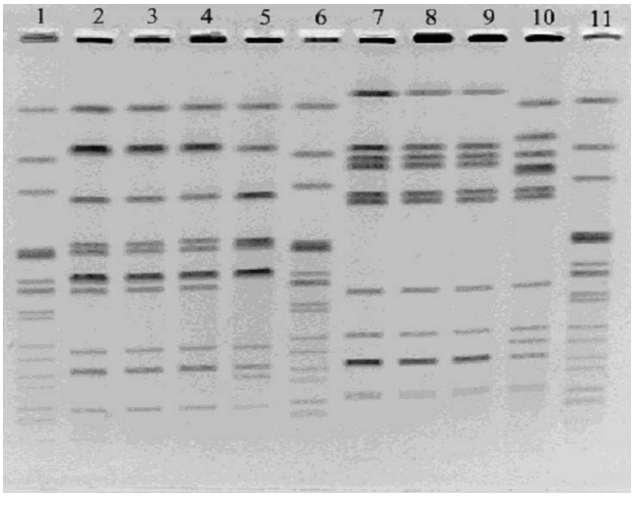

Figure 1. Pulsed-field gel electrophoresis of *Salmonella enterica* serotype Enteritidis isolates from Biorat and Ratin products using *Xba*I (lanes 2-5) and *Bln*I (lanes 7-10). Lanes 1, 6, and 11, molecular weight standard strain AM01144; lanes 2 and 7, Biorat isolate from 1998; lanes 3 and 8, Biorat isolate from 1995; lanes 4 and 9, Biorat isolate from 2001; lanes 5 and 10, Ratin isolate.

Rodenticides containing salmonellae were evaluated during a plague outbreak in San Francisco in 1895 (2); they were found to have no definable impact on the rodent population, but they caused illness and death in humans who prepared and handled them. In 1921, Willfuhr and Wendtland (14) reported several outbreaks of human *Salmonella* infections from rodenticides. In one of these outbreaks, Russian prisoners of war who ate a large number of Ratin potato baits became ill, and two died. In another outbreak in 1918, two persons died and approximately 35 became ill after eating a cake that had been intentionally contaminated with Ratin (14,15). From 1920 through 1940, other outbreaks associated with *Salmonella*-based rodenticides were reported, and several of these outbreaks included deaths (15–18).

The Biorat product insert, as well as information available on the Internet (6,7), claims that the product is not harmful to humans and does not contaminate the environment. Recent newspaper articles have generated interest in using *Salmonella*-based rodenticides as an alternative to chemical rodenticides. Advocates for the use of Biorat claim, "[Biorat] has absolutely no secondary effects on other animals, on the environment, or on humans…. It contains a strain of *Salmonella* that only affects rats" (19).

S. Enteritidis causes severe diarrheal illness, which can be life-threatening, especially among children, the elderly, and immunocompromised persons. We have not identified any peer-reviewed, scientific data on the safety of *Salmonella*-based rodenticides, and to our knowledge, all strains of *S.* Enteritidis are capable of causing human illness. Noting the hazards of *Salmonella*-based rodenticides, many countries have banned their use, and WHO has repeatedly recommended against use of salmonellae in rodenticides.

Current concerns about bioterrorism suggest an additional public health threat posed by a commercially available strain of *S.* Enteritidis. *Salmonella*-based rodenticides have already been used intentionally to cause human illness (14,15); however, human illness may more commonly be caused by inadvertent exposure to *Salmonella*-based rodenticides. These rodenticides are generally mixed with grains to form baits (Figure 2). Biorat, for example, is made with whole rice and can easily be mistaken for food. Ingesting a few grams of bait, with at least 200,000 CFU per gram, could easily cause a severe case of salmonellosis.

To determine why *Salmonella*-based rodenticides are

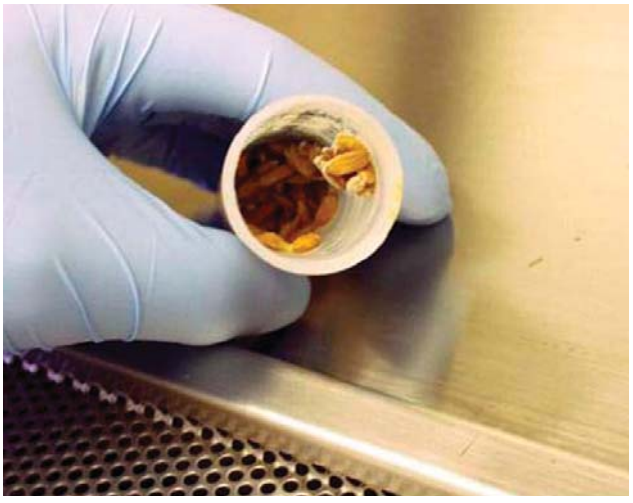

Figure 2. A sample of rodenticide that resembles grains of rice but contains pathogenic *Salmonella enterica* serotype Enteritidis.

still used despite information about the public health hazards, we conducted a literature search with the keywords "*Salmonella*" and "rodenticide" (National Library of Medicine, http://www.ncbi.nlm.nih.gov). We found 10 articles, in addition to the recent public health publications discussed above (2,5); none addressed the public health hazards of *Salmonella*-based rodenticides. Many of the reference materials we used to prepare the present article were not written in English or were not retrievable from current electronic databases. The continued use of *Salmonella*-based rodenticides may likely be related to the fact that the content of important but dated scientific papers is unlikely to be known to current decision-makers.

Salmonella-based rodenticides may contain an approved rodenticide, such as warfarin, in concentrations high enough to kill rats, and the addition of *S.* Enteritidis has not been shown to increase the effectiveness of the poison (2). Extensive use of *Salmonella*-based rodenticides in the past may have increased the prevalence of *Salmonella* in rodents (1) and consequently increased the potential for human salmonellosis by transmission from rodents to food or food animals. Unfortunately, a misperception exists that some strains of *S.* Enteritidis are not pathogenic to humans. We recommend informing rodent-control authorities and the public that *S.* Enteritidis is a known human pathogen and that use of *Salmonella*-based rodenticides has had severe public health consequences. Effective and safe alternatives to *Salmonella*-based rodenticides are available worldwide.

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Dr. Painter is a medical epidemiologist in the Foodborne Outbreak and Surveillance Unit of the Foodborne and Diarrheal Diseases Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention. His research interests include the prevention and control of bacterial enteric diseases.

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Address for correspondence: John Painter, Centers for Disease Control and Prevention, Foodborne and Diarrheal Diseases Branch, 1600 Clifton Road, Mailstop A38, Atlanta, GA 30333, USA; fax: 404-639-2205; email: jpainter@cdc.gov

First Report from the Asian Rotavirus Surveillance Network

Joseph Bresee,* Zhao-Yin Fang,† Bei Wang,‡ E.A.S. Nelson,§ John Tam,§ Yati Soenarto,¶ Siswanto Agus Wilopo,¶ Paul Kilgore,# Jung Soo Kim, Jung Oak Kang,†† Wong Swee Lan,‡‡ Chan Lee Gaik,§§ Kyaw Moe,¶¶ Kow-Tong Chen,## Chuleeporn Jiraphongsa,*** Yaowapa Pongsuwanna,*** Nguyen Van Man,††† Phan Van Tu,‡‡‡ Le Thi Luan,††† Erik Hummelman,* Jon R. Gentsch,* Roger Glass,* and the members of the Asian Rotavirus Surveillance Network[1]**

Rotavirus remains the most common cause of severe, dehydrating diarrhea among children worldwide. Several rotavirus vaccines are under development. Decisions about new vaccine introduction will require reliable data on disease impact. The Asian Rotavirus Surveillance Network, begun in 2000 to facilitate collection of these data, is a regional collaboration of 36 hospitals in nine countries or areas that conduct surveillance for rotavirus hospitalizations using a uniform World Health Organization protocol. We summarize the Network's organization and experience from August 2001 through July 2002. During this period, 45% of acute diarrheal hospitalizations among children 0–5 years were attributable to rotavirus, higher than previous estimates. Rotavirus was detected in all sites year-round. This network is a novel, regional approach to surveillance for vaccine-preventable diseases. Such a network should provide increased visibility and advocacy, enable more efficient data collection, facilitate training, and serve as the paradigm for rotavirus surveillance activities in other regions.

In recent years, several international agencies, including the World Health Organization (WHO), the Global Alliance for Vaccines and Immunization (GAVI), and the Children's Vaccine Program at the Program for Appropriate Technology in Health (PATH), have identified the accelerated development and introduction of a rotavirus vaccine to be among their highest priorities. This decision was made based on the high incidence of rotavirus, the most common cause of severe diarrhea in children worldwide. An estimated 440,000 children die of rotavirus each year (1), and in developing countries, 5% of all deaths in children <5 years of age are due to rotavirus. Furthermore, rotavirus is responsible for 25% to 50% of all hospitalizations of children for diarrhea in both industrialized and developing countries (2). After 2 decades of vaccine development and testing, the principles for making safe and effective, live oral vaccines have been firmly established, and several new candidate vaccines are currently in the late stage of development (3). Given the importance of rotavirus, GAVI has initiated the Accelerated Development and Introduction Program to expedite the development, evaluation, and introduction of rotavirus vaccines into the poorest countries with the goal of preventing most rotavirus deaths and hospitalizations within the next decade.

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China (PRC); ‡Southeast University, Nanjing, PRC; §Chinese University of Hong Kong, Hong Kong, Special Administrative Region (SAR), PRC; ¶Gadjah Mada University, Yogyakarta, Indonesia; #International Vaccine Institute, Seoul, Korea; **Chonbuk National University Medical School, Chonju, Korea; ††Hanyang University School of Medicine, Seoul, Korea; ‡‡Institute of Pediatrics, Kuala Lumpur, Malaysia; §§Kuching Hospital, Kuching, Malaysia; ¶¶Ministry of Health, Yangon, Myanmar; ##Department of Health, Taipei, Taiwan; ***Ministry of Public Health, Nonthaburi, Thailand; †††Poliomyelitis Vaccine Research and Production Center, Hanoi, Vietnam; and ‡‡‡Pasteur Institute, Ho Chi Minh City, Vietnam

[1]Asian Rotavirus Surveillance Network Members–China: Hua-Ping Xie, Jing Zhang, Du Zeng-Qing, Hui Shen, Tian Jian-Mei, Sun Li-Wei, Qing Zhang, Li-Shu Zheng, Hua-Ping Xie, Li-Wei Sun, Jing-Yu Tang, Zeng-Qing Du, Li Ma, Hui Shen, Hong-Ying Gu, Xiao-Bo Song, Zhi-Yi Xu; Hong Kong, SAR: Paul.K.S. Chan, Agnes C.K. Cheng, Tai-Fai Fok, Kin-Sing Ip, Chi-Hang Ng, Kin-Hung Poon, Tracey C.S. Tsen, Joyce S.W. Lee; Indonesia: Abu Tholib Aman, Mega, Rully, Bachryan Eljuta, Nenny Sri Mulyani; Malaysia: Rozita Rosli, Nur Atiqah Ng Abdullah, Shamzarina Mohamad; Korea: Park, Ha-Baik Lee, Nyambat Batmunkh, Jai Myung Yang, Jeong Kee Seo, Jae Sung Ko, Young J. Hur, Tae Hee Park, Moran Ki, Doosung Cheon; Taiwan: Ren-Bin Tang, Ping-Ing Lee, Po-Yen Chen, Yung-Feng Huang, Hour-Young Chen; Thailand: Utcharee Intusoma, Urai Poonawakul, Surasak Kowasupathr, Pipat Klueabwang, Pramot Arpornthip, Nakorn Premsri, Piyanit Tharmaphornpilas, Wanna Harnchaowarakun, Prayura Kunasol, Wandee Warawit, Ratigorn Guntapong; Vietnam: Nguyen Van Trang; Duong, Huong, Nhi; United States: Umesh Parashar, Julie Jacobson, Jon Gentsch; World Health Organization: Bernard Ivanoff, Duncan Steele.

Despite the global awareness about the prevalence of rotavirus, physicians and policymakers in most developing countries, where rotavirus causes the most fatalities and cases of severe disease and where new vaccines could have their greatest value, know little about rotavirus in their location. While these physicians and policymakers may appreciate that diarrhea is the first or second leading cause of death in children <5 years of age, the diagnosis of rotavirus is rarely, if ever, made at the local level. Moreover, acute diarrhea is treated with rehydration, regardless of the cause, so a diagnosis is not required, and no specific means of preventing rotavirus currently exists. Consequently, rotavirus is not often viewed as a priority and is incorrectly considered a disease that can be prevented by improvements in water and sanitation. If a vaccination program against rotavirus is to be successful, local leaders must understand the disease, which might include initiating surveillance of rotavirus, assessing the full incidence of rotavirus disease, and appreciating the impact that a new vaccine might have if added to the existing program of childhood immunizations.

In an effort to create regional awareness of the incidence of rotavirus disease and let countries assess the potential value of rotavirus vaccines currently being developed, we established the Asian Rotavirus Surveillance Network (ARSN), the first regional collaboration of its kind, as a pilot program for epidemiologic surveillance and advocacy. As members of this network, participating countries in the region conduct sentinel hospital surveillance to monitor rates of rotavirus among children hospitalized for diarrhea in several major hospitals by using a common protocol and comparable diagnostic test. The goal is to understand the epidemiology and impact of rotavirus diarrhea in the region, to educate physicians and health leaders about the importance of this problem, and let them assess the potential public health value of introducing vaccines. The same surveillance system could ultimately be used to monitor the impact of a vaccination effort.

Although many of the participating sites had begun surveillance up to 6 months earlier, we report preliminary data for the first year of the network, in which all but one country had completed a full year of surveillance, August 2001–July 2002. Data from Korea are not included because investigators in that country began surveillance in June 2002 and did not have a full year's data to report. We report data from >16,000 children hospitalized for diarrhea and tested for rotavirus in 33 hospitals in eight Asian countries or regions. Our results document the rates of rotavirus in the region, identify features in the epidemiology of the disease that might effect future immunization efforts, and underscore the value of regional surveillance networks to collect data with the same protocol and to test specimens with comparable diagnostic assays.

ARSN

ARSN is a collaboration of investigators from medical centers and public health agencies in nine Asian countries or regions. The goal of the group is to define the epidemiology and rates of rotavirus disease in Asia and to use these data to make informed decisions regarding the possible future use of rotavirus vaccines. ARSN was formed in 2000 in response to a WHO report that called for expedited rotavirus vaccine evaluation and introduction in Asia (4). WHO commissioned a generic protocol for sentinel hospital surveillance of rotavirus that would allow investigators in many countries to assess, in a simple, economical, and timely fashion, the epidemiology and disease prevalence of rotavirus (5). This protocol provides the minimum requirements for hospital-based surveillance, with attention to collecting and testing fecal samples, and includes an appendix to identify the catchment population for the hospital, suggestions to assess the prevalence of fatal rotavirus disease, and a discussion of the methods to characterize rotavirus strains.

Goals and Organizational Structure

The goals of ARSN are to use the generic protocol as a basis to: 1) define the epidemiology and strain distribution of rotavirus in participating countries, 2) estimate the costs associated with rotavirus and its prevalence in these settings, 3) create a surveillance system that can monitor the effectiveness of a vaccine program once introduced, 4) serve as a basis to conduct enhanced surveillance with special studies (e.g., cost-benefit or death rate studies) to inform decision makers considering a new vaccine program, and 5) describe trends in rotavirus activity and strain distribution over a large geographic region over time.

ARSN consists of 13 collaborating institutions in nine countries or regions that conduct hospital-based surveillance to monitor the rate of rotavirus infection among children hospitalized with diarrhea. The network is supported by five international donor groups, and coordination is provided by the Centers for Disease Control and Prevention (CDC) (Table 1). The lead groups in each country are responsible for establishing a hospital surveillance network, ensuring the timely collection of data, creating the laboratory capacity to test fecal specimens for rotavirus and to characterize strains identified, performing the analysis, and writing the report. A summary of all surveillance data is submitted electronically to CDC each month, and CDC prepares and distributes a quarterly report to all participants.

The basic structure for surveillance of children hospitalized for diarrhea is described in the Generic Protocol available from the WHO website (5). In brief, it provides that all children ≤59 months of age admitted to a participating hospital with physician-diagnosed acute diarrhea of

Table 1. Member institutions and participating hospitals of the Asian Rotavirus Surveillance Network

Sites	Lead institution(s)	Participating hospitals
China	Institute of Virology, Chinese Center for Disease Control, Ministry of Health, Beijing Southeast University, Nanjing International Vaccine Institute	Beijing Friendship Hospital Changchun Children's Hospital Lulong County Hospital Lulong Maternal and Child Health Hospital Kunming Hospital Ma-An-Shan Steel Trust Hospital Suzhou University-Affiliated Children's Hospital
Hong Kong	Chinese University of Hong Kong, New Territories	Prince of Wales Hospital Queen Elizabeth Hospital Tuen Mun Hospital Pamela Youde Nethersole Eastern Hospital
Indonesia	Gadjah Mada University, Yogyakarta	Dr. Sardjito Teaching Hospital, Yogyakarta Wirosaban District Hospital, Yogyakarta Purworejo Hospital, Purworejo
Korea	Chonbuk National University International Vaccine Institute, Seoul	ChungEub Asan Foundation Medical Center, Chonbuk National University Hospital Chonju Presbyterian Medical Center
Malaysia	Institute of Pediatrics, Kuala Lumpur University, Kuala Lumpur	Kuala Lumpur Hospital, Kuala Lumpur Kuching Hospital, Kuching, Sarawak
Myanmar	Department of Medical Research, Ministry of Health	Yangon Children's Hospital, Yangon
Taiwan	Taiwan Center for Disease Control, Taipei	National Taiwan University Hospital, Taipei Veteran General Hospital, Taipei Veteran General Hospital, Taichung Veteran General Hospital, Kaohsiung
Thailand	Ministry of Public Health, Bangkok	Nongkhai Hospital Maesod Hospital Prapokklao Hospital Ramathibodi Hospital Hadyai Hospital Srakaew Hospital
Vietnam	National Institute of Hygiene and Epidemiology, Hanoi POLIOVAC, Hanoi Pasteur Institute, Ho Chi Minh City	St. Paul's Hospital, Hanoi Swedish Children's Hospital, Hanoi Children's Hospital, Hai Phong General Hospital, Khan Hoa General Pediatric Hospital #1, Ho Chi Minh City General Pediatric Hospital #2, Ho Chi Minh City

<7 days' duration be surveyed. At admission, simple uniform data are collected from the medical records by using a standard questionnaire that includes date of admission, age and sex of the patient, symptoms of illness, and outcome. A fresh fecal specimen is obtained from each child, placed in a clean container, and stored at 4°C until tested for rotavirus with an enzyme immunoassay (EIA), either Rotaclone (Meridian Diagnostics, Inc., Cincinnati, OH) or DAKOPATTS (DAKO Diagnostics Ltd., Glostrup, Denmark), or by polyacrylamide gel electrophoresis (PAGE). Testing is conducted at the participating hospital laboratory or at the coordinating institution in the country. Each site tracks the proportion of total diarrheal admissions tested for rotavirus as an indicator of the sensitivity of the system to monitor disease. A subset of samples found to be positive for rotavirus is tested for G and P types by using reverse transcriptase–polymerase chain reaction (RT-PCR) according to published methods (6). Samples are selected for strain characterization on the basis of representativeness in terms of seasonal and age distribution and their quality. The generic protocol was pilot tested in Vietnam, and the encouraging results of this survey led to some simplifications and improvements in the study methods (7). Individual country proposals were reviewed and approved by ethical review boards in member institutions.

Progress during the First Year

Surveillance began in Hong Kong in December 2000, and by August 2002, 36 hospitals in 26 cities or towns in

nine Asian countries or regions were actively participating and routinely reporting their results (Table 1). Collaborators from Korea joined the network, with 3 hospitals reporting in June 2002. Of the 36 hospitals conducting surveillance, 19 (53%) are in large urban settings, 14 (39%) are in smaller cities, and 3 (8%) are rural hospitals. Seventeen (47%) are tertiary-care hospitals.

As of July 2002, seven of nine regions had collected 1 full year of data, and two collaborating countries (Korea and Myanmar) had collected data for <1 full year. From August 2001 through July 2002, a total of 11,498 stool samples were obtained from 16,173 patients, which represented more than 71% of all children admitted to participating hospitals with acute gastroenteritis (Table 2). Each month, participating hospitals collected fecal specimens from 679 to 1,556 children <5 years of age. Overall, rotavirus was detected in 45% (n = 5,124) of stools tested, with a range among hospitals of 18% to 67%. When only the sites that contributed 12 full months of data during this period were surveyed (to account for seasonal peaks of rotavirus disease), rotavirus was still detected in 44% of children tested. Rotavirus was detected year-round in all sites; however, wintertime peaks (November to March) were evident in the most northern areas (China, Taiwan, Hong Kong), while seasonal peaks were not as clearly defined in more southern sites, with tropical climates (Vietnam, Thailand, Malaysia) (Figure 1 and 2).

By mid-2002, five sites had begun strain characterization: China, Malaysia, Vietnam, Hong Kong, and Taiwan. CDC sponsored ARSN investigators from Indonesia, Taiwan, Vietnam, and Korea to train in Atlanta, and as a result, these sites have initiated genotyping of specimens from surveillance in these countries as of mid-2003. Finally, arrangements have been made for training a scientist from Myanmar in Hong Kong and will begin soon. CDC provided technical expertise, reagents, and protocols for some of the laboratory work and will provide assistance in resolving nontypeable strains.

Discussion

ARSN was organized to allow for the timely and economic collection of quality data on the effect of rotavirus infections in Asia, to collect these data efficiently, and to facilitate use of the data to inform decision makers about possibly introducing rotavirus vaccines. During the first year of the collaboration, ARSN produced valuable data that also yielded some surprises, and it has contributed to local and regional training, capacity building, advocacy, and development of the infrastructure for surveillance in member sites. Preliminary data from ARSN reaffirm that rotavirus remains a major cause of severe gastroenteritis among infants and young children. Moreover, rotavirus predominates in all sites, whether urban or rural, north or south, industrialized or developing, regardless of a country's gross national product. In addition, unlike rotavirus hospitalizations in the United States (8,9) and Europe (10), those in the ARSN settings occurred year-round, although seasonal peaks in disease were observed in the northernmost sites, areas with more temperate climates. These findings are similar to those found in the summary from the African data of Cunliffe et al. (2). The finding that rotavirus disease occurs with high frequency in settings with a variety of sanitation conditions reinforces the hypothesis that vaccines, not improved hygiene and water quality, are the best strategy to prevent this disease (3).

The preliminary finding of such high rates of rotavirus in sites throughout the region among children hospitalized for diarrhea confirms pilot studies in Vietnam that identified rotavirus in >50% of patients hospitalized with diarrhea (7). These high rates have implications for our global estimates of the prevalence of rotavirus disease. The most recent estimates of the global prevalence of rotavirus disease were determined on the basis of a literature review of studies conducted in the late 1980s and 1990s that estimated the percentage of rotavirus detected among children hospitalized for diarrhea (1). In this study, the authors estimated that from 20% (for low-income countries) to 34%

Table 2. Rates of rotavirus detection in Asian Rotavirus Surveillance Network sites, August 2001–July 2002

Sites	Start of surveillance	No. stool samples tested	% (no.) one rotavirus-positive sample	Range of % rotavirus-positive samples among participating hospitals
China	Aug 2001	2,079	44 (910)	24–65
Korea[a]	Jun 2002	N/A	N/A	N/A
Taiwan	Apr 2001	1,532	49 (744)	43–53
Hong Kong	Dec 2000	2,986	28 (829)	18–35
Vietnam	Feb 2001	1,570	59 (921)	47–67
Myanmar[b]	Dec 2001	388	53 (204)	53[c]
Thailand	Feb 2001	992	44 (436)	38–49
Malaysia	Feb 2001	1,374	57 (778)	52–59
Indonesia	Aug 2001	577	52 (302)	47–57
Overall		11,498	45 (5,124)	18–67

[a]Data from Korea absent due to start of surveillance in June 2002.
[b]Partial year of data.
[c]Single hospital in Myanmar participating in surveillance.

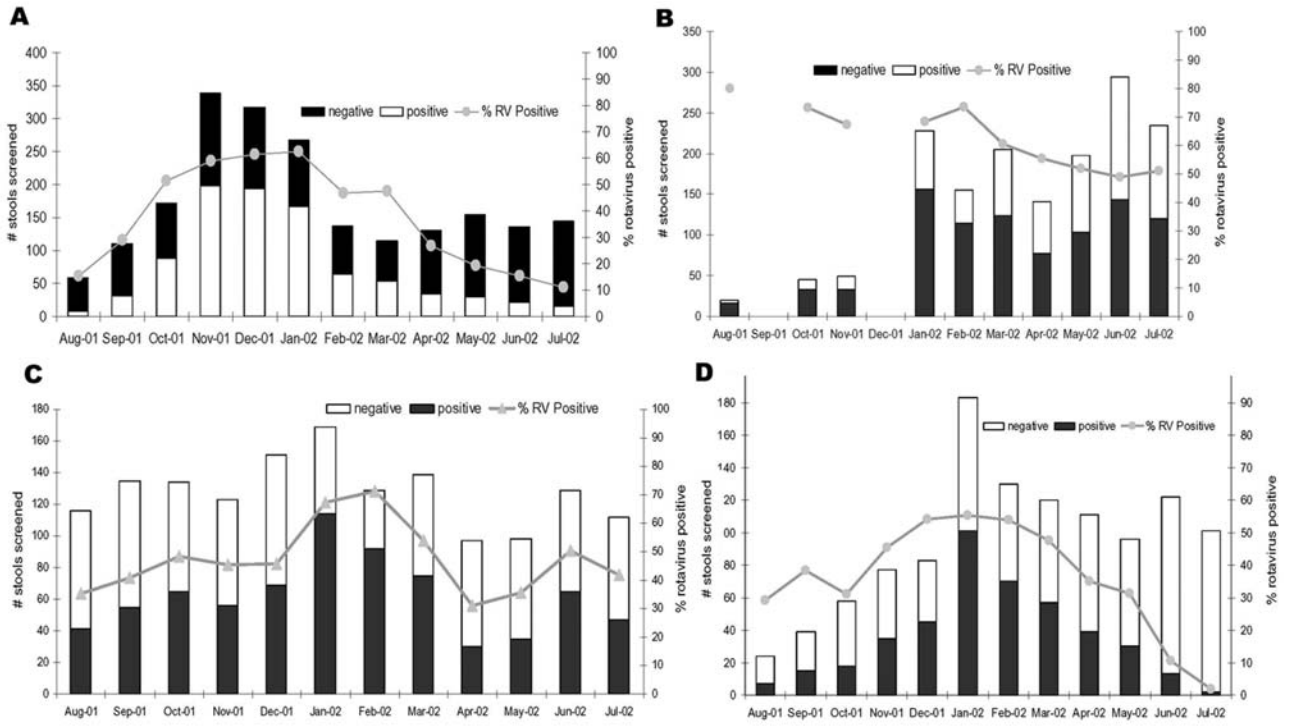

Figure 1. Seasonality of rotavirus (RV) in member countries of the Asian Rotavirus Surveillance Network. A, China; B, Vietnam; C, Taiwan; D, Thailand.

(for high-income countries) of hospitalizations for children >5 years were due to rotavirus infection. Data collected by the ARSN sites indicate higher rates of rotavirus illness in hospitalized children than used in previous models that have estimated rotavirus-associated disease and death globally. Indeed, the findings of the ARSN sites presented here are generally higher than those in previous studies in ARSN member countries that used similar methods (Table 3), but agree with recent data from studies in South America and Africa (31,32), and to recent rates from other investigators in Asia (33). One hypothesis for this difference is that improvements in sanitation and hygiene have reduced the number of diarrheal cases caused by bacteria and parasites, but less so the number due to rotavirus, because of differences in modes of transmission. As a result, the proportional fraction of diarrheal disease due to rotavirus rather than other causes increases as populations gain better access to clean water and sanitation. Among ARSN sites, rates of rotavirus detection in industrialized Hong Kong have changed little over the past decade, while countries with developing economies generally have found estimates higher than those from studies conducted in the 1970s and 1980s. Reports of bacterial enteric disease surveillance from some relatively high-income countries have demonstrated decreasing rates of disease (34–36); however, few data document trends of bacterial and parasitic enteric infections in industrialized countries. In addition, indirect support of the hypothesis comes from diarrheal death rates in Mexico during the 1990s (37) and the United States in the 1980s (38). In both settings, summer and winter peaks of diarrheal death rates have, over time, been replaced by single wintertime peaks. In Mexico, the decrease in summertime diarrheal death rates was associated with improvements in water supply. In both countries, wintertime rotavirus peaks in deaths have declined steadily over time but remain comparatively high.

An alternative explanation for the high detection rates could be the strict adherence to standard stool sample collection and handling procedures or the use of more sensitive tests compared to previous studies. Because rotaviruses are relatively stable in whole stool samples and because the studies chosen for comparison of current data all used comparable enzyme-linked immunosorbent assays, we think that the methods used by our network probably had little impact on detection rates. The principal advantage of the use of the standardized surveillance protocols remains the ease with which it allows for initiation of surveillance and enables collection of comparable data from very diverse settings. Finally, the reasons that data from Hong Kong generally reflected lower rates of disease than other sites remain unclear and deserve additional study. These preliminary data highlight the need to collect data in countries considering rotavirus vaccine introduction.

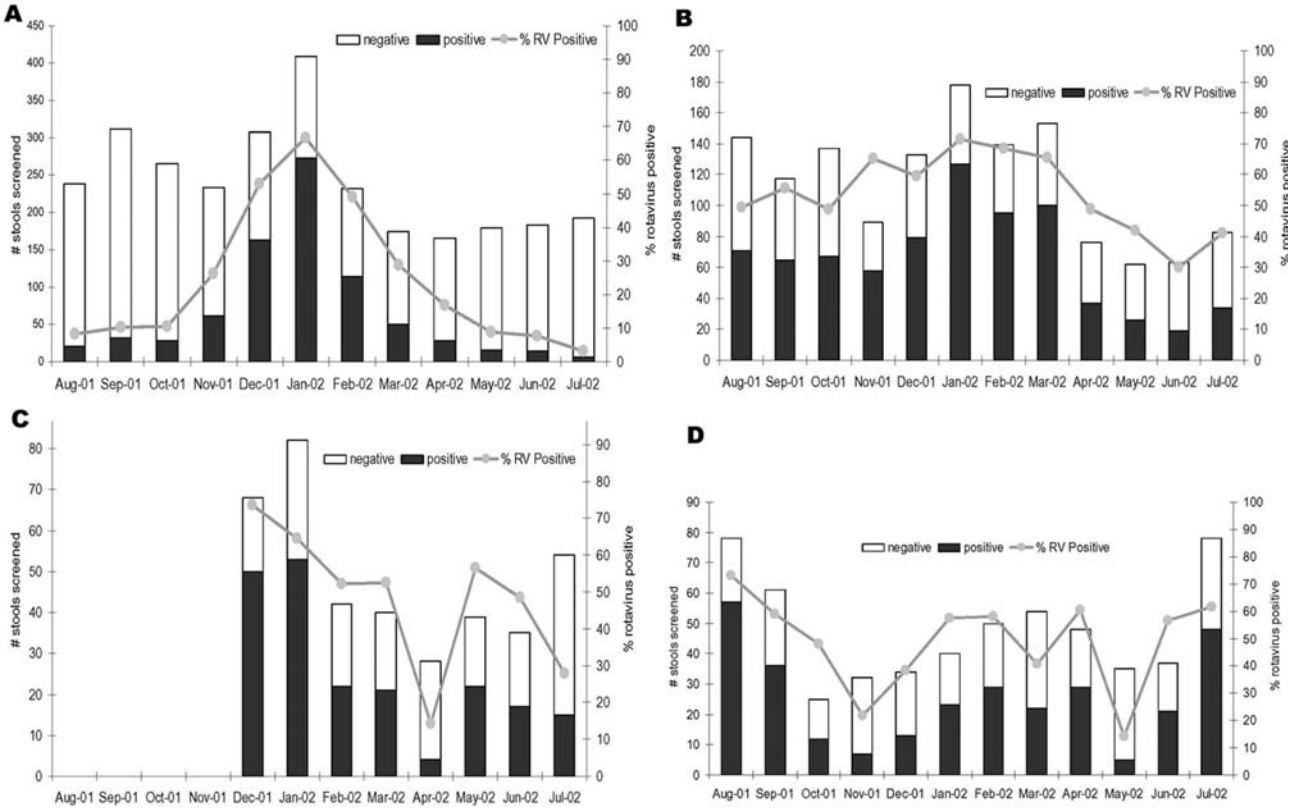

Figure 2. Seasonality of rotavirus (RV) in member countries of the Asian Rotavirus Surveillance Network. A, Hong Kong; B, Malaysia; C, Myanmar; D, Indonesia.

During this study, surveillance for rotavirus was initiated and sustained with ease, even in very large hospitals. The Generic Protocol for Rotavirus Disease Burden Estimation from WHO provides simple guidelines on organizing surveillance and interpreting the data. This protocol formed the basis of this network. Since diagnosis of rotavirus diarrhea is relatively easy compared with other vaccine-preventable diseases, ARSN was able to establish rotavirus testing by using EIA or PAGE at each site. Data collection was simplified by use of a one-page standard data-collection form contained in the Generic Protocol as a template for each site's form and by creation of a premade data entry form and analysis program in EpiInfo (CDC, Atlanta, GA) (available by request from the authors).

The use of regional networks to document rotavirus strain distribution within a region will add to the global understanding of prevalent strains and help in making informed decisions on vaccine composition. Although, strain characterization data from ARSN members during the 12 months of surveillance reported here were not yet complete, knowledge of circulating strains may also help guide local decisions on vaccine introduction and will be important in postlicensure assessment of vaccine effectiveness. Since the leading vaccine candidates employ different strategies, some monovalent human or animal strains and some polyvalent human-animal reassortants, conducting field trials of new vaccines in settings that include diverse, naturally occurring strains will be important. Surveillance networks, such as the ARSN, facilitate data collection for vaccine trials and act as a resource for laboratory scientists.

Regional, cooperative surveillance networks create training and infrastructure, building opportunities for members and creating a mechanism to introduce new technologies. While detecting rotavirus is easy and rapid, characterizing strains requires a higher level of technical skill. ARSN work has been supported by the WHO Collaborating Center for Rotavirus and Other Viral Agents of Gastroenteritis to conduct strain typing for sites without the capacity to do so and to facilitate training and quality control for laboratories interested in performing these tests. When ARSN was formed, four member sites (Thailand National Institute of Health, Chinese University of Hong Kong, National Institute of Hygiene and Epidemiology in Vietnam, and China's Institute of Viral Disease Control and Prevention) had performed rotavirus strain typing. By the end of the first year, strains had been characterized by using RT-PCR by eight of the sites, and

Table 3. Comparison of results of rotavirus detection in hospitalized children from current and past studies

Site	Ref.	Past studies Y of study	% rotavirus positive	ARSN results % rotavirus positive	Difference % increase (decrease)
Taiwan	(11)	1996	27	49	22
	(12)	1991	43		6
	(13)	1984	15		34
	(14)	2002	41		8
China	(15)	1996–99	26	44	18
	(16)	1995	41		3
	(17)	1983–84	13		31
Malaysia	(18)	1988–89	28	57	29
Thailand	(19)	1977–96	30	44	14
	(20)	1983–84	17		27
	(21)	1984–85	55		(11)
	(22)	1985–86	33		11
	(22)	1986–87	25		19
	(23)	1987–88	20		24
	(24)	1995–96	17		26
	Pongsuwanna	1991–94	38		6
Indonesia	(25)	1978–79	38	53	15
Myanmar	(17)	1982–83	22	53	31
Hong Kong	(26)	1994–95	35	28	(7)
	(27)	1984–90	34		(6)
	(28)	1983–84	29		(1)
	(29)	1987–96	26		2
Vietnam	(30)	1981–84	22	59	37

scientists from Myanmar were receiving training in this method. In addition, ARSN has provided a platform for professional development for epidemiologists and healthcare personnel involved in the study.

Future Directions

The final product from ARSN will be data that countries in the region can use to assess their need for a rotavirus vaccine and, where needed, to facilitate their introduction into immunization programs. The work of ARSN can serve as a paradigm of additional regional activities to promote the understanding and appreciation of the disease prevalence and costs associated with rotavirus in the region. Other regional rotavirus surveillance networks are also established in Africa and are being established in Latin America and will help accelerate decisions on vaccine introduction in those regions. The next step is to link these data to advocacy efforts. The value of any surveillance data is in its application or its ability to inform decisions. The new initiative of GAVI, the Accelerated Development and Introduction Program for Rotavirus Vaccines (available from: www.vaccinealliance.org), will serve as a pathway to transform data from networks like ARSN into action.

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Dr. Bresee is a medical officer in the Respiratory and Enteric Virus Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention. His primary research and public health interests are in viral enteric and respiratory diseases, diarrheal diseases, and rotavirus vaccines.

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Address for correspondence: Joseph S. Bresee, Centers for Disease Control and Prevention, Mailstop G04, 1600 Clifton Rd. NE, Atlanta, GA 30333, USA; fax: 404-639 3645; email: jbresee@cdc.gov

Airborne Infection with *Bacillus anthracis*—from Mills to Mail

Kevin P. Fennelly,* Amy L. Davidow,* Shelly L. Miller,† Nancy Connell,* and Jerrold J. Ellner*

The lack of identified exposures in 2 of the 11 cases of bioterrorism-related inhalation anthrax in 2001 raised uncertainty about the infectious dose and transmission of *Bacillus anthracis*. We used the Wells-Riley mathematical model of airborne infection to estimate 1) the exposure concentrations in postal facilities where cases of inhalation anthrax occurred and 2) the risk for infection in various hypothetical scenarios of exposure to *B. anthracis* aerosolized from contaminated mail in residential settings. These models suggest that a small number of cases of inhalation anthrax can be expected when large numbers of persons are exposed to low concentrations of *B. anthracis*. The risk for inhalation anthrax is determined not only by bacillary virulence factors but also by infectious aerosol production and removal rates and by host factors.

The intentional release of *Bacillus anthracis* spores through the mail in the United States in the fall of 2001 was associated with inhalation anthrax in 11 persons, 5 (45%) of whom died (1,2). Seven cases were associated with occupational exposures in the postal service, and two case-patients had documented exposures to contaminated mail in the business office of a media company. No sources of exposure were identified for two women who were presumably exposed to secondarily contaminated mail.

B. anthracis was previously known to have potential as a weapon on battlefields or for large-scale outdoor dissemination. Its delivery through the mail moved the risk indoors. We sought to improve our knowledge of indoor transmission of *B. anthracis* by applying principles and methods used in studies of tuberculosis. Although room ventilation and other environmental factors are known to be important in the airborne transmission of *Mycobacterium tuberculosis*, little attention has been given to these factors in the transmission of *B. anthracis*. We used a mathematical model of airborne infection to theoretically assess risk for infection with *B. anthracis* from indoor exposures; to demonstrate the relative importance of pathogen, environmental, and host factors in transmission; and to estimate a range of infectious doses on the basis of these models and the epidemiology of the 2001 anthrax cases.

*New Jersey Medical School–UMDNJ, Newark, New Jersey, USA; and †University of Colorado, Boulder, Colorado, USA

Methods

We reviewed the literature in English on inhalation anthrax by searching MEDLINE electronically and by obtaining articles cited in references in these and older articles. We focused on occupational cases and outbreaks and on experimental aerosol exposures of nonhuman primates that included environmental sampling data. We used the Wells-Riley modification of the Soper and Reed-Frost models of infection as our mathematical model of airborne infection (3–5). This equation can be arranged so that the probability of airborne infection is given by $P\ (infect) = C/S=1-exp(-Iqtp/Q)$, where C is the number of cases among S persons susceptible to the infection; I is the number of sources of infection; q is the number of "quanta," or units of bacilli necessary to cause infection produced per source per unit of time; t is the time of exposure per unit of time; p is the minute ventilation rate of the exposed susceptible hosts in volume per unit of time; and Q is the volumetric rate of fresh air ventilation that removes the infectious aerosol in volume per unit of time. Units used were cubic feet and minutes. Biologically, a quantum may be one or more spores in one or more airborne particles, analogous to a colony-forming unit on a culture plate. Statistically, a quantum is the amount of infectious material needed to produce infection in 63% of uniformly exposed animals, or 1.25 times the median infectious dose, i.e., $q = 1.25 \times ID_{50}$ (5). We modeled transmission of infection from contaminated mail in two general environments: 1) the workplace of mail distribution centers and 2) home or office exposures.

Using published attack rates of inhalation anthrax of 0.25% (2/750) and 0.66% (4/610) in two U.S. Postal Service (USPS) processing and distribution centers in New Jersey (6) and Washington, D.C. (7), respectively, we estimated the quanta produced for a range of available workplace ventilation rates by using the Wells-Riley equation above and solving for q. Data on the size and ventilation of

the processing and distribution center workrooms were obtained from a USPS ventilation engineering contractor (C. Hong, pers. comm.). For the exposures in the USPS postal distribution centers, we assumed an exposure time of 8 hours to represent a work shift and a pulmonary ventilation rate of 14.6 L/min (0.5156 ft^3/min), as might be expected with moderate work. This rate is comparable to the rate used to estimate inhaled doses in a study of an anthrax outbreak that occurred in a goat hair–processing mill in Manchester, New Hampshire, in 1957 (8).

We also estimated the risks for airborne infection associated with hypothetical exposures to *B. anthracis* that might have occurred during the handling and opening of contaminated mail in a home or office. For the first of these modeling scenarios, we used room dimensions of 12 ft x 18 ft x 8 ft (3.7 m x 5.5 m x 2.4 m), or a volume of 2,160 ft^3 (61 m^3), which is a reasonable size for an office or room in a home and allows for comparison to modeling data on occupational tuberculosis. We then modeled the risks for exposure in a smaller room of 56 ft^2 x 8 ft high (432 ft^3, 12.2 m^3) and for exposure as a result of dispersion throughout a median-sized U.S. house, which has a volume of 10,947.5 ft^3 (310 m^3) (9).

We modeled room ventilation rates of 0.5 and 2 air changes per hour that correspond to 18 ft^3 and 72 ft^3 (0.5 m^3 and 2.0 m^3) per minute. This rate approximates a current standard for fresh air ventilation of 20 ft^3/min (0.57 m^3/min) per occupant for office space and 15 ft^3/min (0.42 m^3/min) per person for residential living areas, if one assumes that only one person occupied the room (10). We assumed duration of exposure of 1 hour to simulate an exposure occurring with the opening of contaminated mail with gradual dispersion of the aerosol. We included pulmonary ventilation rates of 4 L/min, 6 L/min, and 10 L/min for exposed persons to span the range of minute-ventilation reported in the literature for persons at rest (11–13).

Because our review of the literature did not provide a conclusive infectious dose for humans, we followed the approach first suggested by Wells (4,5) of using units of quanta to represent the unit required to establish infection, as has been used in studies of tuberculosis and measles (4,14). Since the infectious dose of inhaled virulent anthrax spores cannot ethically be determined experimentally in humans, we estimated the 50% infectious dose (ID_{50}) based on data from the 8-hour inhaled dose measured in Manchester in 1957. The inhalation dose for workers in the area where four of the five cases of inhalation anthrax had occurred was estimated to be 140–690 spores <5 μm in size (and 620–2,200 total spores), based on air sampling data (8). Sixteen presumably susceptible workers were in this area (15), so this estimate may be considered an ID_{25}, since 25% of the exposed persons were infected. On the basis of studies of 1,236 monkeys, Glassman estimated the median lethal dose to be 4,130 spores, and using the probit model he suggested that the LD_{25} was associated with a 10-fold decrease in dose (16). When this principle is applied, a 10-fold increase of the ID_{25} for the Manchester mill outbreak would result in an ID_{50} of 1,400 to 6,900 spores <5 μm, or 6,200–22,000 total spores (8). This number is similar to Glassman's estimate and to the lower range of the LD_{50}, 2,500–55,000, accepted by an expert panel (17). Since $q = 1.25 \times ID_{50}$, then one quanta under these assumptions is 1,750–8,625 spores <5 μm (7,750–27,500 total spores).

The number of persons exposed to *B. anthracis* spores through the mail in the fall of 2001 is unknown, so we arbitrarily expressed the probability for infection as the number infected per 10,000 population. Estimates of the numbers of persons infected can be calculated by using these estimates. For example, if approximately 5,000 persons were exposed to contaminated letters as might be suggested by the modeling data of Webb and Blaser, these risks can be divided by two. All data were entered, stored, and analyzed by using JMP-SAS software (version 4.0.4, SAS Institute, Cary, NC.)

Results

Inhalation anthrax developed in two of the 750 persons working in the USPS processing and distribution centers in Trenton, New Jersey, for an overall attack rate of 0.25% (6). The workroom is 151,200 ft^2, with a height of 15 ft, for a total volume of 2,268,000 ft^3 (C. Hong, pers. comm.). The area is ventilated with 5.29 air changes per hour, with a minimum of 11.8% and a maximum of 100% of fresh air. Therefore, the nonrecirculated air-ventilation rate varies from a minimum of 23,596 ft^3/min to a maximum of 199,962 ft^3/min. Solving for q results in an exposure of 0.238 to 2.08 infectious quanta per minute, or 114 to 998 infectious quanta generated per 8-hour shift. If one assumes that one quantum is 1,750–8,625 spores <5 μm (7,750–27,500 total spores), then the 8-hour cumulative production of infectious aerosol is estimated to have ranged from 199,500 to 8,607,750 spores <5 μm (883,500–27,445,000 total spores).

Similar calculations were performed for the processing and distribution center in Washington, D.C., where inhalation anthrax developed in 4 of 610 workers (attack rate 0.66%). The workroom is 4,605,000 ft^3 (C. Hong, pers. comm.) and is ventilated at 2.88 air changes per hour, with a minimum of 16.2% outside air. The calculated ventilation rate is 35,809–221,040 ft^3/min, resulting in a range of q of 0.96–6.0 infectious units per minute, or 461–2,880 quanta per 8-hour shift. When the same assumptions of the number of bacilli per quantum are used, the 8-hour cumulative production of infectious

aerosol is 806,750–24,840,000 spores <5 μm (3,572,750–79,200,000 total spores).

The risks for airborne infection to susceptible occupants in our hypothetical scenarios of exposure in a home or office are summarized in the Table and plotted in the Figure. The risks for infection associated with exposure to one quantum in these models is 6–936 per 10,000 persons. If the exposure concentration decreases by one log, i.e., to 0.1 quantum, the risk decreases to 0.6–98 per 10,000 persons. At exposure concentrations ≥0.1 quantum, the risks are ≥6 cases per 10,000 and increase to very large magnitudes.

If 1 quantum is 1,750–8,625 spores <5 μm, then inhaled doses corresponding to lower exposures can be calculated: 0.1 quantum would be 175–863 bacilli, 0.01 quantum would be 18–86 bacilli, and 0.001 quantum would be 2–9 bacilli. Exposures estimates <0.001 quantum are biologically meaningless under these assumptions since fractions of spores are not viable.

Changes in room size and ventilation rate affect risk more than changes in pulmonary ventilation in these scenarios. The relative risk reduction associated with an increase of room ventilation from 0.5 to 2 air changes per hour is highest for low-concentration exposures and decreases with increasing concentration of the aerosol exposure. For example, in the scenario of a high-concentration exposure of 100 quanta and the exposed person's breathing 10 L/min in the moderate-size room with 0.5 air changes per hour, improving ventilation to 2.0 air changes per hour decreases the risk by 55%. However, if the same person is exposed to 0.1 quantum in the same room with the same changes in ventilation, the risk decreases by 75%. As expected, for a given exposure concentration and pulmonary ventilation rate, the lowest risk is in the large, well-ventilated space and the highest risk is in the small, poorly ventilated room. For example, if exposure is 1 quantum of infection to a person breathing 6 L/min, the risk in the small, poorly ventilated room is 58.9 per 10,000 and in the large, well-ventilated space, the risk is 0.6 per 10,000.

The Figure illustrates that, although the production of infectious aerosol and the duration of exposure were higher for postal workers in the postal distribution center facilities, the greater rate of removal of the infectious aerosol by dilution ventilation in such facilities results in a lower risk for infection for a given aerosol production rate (q) than in the home exposure scenarios.

Discussion

These analyses emphasize that the risks for airborne infection are determined not only by the virulence of the agent but also by environmental factors, e.g., room size and ventilation rates, and host factors, e.g., pulmonary ventilation rate. Little can be done to change one's pulmonary ventilation rate, but these data suggest that risks for inhalation anthrax are likely to decrease considerably with fairly modest increases in room ventilation for low-concentration exposures. These data might be helpful in educating both the general public and policymakers regarding strategies to reduce risks from aerosolized bioweapons.

General dilution ventilation reduces the risk of transmitting airborne pathogens; however, as described for *M. tuberculosis*, room ventilation is theoretically limited in decreasing this risk, especially when the exposure concentration is high (14). UV germicidal irradiation has been suggested as a potential bioterrorism countermeasure (18). Although we are not aware of data regarding the use of irradiation to inactivate *B. anthracis*, Peng and colleagues found that UV germicidal irradiation of upper-room air reduced *B. subtilis* spore concentrations by 46% to 80%, depending on the ventilation rate (19). Thus, further research on the use of UV germicidal irradiation and other interventions seems warranted.

These modeling data suggest that approximately 0.7–63.4 million total airborne spores and approximately 0.2–20 million spores <5 μm may have been produced by contaminated mail during work shifts at the two USPS processing and distribution centers where inhalation anthrax developed in postal workers. These data are plausible, given the contamination with *B. anthracis* documented by environmental sampling of both the New Jersey and the Washington, D.C., facilities (6,20). Although the results of

Table. Risk for airborne infection with *Bacillus anthracis* modeled for various scenarios of exposure to secondarily contaminated mail for 1 hour in a home or office, expressed as number of cases per 10,000 susceptible persons exposed[a]

	Small room 0.5 ACH	Moderate-size room 0.5 ACH			Moderate-size room 2 ACH			House 2 ACH
Quanta	PV 10 L/min	PV 10 L/min	PV 6 L/min	PV 4 L/min	PV 10 L/min	PV 6 L/min	PV 4 L/min	PV 4 L/min
0.001	1.0	0.2	0.1	0.1	0.05	0.03	0.02	0.006
0.01	9.8	2.0	1.2	0.8	0.5	0.3	0.2	0.1
0.1	97.8	19.6	11.8	7.9	4.9	3.0	2.0	0.6
1	936	195	117	78	49	30	20	6
10	6,256	1,784	1,113	756	479	291	195	61
100	10,000	8,599	6,928	5,443	3,881	2,555	1,784	596
1,000	10,000	10,000	9,999	9,996	9,926	9,477	8,598	4,588

[a]ACH, air changes per hour; PV, pulmonary ventilation.

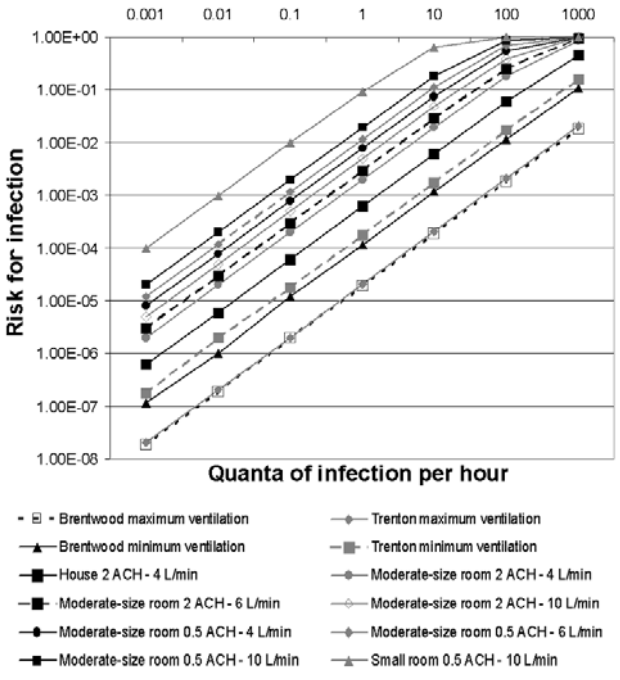

Figure. Risk for airborne infection with *Bacillus anthracis* in various scenarios. Home and office exposures are for 1 hour, and postal facility exposures are for 8 hours; for postal facilities, the models assume a 14.6 L/min pulmonary ventilation rate with moderate work, comparable to the rate used to estimate inhaled doses in the Manchester study. ACH, air changes per hour.

air sampling were negative, dust samples grew 3–9.7 million CFU/g (20). A contaminated letter in Washington, D.C., purportedly contained 2 g powder with 100 billion to 1 trillion spores per gram (21), which suggests that letters can be a source of infectious material.

These data also suggest that a few cases of inhalation anthrax can be expected if large numbers of people are exposed to a virulent strain at low concentrations within homes or offices. We will probably never know how many persons were exposed to mail contaminated with *B. anthracis* in the fall of 2001. If 10,000 persons were exposed in a home or office to secondarily contaminated mail, and two persons were infected, these models suggest that exposure was probably to 0.1 or 0.01 quantum, or 18–863 airborne spores. Above and below that range of exposures, the number estimated to be infected is very large or very small. Even if 1,000 persons were exposed, this range of exposures appears most reasonable, except for exposures to 1 quantum in larger or well-ventilated environments.

Glassman suggested that 100 spores may be sufficient to cause infection (16), and a more recent analysis suggested that as few as 1–3 spores may be sufficient to cause infection (22). Our data are consistent with these conclusions, as 0.001 quantum would be 2 to 9 bacilli in our model.

We assumed in our analyses that the strain used in the mailings was at least as virulent as the strain reported in the outbreak of inhalation anthrax at the Manchester mill (15). Genomic analysis recently identified the isolate used in the mailings as the Ames strain, which is used in multiple research laboratories (23). However, the strain was prepared for aerosolization in powder form, and we are not aware of published data on infectious doses for powdered preparations. The method of preparing bacilli for aerosolization may affect retention of aerosolized agents and their virulence of *B. anthracis*. For example, the surface-active compound Tergitol was found to increase virulence 10-fold in guinea pigs (24).

The two women with no identified exposures in the recent bioterrorism-related outbreak may have been unusually susceptible to inhalation anthrax. Risk factors for increased susceptibility or resistance to inhalation anthrax are not known, although age has been suggested (25). One of these women was 61 years old (2), and the other was 94 years old (1). Aging is associated with a decrease in mucociliary clearance as well as alterations in immune responses, but given the large numbers of elderly persons who were likely exposed to contaminated mail, age alone seems inadequate to explain the epidemiology of this outbreak. We are reluctant to accept that children are less susceptible than elderly adults without additional data. Previous reports of cases associated with industrial sources or with materials contaminated by *B. anthracis* suggest that some susceptible persons, including children, were infected during relatively brief exposures (26–28). We speculate that the combination of thin body type, age, and female sex might be a risk factor for inhalation anthrax, as it is for pulmonary infections with environmental nontuberculous mycobacteria, e.g., with *M. avium* complex (29). Women with asymptomatic bronchial hyperresponsiveness have increased deposition of inhaled particles of ≈1 μm (30), but we are not aware of data that show this to be a risk factor for respiratory infections.

Modeling studies of transmission of tuberculosis or measles have usually assumed a single uniform pulmonary ventilation rate in exposed persons as well as a homogenous concentration of infectious aerosol over time and space. Riley's choice of 10 L/min to model human ventilation has been used by other modelers (14), but we have added a broader range of values to assess the relative importance of variability in minute ventilation. However, human ventilation is not uniform, being punctuated by sighs in which the tidal volume may be three times greater than the volume at rest (12). If a piece of contaminated mail were opened, aerosol was likely concentrated immediately afterwards and in the immediate vicinity. Thus, if a person were to sigh when exposed to the maximum number of airborne spores, spores would likely deposit in

greater concentration. However, we are not aware of data documenting deposition associated with short-term irregularities in ventilation. This is another area for further research.

Druett first applied the probit model in early studies of inhalation anthrax in animal models (31). He acknowledged that this model best fit the data within the range of the LD_{25}–LD_{75} but cautioned about interpreting data outside that range. Our concern with estimating the number of bacilli required to kill roughly 0.1%–1.0% of exposed persons (10–100/10,000) was one of the reasons we chose not to apply the Druett model. We selected the Wells-Riley model because of a number of clear and compelling traits. First, data generated by the Wells-Riley model agree with observed data in airborne infection with measles and tuberculosis (4,5). Neither the Druett model nor other models of airborne infection with more specified variables and more complex mathematical forms (32,33) have been validated against epidemiologic data of any airborne infection. Second, the Wells-Riley model includes variables for environmental conditions and aerosol production rates not accounted for in the Druett model. Alternative models might allow for a sigmoidal growth in the probability for infection, i.e., the probability of infection increases monotonically; however, the rate of change first increases to a maximum and then decreases steadily to zero. While such a model is biologically plausible, experimental data to justify such a model are lacking. No models have been validated for inhalation anthrax in humans, which differs markedly from pulmonary tuberculosis and measles in its pathogenesis.

One possible approach to extending the Wells-Riley model in the future would be adding a susceptibility factor, y. With the current model formulation, as q increases, airborne infection approaches 100% certainty, i.e., the probability approaches 1. Increases in the other parameters (I, t, p and 1/Q) have a similar effect on the probability for airborne infection. An alternative model might be given by $C/S = y[1-\exp(-Iqtp/Q)]$ where $0 < y < 1$. With such a model, as any of the parameters I, q, t, p, or 1/Q increase, the probability of airborne infection approaches 1. The factor y may be conceived as a fixed or random effect, or one that is conditional on measurable, individual-level susceptibility factors such as age or coexisting conditions. Additional data on susceptibility are needed, however, before such a parameter can be included with confidence.

Modeling depends on the assumptions used; the most critical of our assumptions is that all affected persons were exposed to similar quanta of *B. anthracis* from contaminated mail. More likely, the number of quanta decreased with "postal distance" from the index letters. Webb and Blaser allowed for this possibility by modeling the cross-contamination of letters resulting from contact with index letters passing through a prototypical postal system (25). Their model, however, did not allow for the environmental considerations that can mediate between aerosolization and infection. The Wells-Riley model allows for environmental considerations, and it can be adapted to variable exposure scenarios by stratifying by persons sharing a common quantum of exposure. To do that would require further modeling assumptions in the fashion of those made by Webb and Blaser. Epidemiologic data will likely never become available to test the validity of any of these models, but this modeling exercise demonstrates how the risk for infection is sensitive not only to the infectious dose but also to environmental parameters.

Conclusion

The risk for airborne infection with *B. anthracis* is determined not only by the virulence of the organism but also by the balance between infectious aerosol production and removal, pulmonary ventilation rate, duration of exposure, and host susceptibility factors. Dilution ventilation of the indoor environment is an important determinant of the risk for infection. Enhanced room ventilation, UV germicidal irradiation, and other engineering control measures may be used to decrease the risk for infection. Although much research focuses on bacillary factors to improve our understanding of the pathogenesis of inhalation anthrax, our modeling data emphasize the need to better understand the complex interactions among host susceptibility factors, environmental factors, transmission mechanisms, and dose-response relationships in determining the risk of airborne infection with *B. anthracis* and other agents of bioterrorism.

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Dr. Fennelly is assistant professor of medicine in the Center for Emerging Pathogens and the Division of Pulmonary and Critical Care Medicine at the New Jersey Medical School in the University of Medicine and Dentistry of New Jersey. His research interests include airborne transmission of *Mycobacterium tuberculosis* and host determinants of infectiousness in tuberculosis.

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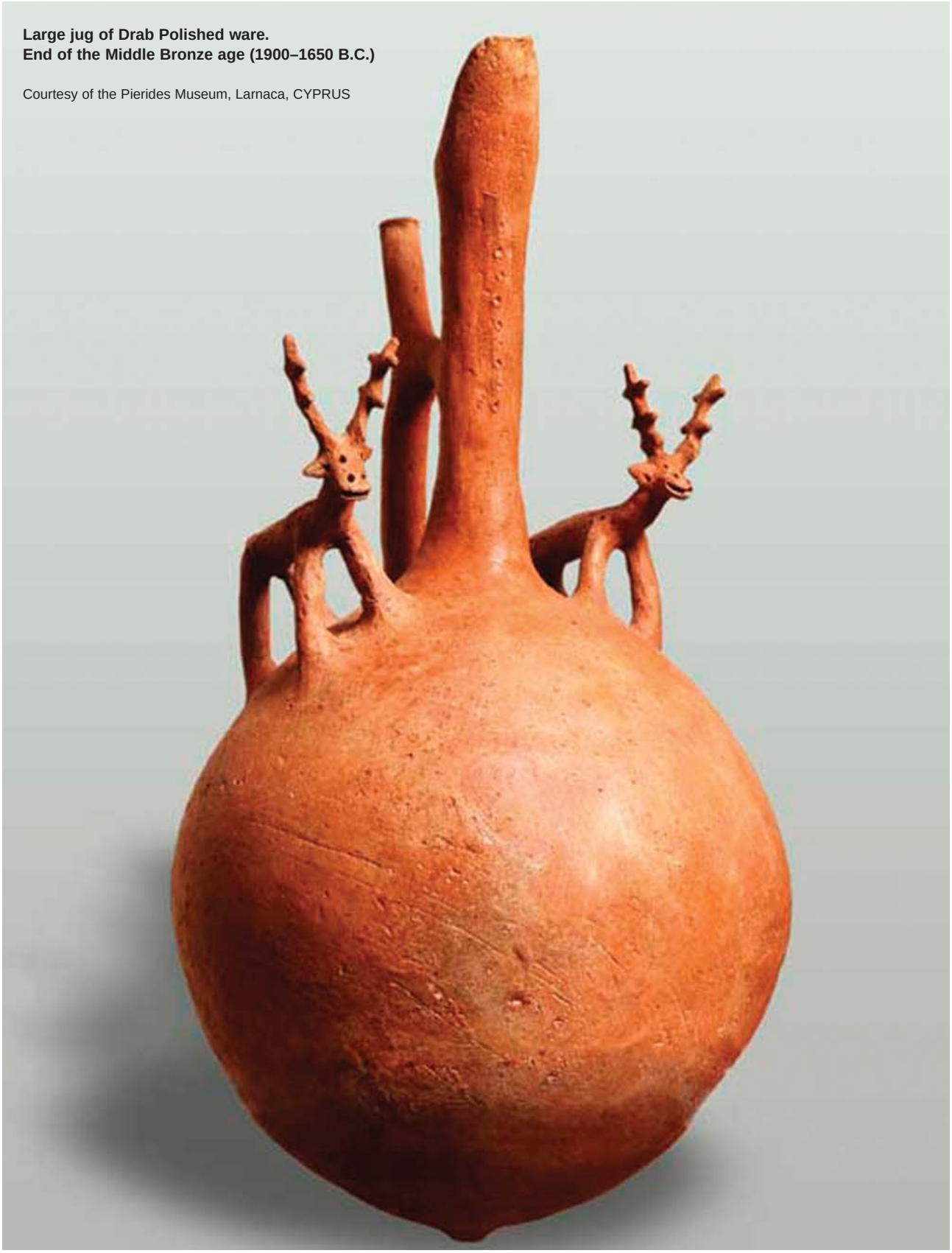
Address for correspondence: Kevin P. Fennelly, Center for the Study of Emerging and Re-Emerging Pathogens, Department of Medicine, New Jersey Medical School–UMDNJ, 185 South Orange Ave., MSB A-901, Newark, NJ 07107-3000, USA; fax: 973-972-8716; email: fennelkp@umdnj.edu

Large jug of Drab Polished ware.
End of the Middle Bronze age (1900–1650 B.C.)

Courtesy of the Pierides Museum, Larnaca, CYPRUS



Ovoid body, rounded base with a mastoid projection, long cylindrical neck, beak-shaped mouth, handle from neck to shoulder with vertical cylindrical projection at the top. Two stags in the round on the shoulder on either side of the handle. Impressed decoration on the animals' bodies, vertical band in relief along the neck opposite handle. (48 cm height).

Environmental Sources of Prion Transmission in Mule Deer

Michael W. Miller,* Elizabeth S. Williams,† N. Thompson Hobbs,‡ and Lisa L. Wolfe*

Whether transmission of the chronic wasting disease (CWD) prion among cervids requires direct interaction with infected animals has been unclear. We report that CWD can be transmitted to susceptible animals indirectly, from environments contaminated by excreta or decomposed carcasses. Under experimental conditions, mule deer (*Odocoileus hemionus*) became infected in two of three paddocks containing naturally infected deer, in two of three paddocks where infected deer carcasses had decomposed in situ ≈1.8 years earlier, and in one of three paddocks where infected deer had last resided 2.2 years earlier. Indirect transmission and environmental persistence of infectious prions will complicate efforts to control CWD and perhaps other animal prion diseases.

Controlling and possibly eradicating animal prion diseases (1) are goals shared by the international community (2,3). However, progress toward eliminating prion diseases from food-producing animals worldwide has been hampered by incomplete knowledge about transmission and environmental persistence of these novel proteinaceous pathogens. Two prion diseases, scrapie of sheep and goats (4–8) and chronic wasting disease (CWD) of deer (*Odocoileus* spp.) and elk (*Cervus elaphus nelsoni*) (9–14), are particularly difficult to control because both are contagious among susceptible hosts. In contrast, bovine spongiform encephalopathy (BSE) does not appear to be contagious in cattle, but epidemics are sustained artificially through exposure to feed contaminated with infected bovine tissues (15); whether BSE in sheep is contagious remains undetermined (16). Both infected animals and environments apparently contaminated with the causative agent contribute to scrapie epidemics (4,6,8), and under some conditions, scrapie agents may persist in contaminated environments for years (7). Similarly, CWD is transmitted in the presence of infected mule deer (*O. hemionus*) (10), and circumstantial evidence exists for transmission from environments contaminated with the CWD agent (9,11,14). CWD epidemics do not appear to have been perpetuated by exposure to contaminated feed, but because ingestion of brain tissue can transmit CWD experimentally to deer (11,17), decomposed carcasses could serve as sources of infection in the environment.

Environmental sources of CWD infection represent potential obstacles to control in natural and captive settings. To investigate their role in transmission of this disease, we compared three potential sources of infection: infected live deer, decomposed infected deer carcasses, and an environment contaminated with residual excreta from infected deer.

Materials and Methods

We conducted a replicated experiment to compare CWD transmission from three infection sources: naturally infected captive mule deer (one infected deer/paddock), carcasses from naturally infected captive mule deer that had decomposed in situ ≈1.8 years earlier (one carcass/paddock), or undisturbed paddock environments where infected mule deer had last resided 2.2 years earlier. Each exposure source was replicated in three separate paddocks; two clean paddocks served as unexposed controls. Control paddocks and paddocks where live infected deer were added or where carcasses decomposed were constructed specifically for this experiment; these paddocks had never housed captive deer or elk and had been closed to access by free-ranging cervids for ≈17 years. Because clinical courses varied in naturally infected deer that served as sources of direct exposure, actual exposure periods varied from 0.75 year (replicate 3) to 1 year (replicate 1). Excreta-contaminated paddocks previously held 19 mule deer that had been orally inoculated during a 2-year pathogenesis study (11) that ended 2.2 years before our study began (≈3.8 infected deer x years of excreta/paddock, assuming equal distribution) but that had not held deer or elk in the interim. All three carcasses were from mule deer euthanized in end-stage clinical CWD. They had been left to decompose in intact form except for the removal of small pieces of brainstem used to confirm CWD infection; only the skeletal remains of carcasses were present at the start of the study.

*Colorado Division of Wildlife, Fort Collins, Colorado, USA; †University of Wyoming, Laramie, Wyoming, USA; and ‡Colorado State University, Fort Collins, Colorado, USA

Experimental animals included 31 free-ranging mule deer from two donor populations distant to endemic CWD foci. Experimental animals were captured from the grounds of the Rocky Mountain Arsenal National Wildlife Refuge (n = 17) and the U.S. Air Force Academy (n = 14), Colorado. We assumed that all experimental animals were free from CWD when they were introduced into the experiment, and surveillance data provided evidence that deer obtained from these herds were uninfected before exposure. Surveillance for CWD in the source populations (10,18) showed 0 positive cases in a sample of 210 adult deer from the refuge and 0 positive cases in a sample of 65 adult deer from the academy.

We used these data to estimate the probability that infection could have been caused by transmission from animals from the source herds. To do so, we estimated one-sided, exact 99% binomial confidence intervals (BCI) on the proportion of each population that could be positive for CWD (refuge = 0–0.022, academy = 0–0.068). We then used the upper limit of this interval to estimate the maximum prevalence, $\hat{p}_{max}$, that could be reasonably expected in each of the source populations, given the inability to detect infections through surveillance. To assess whether observed results were likely due to preexisting infections, we treated each replicate (i.e., paddock) as an independent binomial experiment because the conditions in one paddock had no opportunity to influence the events in another paddock. Thus, for each replicate where infection occurred, we calculated the probability of at least one positive (i.e., "success") given the number of animals introduced to that replicate from the source population (i.e., "trials"), on the assumption that the probability of drawing a positive from the source population was $\hat{p}_{max}$. When two replicates within an exposure category showed infections, we estimated the probability that cases in both replicates resulted from introducing infected animals (and not from our experiment) as the product of the individual replicate probabilities.

We captured deer during March and May 2002 and transported them to the Colorado Division of Wildlife's Foothills Wildlife Research Facility, where they were confined in outdoor paddocks of ≈800 m^2 (three replicate paddocks/exposure route, three deer/paddock); four deer were held in the two clean paddocks as unexposed controls. Each replicate of exposure paddocks was initially stocked with three mule deer. Shortly after arrival, one deer was moved to a different paddock within the same exposure condition to resolve social strife, and four fawns were born into three other paddocks; these changes are reflected in denominators in the Table. The distribution of prion protein genotype at codon 225 (serine [S]/phenylalanine [F] [19]) did not differ (Fisher exact test p = 0.6) among the four groups (three exposure groups + control).

Deer were fed alfalfa hay and a pelleted supplement; diets contained no animal protein or other animal byproducts. Individual paddocks and exposure blocks were physically segregated to prevent cross-transmission within and among exposure categories; dedicated clothing and equipment were used to minimize potential cross-contamination, but other potential fomites, like small mammals, birds, and insects, could not be controlled. However, transmission by routes such as these would be consistent with hypothesized transmission from environmental sources rather than direct animal-to-animal contact. After the animals had undergone ≈1 year of exposure to respective sources of infection, we obtained biopsied tonsil specimens from each participant deer and conducted an immunohistochemical analysis using anti-PrP MAb 99/97.6.1 (20,21). Upon detecting ≥1 infected deer in a paddock, we removed all inhabitants of that paddock and confirmed CWD infection in animals with positive biopsy results (20). Study protocols were reviewed and approved by the Colorado Division of Wildlife Animal Care and Use Committee

Results

Mule deer exposed to contaminated environments or to infected deer contracted CWD (Table). None of the unexposed deer were infected. One or more introduced deer became infected in two of three paddocks containing a naturally infected deer, in two of three paddocks containing a decomposed deer carcass, and in one of three paddocks contaminated with residual deer excreta (Table) within 1 year of exposure. Infected deer included unrelated animals from both donor herds (2/17, 3/14; Fisher exact test p = 0.64), as well as one of four fawns born during the study.

Table. Chronic wasting disease arising in mule deer exposed to environments contaminated by residual excreta, carcasses, or other infected deer

	Exposure source			
Replicate	Infected deer	Infected carcass	Residual excreta	Unexposed
1	1/4[a]	0/3	1/3	0/2
2	0/2	2/4	0/3	0/2
3	1/4	1/5	0/3	NA[b]
Total	2/10	3/12	1/9	0/4

[a]Number positive/number exposed (not including infected source deer).
[b]Not applicable; controls included only two replicate paddocks.

Males (4/16) and females (2/15) were infected at equivalent rates (Fisher exact test p = 0.65); similarly, deer of all three codon 225 genotypes (SS = 6/26, SF = 0/7, FF = 0/2) were infected at equivalent rates (Fisher exact test p = 0.52). Deer with positive biopsy results appeared healthy and did not show signs of CWD, consistent with early (<1 year in duration) infections (11,17).

On the basis of prior data from surveillance of source populations, our results were not likely explained by the null hypothesis of infections introduced from the source populations ($p = 0.036$ for academy source deer and $p < 0.0001$ for refuge source deer). The probability of prior infection accounting for our results in the pattern observed (Table) was $p \leq 0.0013$ for the infected animal exposure, $p \leq 0.037$ for the carcass exposure, $p \leq 0.064$ for the excreta exposure, and overall $p \approx 0.000003$ for the observed results arising from preexisting infections. Because these probabilities were based on one-sided, upper 99% BCIs, we can conservatively reject the null hypothesis of infection arising from the source populations. The only remaining possibility is that infections arose from experimental exposures that included environments harboring the infectious agent from excreta or decomposed carcasses.

Discussion

Prions cannot be directly demonstrated in excreta or soil. However, CWD infection–specific protease-resistant prion protein (PrP^{CWD}) accumulates in gut-associated lymphoid tissues (e.g., tonsils, Peyer patches, and mesenteric lymph nodes) of infected mule deer (11,17,22), which implicates alimentary shedding of the CWD agent in both feces and saliva (10,11,17). Because PrP^{CWD} becomes progressively abundant in nervous system and lymphoid tissues through the disease course (11), carcasses of deer succumbing to CWD also likely harbor considerable infectivity and thus serve as foci of infection. We could not determine the precise mechanism for CWD transmission in excreta-contaminated paddocks, but foraging and soil consumption seemed most plausible. Deer did not actively consume decomposed carcass remains, but they did forage in the immediate vicinity of carcass sites where a likely nutrient flush (23) produced lush vegetation (Figure).

Our findings show that environmental sources of infectivity may contribute to CWD epidemics and illustrate the potential complexity of such epidemics in natural populations. The relative importance of different routes of infection from the environment cannot be discerned from our experiment, but each could play a role in sustaining natural epidemics. Although confinement likely exaggerated transmission probabilities, conditions simulated by this experiment do arise in the wild. Mule deer live in established home ranges and show strong fidelity to historic home ranges (24–26). As a result of such behavior, encounters with contaminated environments will occur more frequently than if deer movements were random. Feces and carcass remains are routinely encountered on native ranges, thus representing natural opportunities for exposure. Social behavior of deer, particularly their tendency to concentrate and become sedentary on their winter range, also may increase the probability of coming into contact with sources of infection in their environment.

Figure. Green forage growing at the site where a deer carcass infected with chronic wasting disease had decomposed. Such sites were attractive to deer, as illustrated by the grass blades recently cropped by deer in the experiment.

The ability of the CWD agent to persist in contaminated environments for ≥2 years may further increase the probability of transmission and protract epidemic dynamics (8). Because infectivity in contaminated paddocks could not be measured, neither the initial levels nor degradation rate of the CWD agent in the environment was estimable. However, the observed persistence of the CWD agent was comparable to that of the scrapie agent, which persisted in paddocks for ≈1 to 3 years after removal of naturally infected sheep (7). Similarities between the CWD and scrapie agents suggest that environmental persistence may be a common trait of prions. Whether persistence of the BSE prion in contaminated feed production facilities or in environments where cattle reside contributed to BSE cases in the United Kingdom after feed bans were enacted (27) remains uncertain but merits further consideration.

Indirect transmission and environmental persistence of prions will complicate efforts to control CWD and perhaps other animal prion diseases. Historically, control strategies for animal prion diseases have focused on infected live animals as the primary source of infection. Although live deer and elk represent the most plausible mechanism for geographic spread of CWD, our data show that environmental sources could contribute to maintaining and prolonging local epidemics, even when all infected animals

are eliminated. Moreover, the efficacy of various culling strategies as control measures depends in part on the rates at which the CWD agent is added to and lost from the environment. Consequently, these dynamics and their implications for disease management need to be more completely understood.

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Dr. Miller is a wildlife veterinarian with the Wildlife Research Center, Colorado Division of Wildlife. His research has focused on the ecology and management of infectious diseases in free-ranging wildlife.

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Address for correspondence: Michael W. Miller, Colorado Division of Wildlife, Wildlife Research Center, 317 West Prospect Road, Fort Collins, CO 80526-2097, USA; fax: 970-472-4457; email: mike.miller@state.co.us

Sporadic Cryptosporidiosis, North Cumbria, England, 1996–2000

Stella Goh,* Mark Reacher,† David P. Casemore,‡ Neville Q. Verlander,† Rachel Chalmers,§ Margaret Knowles,¶ Joy Williams,* Keith Osborn,# and Sarah Richards**

Risk factors for sporadic cryptosporidiosis were determined in 152 patients and 466 unmatched controls who resided in two local government districts in North Cumbria, North West England, from March 1, 1996, to February 29, 2000. Risk was associated with the usual daily volume of cold unboiled tap water drunk (odds ratio [OR] 1.40, 95% confidence intervals [CI] 1.14 to 1.71 per pint consumed per day [p = 0.001]) and short visits to farms (OR 2.02, 95% CI 1.04 to 3.90, p = 0.04). Fifty-six (84%) of 67 fecal specimens from patients obtained from January 1, 1998, and February 29, 2000, were *Cryptosporidium parvum* genotype 2 (animal and human strain). Livestock fecal pollution of water sources appears to be the leading cause of human sporadic cryptosporidiosis in this population and shows the need for better protection of water catchments from livestock and improved drinking water treatment in this area of England.

The protozoan parasite *Cryptosporidium parvum* is a leading cause of infectious diarrhea in humans and livestock with fecal-oral transmission by ingestion of oocysts (1,2). Infection is generally self-limiting, followed by variable protective immunity involving humoral and cell-mediated responses, except in the immune-suppressed, when infection may be prolonged and fatal (2,3). *Cryptosporidium* oocysts remain viable in water and damp soils for prolonged periods and are resistant to disinfectants at concentrations usually used in water treatment (4,5). Although sound, conventional water treatment is believed to substantially reduce the risk of viable oocysts passing into treated water, the possibility of low-level intermittent contamination has been recognized; whether such contamination affects public health is uncertain (4,6). Outbreak investigations have shown diverse modes of transmission, including contact with livestock (7,8); person-to-person transmission in households and care settings (9); consumption of contaminated foods and drinks, including milk (1,10); water from private supplies (11); and recreational water exposure (12). Infection may also be associated with travel to countries with higher incidence of cryptosporidiosis (13).

In 1992, a community outbreak of cryptosporidiosis occurred in residents of Allerdale and Copeland local government districts in North Cumbria, North West England, which compose part of the Lake District National Park; these areas have a predominantly agricultural and tourism-based economy and a population of approximately 160,000. The lakes have livestock farms and open grazing land abutting them. Approximately one third of the population received public water supplies from Ennerdale Lake, one third from Crummock Lake, and one third from a number of smaller sources. Water from Ennerdale and Crummock Lakes was disinfected with chlorine but unfiltered because the low level of particulate matter in these sources precluded chemically assisted flocculation. The smaller sources used for public supplies received a variety of conventional treatments, including coagulation, filtration, and chlorination, and chlorination alone. A small number of households had private supplies. A case-control study conducted during the outbreak showed a significant association between cryptosporidiosis and consuming cold unboiled mains tap water for persons served by water from Ennerdale Lake but no such association for those served by water from Crummock Lake and other water sources (North Cumbria Health Authority, Carlisle, 1992, unpub. data). After the outbreak, rates of laboratory-confirmed cryptosporidiosis from 1993 to 1995 were 31.2–44.2 per 100,000 in Allerdale and Copeland compared to 19.8 to 23.9 per 100,000 in the neighboring local government districts of Carlisle and Eden. Cases were not obviously clustered in time and could not be linked. A prospective case-control study was therefore undertaken to test the hypothesis that no dose-response relationship existed between consuming unboiled tap water from public water supplies and risk for sporadic *Cryptosporidium* infection in Allerdale and Copeland.

*Carlisle and District Primary Care Trust, Rosehill, Carlisle, United Kingdom; †Communicable Disease Surveillance Centre, London, United Kingdom; ‡University of Wales, Aberystwyth, Ceredigion, Wales, United Kingdom; §Singleton Hospital, Swansea, Wales, United Kingdom; ¶Cumberland Infirmary, Carlisle, United Kingdom; #United Utilities, Great Sankey, Warrington, United Kingdom; and **West Cumberland Hospital, Whitehaven, United Kingdom

Medical and diagnostic microbiology services for Allerdale and Copeland residents were provided free at the point of use by the United Kingdom National Health Service and managed by North Cumbria Health Authority (14). The Authority was also responsible for maintaining the computer patient register and updating it for births, deaths, migration, and surveillance and control of infectious disease (14). The register held persons' name, sex, date of birth, home address, and postal (zip) code but not medical information. The postal codes were geographically referenced but did not share boundaries with public water supply distribution zones, government boundaries, or census enumeration districts.

Methods

Local ethical committees approved the protocol, and a study center was set up at North Cumbria Health Authority. All fecal specimens were examined for *Cryptosporidium* oocysts, regardless of whether this test was requested by the clinician. Family physicians and hospital clinicians were informed of the study, but not the main hypothesis, and were reminded to ensure best practice in investigating cases of diarrhea with the assistance of the three local microbiology laboratories. Laboratory staff was requested to immediately report *Cryptosporidium*-positive fecal smear results to the study coordinator by telephone.

Epidemiology

Five workers were trained to conduct the study in a standard manner by using written protocols and questionnaires. Both methods were pilot tested and their techniques refined before enrollment (15).

Definitions

Case-patients were defined as residents of Allerdale or Copeland with 1) diarrhea (three or more loose stools in a 24-hour period), 2) onset from March 1, 1996, to February 29, 2000, and 3) a fecal smear positive for *Cryptosporidium* oocysts but negative for other enteric pathogens. Patients were excluded if they had, within 14 days of onset of illness, contact with another household member with cryptosporidiosis or any diarrhea illness, traveled outside the United Kingdom, stayed away from home outside the study area within the United Kingdom for >7 nights, or if they or a household member had already been enrolled as a patient or control at any time during the study.

Controls were defined as residents of Allerdale or Copeland with no history of diarrhea (three or more loose stools in a 24-hour period) in the 14 days before interview. Potential controls were excluded if they had, within 14 days of interview, traveled outside the United Kingdom, stayed away from home outside the study area but within the United Kingdom for >7 nights, or if they or a household member had already been enrolled as a patient or control at any time during the study.

Three controls were randomly selected, by using a computer algorithm, from subtables of the health authority patient register with the same age span (0–5, 6–15, and 16+ years) and the same lead characters of the postal code as the case-patient. The process was repeated when necessary to replace persons who declined, could not be contacted, or met exclusion criteria. The patient register was compared with population estimates from the 2001 census.

Interviews

Interviews were conducted face-to-face at home. Participants <18 years of age were interviewed with parents or guardians, who acted as proxies for younger children, as appropriate. Informed consent was obtained and recorded. Case-patients and associated controls were enrolled as close to each other in time as possible.

For unboiled cold tap water, bottled water, and soft drinks, we asked about "usual" consumption without a time restriction. For all remaining exposures, including consuming pasteurized and unpasteurized milk, ice, and foods, exposure was sought for the 2 weeks before onset of illness for patients and before date of interview for controls.

The volume of different fluids drunk by study participants was determined by showing a standard picture card of a glass, cup, and mug; the volume was recorded according to the calibration on the card. The usual daily volume of water as cold unboiled tap water drunk at home was determined as water alone and as a diluent in cold fruit-squash type drinks. The usual daily volume of cold unboiled tap water drunk at work, school, or nursery at locations within Copeland and Allerdale District boundaries was determined the same way. The two usual daily volumes consumed at and away from home were added to give the total usual daily volume of cold unboiled tap water consumed within Copeland and Allerdale Districts. The usual daily volume consumed of bottled drinking water and soft drinks not diluted with water was determined separately without distinction between consuming it at home or away from home. Questions about consumption of ice in cold drinks were asked separately for ice made at home and ice consumed at work, school, or nursery.

The following types of contact were recorded: farms, farmed animals, and handling and feeding farm animals; slurry; household pets and feeding household pets; children's nurseries; and recreational exposure to water in swimming pools, rivers, and streams. Information about consumption and frequency of consumption of uncooked salad items, uncooked meat, uncooked sausage meat and sausages, yogurt, cheese, and cream was also elicited.

The nature of the water supply (public or private) and sewage services (public or septic tank) to the home were recorded and corroborated against water company records. Disruption to tap water or change in tap water color or taste in the week before onset of illness for patients, and in the week before date of interview for controls, was recorded. Information on sources, water treatment works, and the water that supplied each address's postal code was obtained from water company records and linked to individual patient and control records.

Knowledge about *Cryptosporidium* Infection

A series of television and newspaper articles on *Cryptosporidium* occurred in North Cumbria in April and May 1999. Study participants enrolled from April 28 to November 30, 1999, were also asked if they had heard of *Cryptosporidium* and what they knew about it.

Data Entry and Analysis

Data were double entered and differences edited and corrected in EpiInfo version 6.0.4.d. (Centers for Disease Control and Prevention, Atlanta, GA). Fluid intakes were analyzed as yes/no responses; within categories (<1/4 pint, 1/4–1 pint, >1–2 pints, and >2 pints) with χ^2 tests for trend in single variable analysis; and as actual volume consumed in multivariable analysis (16).

Variables positively associated with infection at the $p \leq 0.2$ level in the single variable analysis, age, sex, and water supply zone were included in the initial multivariable model. Backward stepwise logistic regression was undertaken by comparing nested models using Likelihood Ratio Tests and GLIM software (16,17). Variables with significance $p \geq 0.3$ in iterations of the multivariable model were removed in stepwise fashion except for age, sex, and water supply zones, which were retained in all models regardless of their significance. Subsidiary analyses modeled usual daily consumption of tap water at home and usual total daily tap water consumption; cases and controls served by mixes of water from more than one source were omitted.

Microscopy of stained fecal smears for *Cryptosporidium* oocysts (18), *Giardia*, and culture for pathogenic enteric bacteria were undertaken by using standard methods at each of the three local microbiology laboratories. Smears in which *Cryptosporidium* oocysts were identified from January 1998 to February 2000 were also analyzed by polymerase chain reaction and restriction fragment length polymorphism typing of a region of the *Cryptosporidium* oocyst wall protein gene (19,20).

Results

None of the incident cases arising from the study population during the 4 years of the study were linked or clustered in time and space. All were considered sporadic infections eligible for inclusion in the study. No changes occurred in livestock farming, livestock densities, water sources, or water treatment within the study area during the study period.

Potential Study Cases and Exclusions

Two hundred seven case-patients were ascertained during the study period; 152 (73.4%) were enrolled, and 55 (26.6%) were excluded (Table 1). One refused to participate, one was unable to complete the interview, and two did not respond. Thirty-six (17.4%) were secondary to a laboratory-confirmed case in the household, 8 (3.9%) had traveled outside the United Kingdom, and 1 (0.5%) had traveled within the United Kingdom and stayed outside the study area >7 nights. Further single case-patients were excluded for having no history of diarrhea, mixed enteric infection, and being a visitor to, or resident outside, the study area. Two additional case-patients were excluded because a member of their household had already been interviewed as a case-patient or control earlier in the study.

Potential Controls and Exclusions

Seven hundred seventy-eight potential controls were identified; 466 (59.9%) were enrolled, and 312 (40.1%) were excluded (Table 1). One hundred eighty-three (23.5%) refused to participate or were unavailable for interview. The address of three (0.4%) could not be found. Forty-six (5.9%) had a history of diarrhea, 8 (1.0%) had traveled outside the United Kingdom, and 2 (0.3%) had traveled within the United Kingdom away from the study area for >7 nights in the 2 weeks before interview. Twenty-seven (3.5%) had moved from the study area, 2 (0.3%) had an address outside the study area, and 7 (0.9%) shared a household with a patient or a control. Nineteen (2.4%) were not enrolled because 3 controls had already been recruited in association with the case, 9 (1.2%) were in the wrong age band, and 3 (0.4%) had no reason recorded.

Study Population

Cases

Of the 152 study case-patients, 86 (56.6%) were <6 years of age; 47 (30.9%) were 6–15; and 19 (12.5%) were ≥16 years. Eighty-two (53.9%) were male. More cases were detected in the first half of each year of the study (Figure). The average annual incidence rate was similar in populations served by water from Crummock Lake, Ennerdale Lake, and the other water sources combined (Table 2).

In addition to diarrhea, the 152 patients reported abdominal pain (110 [72.4%]), vomiting (94 [61.8%]), fever (69 [45.4%]), anorexia (68 [44.7%]), and weight loss (56 [36.8%]). Thirty-seven patients were ill at interview. In

Table 1. Recruitment and reasons for exclusion from study, United Kingdom (UK)

Exclusion criteria	No. (%) excluded
Case-patients (n = 207)	
Refusal to participate	1 (0.5)
Could not complete adequate interview	1 (0.5)
Did not respond to letters or phone calls	2 (1.0)
Not meeting the study case definition	
No history of diarrhea	1 (0.5)
Mixed enteric infection	1 (0.5)
Secondary case	36 (17.4)
Travel outside the UK in 14 days before onset	8 (3.9)
Travel in UK outside study area for >7 nights in the 14 days before onset	1 (0.5)
Visitor to study area	1 (0.5)
Residence outside study area	1 (0.5)
Case-patient or household member previously interviewed as case-patient or control	2(1.0)
Total potential cases excluded	55 (26.6)
Total cases enrolled	152 (73.4)
Controls (n = 778)	
Refusal to participate in interview	23 (3.0)
Unavailable at requested interview times	125 (16.1)
Said interview times were not convenient	35 (4.5)
Subtotal: refused, unavailable for interview	183 (23.5)
Address not found	3 (0.4)
History of diarrhea	46 (5.9)
Travel outside UK in the 14 days before interview	8 (1.0)
Travel in UK outside study area for >7 nights in the 14 days before interview	2 (0.3)
Not resident in study area in the 14 days before interview	3 (0.4)
Moved from study area	27 (3.5)
Resident outside study area	2 (0.3)
Control or household member already interviewed as a case-patient or control	7 (0.9)
Subtotal: did not meet study control definition	95 (12.2)
Interview cancelled by study team because three controls already enrolled for associated case	19 (2.4)
Interview cancelled by study team as potential control found to be in wrong age group	9 (1.2)
Reason for exclusion not recorded	3 (0.4)
Subtotal: not enrolled for administrative reasons or reason not recorded	31 (4.0)
Total potential controls excluded	312 (40.1)
Total controls enrolled	466 (59.9)

115 patients who had recovered when interviewed, the median duration of illness was 9 days (range 2–21). Nineteen (22.1%) of the 86 patients ≤5 years of age and 4 (8.5%) of 47 case-patients ages 6-15 years were admitted to hospital.

Controls

Three or four controls were recruited in association with 131 (86.2%) patients and one or two in association with the remainder. Patient and control groups were comparable by sex, local government district of residence, water sources and water supply zones, disruption and discoloration of tap water, nights spent away from home within the United Kingdom in the 2 weeks before onset or interview, and sewage services to the home (Table 3).

Time until Study Recruitment

One hundred twenty-eight (84.2%) patients were interviewed within 1 week and 151 (99%) within 2 weeks of the date of the *Cryptosporidium*-positive fecal smear test report. The delay between reporting a case and enrolling the patient and associated controls was a median of 2.3 weeks (range 1–8).

Knowledge about *Cryptosporidium*

Thirty six (75%) of 48 patients and 113 (67.7%) of 167 controls recruited from April 29 to November 30, 1999, stated that they had not heard of *Cryptosporidium* before being contacted for the study. The proportion without knowledge was similar for patients and controls recruited before and after July 8, 1999. Of the 66 persons who had previously heard of *Cryptosporidium*, 16 had knowledge of modes of transmission: 4 reported transmission could occur through water, drinks, or contact with farms and animals; 6 reported that transmission was by water only; and 6 reported transmission was by farm contact only.

Single Variable Analysis

Significant associations were seen with consuming cold unboiled tap water (odds ratio [OR] 2.12, 95% confidence interval [CI] 1.16 to 3.91, p = 0.012) with a significant dose-response relationship (χ^2 test for trend p = 0.017). A significant dose-response relationship was also seen for the usual volume of cold unboiled tap water consumed at home (χ^2 test for trend p = 0.005), but not for that consumed at the workplace, nursery, or school (χ^2 test for trend p = 0.495) (online Appendix available from

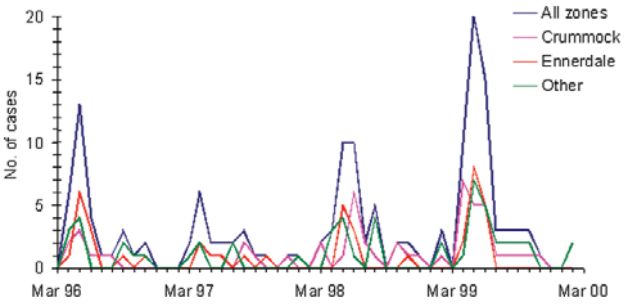

Figure. Case-patients recruited to the study by month of onset and water supply zone.

http://www.cdc.gov/ncidod/EID/vol10no6/03-0325_app.htm). No association was found between consuming bottled water, ice, soft drinks, and pasteurized or unpasteurized milk. Consuming lettuce, tomatoes, mixed salad, and cream was associated with lower risk ($p < 0.05$).

Any contact with a farm was associated with a twofold increase in risk (OR 2.11, CI 1.4 to 3.2, $p < 0.001$). Risk was higher for short farm visits (OR 2.56, CI 1.57 to 4.17) and increased with the frequency of farm visits (χ^2 test for trend $p = 0.003$) (online Appendix). Risk was also increased by contact with farm animals (OR 2.23, CI 1.45 to 3.43), eating food within 2 hours of contact with farm animals (OR 3.11, CI 1.79 to 5.38), and stroking farm animals (OR 2.01, CI 1.17 to 3.42). Walking near slurry applied to fields was not associated with increased risk, but contact with slurry showed some evidence of increased risk (OR 2.0, CI 0.99 to 4.02).

Contact with pets at home or contact with pets with diarrhea did not increase risk. Risk was increased for feeding pets leftovers (OR 3.79, CI 1.0 to 14.69), with marginal evidence of risk for feeding pets raw vegetables (OR 2.09, CI 0.95 to 4.56) and biscuits (OR 1.76, CI 0.95 to 3.21). Contact with animals other than farm animals and home pets was not associated with infection (OR 0.84, CI 0.54 to 1.30). Risk was increased by having accidentally touched feces from any animal (OR 3.04, CI 1.33 to 6.94). Attendance at a playgroup or nursery and recreational exposure to water was not associated with infection (online Appendix).

Multivariable Analysis

The usual volume of cold unboiled tap water consumed was independently associated with cryptosporidiosis (OR 1.40, CI 1.14 to 1.71 per pint consumed per day) in the final multivariable model (Table 4). A short visit to farms (OR 2.02, CI 1.04 to 3.9) was also significant. No difference in risk was found between the different water supply zones, irrespective of whether the zones received unfiltered water from Crummock and Ennerdale Lakes, other public supplies with a variety of conventionally filtered and unfiltered water, or private water supplies (Tables 3 and 4). Slight evidence was found for increased risk for feeding pets raw vegetables (OR 2.11, CI 0.98 to 4.56) and biscuits (OR 1.77, CI 0.94 to 3.35) but not for age, sex, consuming nonlocally produced cheese, contact with farms without cattle or sheep, or with cattle (Table 4). Subsidiary analysis showed that usual volume of cold unboiled tap water consumed at home was also a significant risk factor. A further analysis that excluded persons whose house was served by a mixed public water supply found similar results (not shown).

Genotyping Results

Genotyping was undertaken in 67 of 101 cases from 1998 to 2000. All the smears were confirmed positive, and 56 (83.6%) of tested smears were *C. parvum* genotype 2 (animal and human) strain, 1 (1.5%) was genotype 1 (human strain), and 10 (14.9%) could not be typed.

The primary care patient register was reviewed after patients were recruited for the study. The register contained 166,376 names of Allerdale and Copeland residents compared to a population of 162,809 enumerated at the 2001 census (available from www.statistics.gov.uk/census2001). The computer algorithm used to randomly select potential controls generated 125 tables of registered patients' names, where registered patients were within the same age category as study case-patients and had the same lead characters of the postal code of residence as study case-patients. The tables contained a median of 496 (range 9–7,800) names.

Discussion

Drinking cold unboiled tap water from public drinking water supplies was a highly significant risk factor for sporadic human cryptosporidiosis, regardless of the water source. To our knowledge, this study is the first to show that drinking from public water supplies is an important risk factor for sporadic human *Cryptosporidium* infection. Most cases were in children, consistent with previous reports from England and Canada (21,22). Many patients required admission to hospital, showing the seriousness of illness. Infection in study patients was also associated with short visits to farms and predominantly with the *C. parvum*

Table 2. Estimated average annual incidence of primary *Cryptosporidium* cases by water source, 1996–1999[a]

Water source	Population	Incidence (per 100,000 per y)
Crummock	58,295	24
Ennerdale	47,780	22
Crummock and Ennerdale	106,075	23.1
All other sources	59,699	22.6

[a]Population denominators from water company records.

Table 3. Baseline characteristics of study population

Characteristics	Cases (%) 152 (100)	Controls (%) 466 (100)	p value
Sex			
M	82 (53.9)	236 (50.6)	0.539
F	70 (46.1)	230 (49.4)	
Age, y			
0–5	86 (56.6)	273 (58.6)	0.904
6–15	47 (30.9)	136 (29.2)	
16+	19 (12.5)	57 (12.2)	
Local government district			
Allerdale	83 (54.6)	248 (53.2)	0.838
Copeland	69 (45.4)	218 (46.8)	
Source of water/zone			
Crummock Lake/ Crummock North	37 (24.3)	103 (22.1)	0.941
Crummock Lake/ Crummock South	19 (12.5)	49 (10.5)	
Ennerdale Lake/ Ennerdale North	29 (19.1)	101 (21.7)	
Ennerdale Lake/ Ennerdale South	13 (8.6)	43 (9.2)	
Millom/Millom	19 (12.5)	54 (11.6)	
Quarry Hill/Quarry Hill	16 (10.5)	41 (8.8)	
Hausegill/Hausegill	3 (2.0)	5 (1.1)	
Hayknott/Hayknott	2 (1.3)	6 (1.3)	
Underscar/Underscar	1 (0.7)	3 (0.6)	
Fellside/Fellside	0	1 (0.2)	
Multiple sources/Mixed from >1 zone	11 (7.2)	51 (10.9)	
Different private water supplies	2 (1.3)	9 (1.9)	
Disruption of main supply in week before illness or outbreak?			
Y	8 (5.3)	14 (3.0)	0.294
N	143 (94.1)	448 (96.1)	
Not recorded	1 (0.7)	4 (0.9)	
Water discolored in the week before illness or outbreak?			
Y	14 (9.2)	33 (7.1)	0.398
N	130 (85.5)	431 (92.5)	
Not recorded	8 (5.3)	2 (0.4)	
Away from home in the 2 weeks before illness or outbreak?			
Y	49 (32.2)	163 (35.0)	0.603
N	103 (67.8)	303 (65.0)	
Sewage services to home			
Mains	130 (85.5)	411 (88.2)	
Septic tank	21 (13.8)	51 (10.9)	
Chemical toilet	1 (0.7)	0	
Not recorded	0	4 (0.9)	

genotype 2 (animal and human) strain, consistent with farmed livestock's being a major source of infection. Risk was not increased by contact with pets.

Excluding study participants by recent travel ensured that environmental exposures most likely occurred within the study area. Excluding household contact with an earlier onset case ensured that person-to-person transmission within the household was unlikely to have occurred in study case-patients.

The number of patients who sought medical attention was likely to have been high because the National Health Service provided free medical care within the study area at the point of use and because all fecal specimens were tested for *Cryptosporidium* by National Health Service laboratories without charge. Although only half of patients with cryptosporidiosis may seek medical attention in the United Kingdom (23), we have no reason to suppose that risk factors for patients who do not visit healthcare facilities would differ substantially from those that did.

Matching refers to pairing cases with one or more controls on the basis of their similarity in selected variables, with the objective of eliminating bias (24,25). We undertook stratified random sampling from a population list to select potential controls and adjusted by using multiple regression analysis, which is one of a number of alternative designs to matching (24,25).

Refusal to participate was low at 4 (1.9%) of 205 cases and 183 (23.5%) of 778 potential controls. Lower response rates in controls compared to cases is expected (24). Care was taken during the design and conduct of the study to mask interviewers to the tap water hypothesis. Interviewer training emphasized that all risk factors were plausible and required equal care in measurement. A survey after media

Table 4. Final multivariable model[a]

Variables	Cases	Controls	Adjusted OR	CI	p value
Total	152	466			
Sex					
M	82	236	1.1	0.73 to 1.67	0.64
F	70	230	1		
Age			0.99/y	0.97 to 1.01	0.29
Water supply zones					
Crummock North	37	103	1		0.71
Crummock South	19	49	1.28	0.61 to 2.67	
Ennerdale North	29	101	0.95	0.51 to 1.78	
Ennerdale South	13	43	0.9	0.39 to 2.09	
Millom	19	54	1.04	0.51 to 2.13	
Quarry Hill	16	41	1.36	0.61 to 3.05	
Hausegill	3	5	1.37	0.26 to 7.11	
Hayknott	2	6	0.89	0.16 to 4.85	
Underscar	1	3	1.83	0.16 to 20.37	
Fellside	0	1	0.002	0 to ∞	
Mixed from >1 zone	11	51	0.77	0.34 to 1.77	
Private water supplies	2	9	0.004	0 to 203.4	
Usual daily volume of cold unboiled tap water drunk[b]			1.40/pint	1.14 to 1.71	0.001
Any short visit to a farm					
Y	40	57	2.02	1.04 to 3.9	0.04
N	107	391	1		
Fed pet raw vegetables					
Y	13	20	2.11	0.98 to 4.56	0.06
N	139	446	1		
Fed pet biscuits					
Y	21	39	1.77	0.94 to 3.33	0.08
N	131	427	1		
Ate nonlocally produced cheese					
Y	113	322	1.49	0.91 to 2.43	0.1
N	34	139	1		
Contact with farms without cattle or sheep					
Y	15	19	1.96	0.79 to 4.88	0.15
N	125	417	1		
Contact with a cattle farm					
Y	15	29	1.67	0.73 to 3.82	0.23
N	125	407	1		

[a]OR, odds ratio; CI, confidence interval.
[b]At home and away from home in the Allerdale/Copeland area.

coverage indicated little knowledge about risk factors for cryptosporidiosis by patients or controls. Patients may have increased fluid consumption after the onset of illness. Study participants were therefore asked to report their "usual consumption" of unboiled tap water, bottled water, and soft drinks, without a time restriction. Although a bias towards a patient's recalling consumption of fluids after onset of illness could explain some of the association with tap water that we observed, we do not think it can explain it entirely. In particular, fluid volumes were measured in the same way for unboiled tap water, bottled water, and soft drinks, but the highly significant association and dose-response relationship were observed only for unboiled tap water. An interview date bias was avoided because patients and associated controls were enrolled within a short time of each other.

Cryptosporidiosis in HIV-infected persons is associated with consuming unboiled tap water. We do not believe that undetected HIV infection or other causes of immune suppression could have been a major confounding factor. Only two new cases of HIV infection would be expected in Allerdale and Copeland each year even if rates for the whole North West of England were applied to the study population. However, our findings reinforce the need for immune-compromised persons, including those with HIV infection, to avoid drinking unboiled tap water (26,27). Allerdale and Copeland had very similar levels of social deprivation (28). Moreover, controls resided in approximately the same locality as patients, as defined by shared lead characters of the postal code. Therefore, a systematic difference in social deprivation between patients and controls was unlikely and would not have explained the associations we observed.

The spring peak and smaller autumn peak in our cases are consistent with previous reports from England and Wales. These peaks are attributed to lambing, calving, and runoff from spring rains and to summer travel to countries with higher incidence of cryptosporidiosis (4,13).

Our findings contrast with a case-control study in Adelaide and Melbourne, which did not detect increased risk for sporadic cryptosporidiosis associated with the public water supplies (29). This difference may reflect the quality of the source waters, of water treatment, or both in these cities. Swimming pool exposure was the most significant risk factor in the Australian study. Regularly consuming raw vegetables was protective in that study. We also observed a protective association with lettuce, tomatoes, and mixed salad, and additionally for cream, in single variable analysis. These foods might have conferred a direct protective nutrient effect, been markers for more favorable general nutrition, or contained small numbers of oocysts derived from water used for irrigation and preparation sufficient to induce immune boosting (3,30,31). A recent study of sporadic cryptosporidiosis in the San Francisco Bay Area also failed to show an association with tap water, but the study was small (32).

Most of our patients were children, which suggests that older members of our study population were mainly immune, probably because of long-term immune boosting from low-level intermittent contamination of water supplies and contact with livestock (31,33). This observation is consistent with recent seroprevalence studies in blood donors resident in midwestern American cities and indicates lower seroprevalence of *Cryptosporidium* antibodies in populations served by deep borehole water compared to lake and river water supplies (34,35).

No association was seen for contact with pets at home, pets with diarrhea, or feeding tinned pet meat, raw meat, or pellets. However, feeding pets biscuits and raw vegetables was associated with slightly increased risk in single variable analysis and in the final multivariable model. These food types may be markers for more intimate contact with animal secretions; feeding raw vegetables may indicate contact with contaminated water in preparation. Although contamination of pet biscuits is possible, these products are dry and manufactured at high temperature; thus, survival of oocysts within these foods seems unlikely. Accidental hand contact with the feces of any type of animal was significant in single variable analysis but not in the final multivariable model. These results suggest that sound hygiene in cleaning animal feeding utensils, avoiding cross-contamination between pet and human food preparation areas, and good hand hygiene are desirable but that pets and pet feeding were not major risk factors for cryptosporidiosis in this population.

The findings of this study are consistent with the decline in human *Cryptosporidium* infection throughout England and Wales, coincident with the foot and mouth disease epidemic in livestock during 2001 (13). These two facts strongly suggest that livestock reservoirs of *Cryptosporidium* contribute substantially to sporadic human cryptosporidiosis in North Cumbria and in England and Wales as a whole, through low-level intermittent *Cryptosporidium* oocyst contamination of public drinking water supplies. Our results support the need for rigorous risk assessment of water sources and, where indicated, improved catchment control. Our results are also in accord with recent U.K. legislation that requires continuous monitoring of *Cryptosporidium* oocyst concentrations in treated water from at-risk supplies (36). Advanced methods of filtration, disinfection, and UV light treatment may be required to further decrease the risk for cryptosporidiosis from public water supplies (5,37,38).

The water company installed membrane filtration during 2000 at works served by Crummock and Ennerdale Lakes, which previously provided chlorination alone. The impact of this intervention will be presented in a separate article.

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Dr. Goh is an honorary consultant to the Carlisle and District Primary Care Trust and was formerly the consultant in Communicable Disease Control to North Cumbria Health Authority, in North West England. She specialized in public health medicine and developed an interest in childhood immunization, enteric infection, *Cryptosporidium*, and water supplies.

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Address for correspondence: Mark Reacher, Consultant Epidemiologist, Health Protection Agency-Communicable Disease Surveillance Centre, 61 Colindale Avenue, London NW9 5EQ, U.K.; fax: 44-0-20-8200-7868; email: Mark.Reacher@hpa.org.uk

Environmental Exposure and Leptospirosis, Peru

Michael A.S. Johnson,*† Hannah Smith,† Priya Joseph,† Robert H. Gilman,*† Christian T. Bautista,*‡ Kalina J. Campos,*§ Michelle Cespedes,† Peter Klatsky,† Carlos Vidal,¶ Hilja Terry,† Maritza M. Calderon,*§ Carlos Coral,† Lilia Cabrera,† Paminder S. Parmar,† and Joseph M. Vinetz#

Human infection by leptospires has highly variable clinical manifestations, which range from subclinical infection to fulminant disease. We conducted a population-based, cross-sectional seroepidemiologic study in Peru to determine potential relationships of environmental context to human exposure to *Leptospira* and disease associated with seroconversion. Three areas were studied: a flooded, urban slum in the Peruvian Amazon city of Iquitos; rural, peri-Iquitos villages; and a desert shantytown near Lima. Seroprevalence in Belen was 28% (182/650); in rural areas, 17% (52/316); and in a desert shantytown, 0.7% (1/150). *Leptospira*-infected peridomestic rats were found in all locales. In Belen, 20 (12.4%) of 161 patients seroconverted between dry and wet seasons (an incidence rate of 288/1,000). Seroconversion was associated with history of febrile illness; severe leptospirosis was not seen. Human exposure to *Leptospira* in the Iquitos region is high, likely related both to the ubiquity of leptospires in the environment and human behavior conducive to transmission from infected zoonotic sources.

Leptospirosis is a zoonotic disease of global importance (1–5) that occurs in both urban and rural settings (2,6–8) and causes both endemic and epidemic illness, including pulmonary hemorrhage and death (7,9–11). Transmission of *Leptospira* and the clinical expression of leptospirosis seem to vary in different environmental and socioeconomic contexts. Epidemic leptospirosis associated with pulmonary hemorrhage, renal failure, and jaundice seems to predominate in the urban setting, where baseline clinical immunity in humans is likely to vary (7,8,12–15). In contrast, a substantial prevalence of seropositivity associated with subclinical leptospiral infection has been shown in many rural places throughout the developing world, including, for example, Nicaragua (16).

The environment of Iquitos, Peru, in the Amazon Basin, is ideal for the transmission of *Leptospira* with its hot, humid tropical conditions and dense human and potential mammalian reservoir populations (17). We have observed that ≈30% of patients in this region seen with acute undifferentiated fever have serologic results suggestive of acute leptospirosis (microscopic agglutination test with titers >1/400, seroconversion, or fourfold rise in titer; Vinetz et al. unpub. data).

Our objective was to determine potential relationships of environmental context to human exposure to *Leptospira*. A cross-sectional, population-based seroepidemiologic study was conducted in three contrasting epidemiologic contexts in Peru, where leptospirosis transmission would be predicted to be high (Belen, an urban slum in Iquitos), intermediate (rural peri-Iquitos villages), and low (the Pampas de San Juan de Miraflores, a desert shantytown outside of Lima). Peridomestic rats, one potentially important source of leptospiral transmission, were also surveyed for the leptospiral carrier state to assess their potential for transmission.

Patients and Methods

Study Sites

Iquitos, Loreto, is located in the Amazon rainforest of northeastern Peru. Its environment is tropical: rainfall averages 288 cm/year; temperatures range from 21.8°C to 31.6°C. The region has a population of ≈400,000. Belen (Figure 1), an urban slum area of Iquitos on the floodplain of the Itaya and Amazon Rivers, annually floods during January to May from Andean run-off. Many residences are built on floats; during flooding, these houses rise with the river. Belen has a population of 40,000, primarily mestizos of mixed European and Amerindian ancestry (Dirección Regional de Salud, Iquitos, Peru, pers. comm.). Main

*Asociación Benéfica Prisma, Lima, Peru; †Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA; ‡U.S. Naval Medical Center Research Detachment, Lima, Peru; §Universidad Peruana Cayetano Heredia, Lima, Peru; ¶Ministry of Health, Iquitos, Peru; and #University of California San Diego School of Medicine, La Jolla, California, USA

Figure 1. Typical views of the residential areas of Belen, Iquitos, Peru. A. Houses near the river edge are built on stilts. B. Conditions abound for the proliferation of peridomestic rats in the same places where people live and play. This view is adjacent to the major market area of Belen, where *Rattus* spp. are commonly observed, even during the day in the middle of commercial activities.

occupations include fishing, small-scale commerce, and informal day labor.

The peri-Iquitos rural communities studied were Moralillo, Villa Buen Pastor, and San Carlos, located 16, 21, and 34 km, respectively, south of the center of Iquitos (Figure 2). Combined, the population of these villages at the time of sampling was 1,197, mainly mestizo. These villages are rural, located upriver away from the city. Most inhabitants live in poor housing with electricity but without household connections to water or sewer systems. The primary occupation is small-scale agriculture. These villages do not flood seasonally because they are above the floodplain. Water is obtained from village pumps or springs. Residents typically bathe in local rivers or ponds.

Las Pampas de San Juan de Miraflores is a pueblo joven (shantytown) located in the desert 25 km south of Lima. The population at the time of sampling was 40,000. Basic demographic and environmental features of this area have been previously described (18–20). Residents are primarily mestizo immigrants from the Peruvian Andes who earn money through informal day labor. Annual rainfall averages <1 cm.

Study Design

Belen

A census of 3,704 people from seven communities was conducted and coded in February 2001. The census was divided into four age groups before random selection: 1) 1–5 years; 2) 6–11 years; 3) 12–17 years; and 4) 18–65 years. From the last group, 1,210 adults were randomly selected to participate in a seroprevalence survey from February to October 2001. Eighty children from each of groups 1 to 3 were also recruited. Standardized questionnaires were completed, including questions pertaining to potential household, occupational, and social exposures. Serum was collected and preserved at –20°C for serologic analysis. A subgroup of participants tested in February and March 2001 were retested from July to October 2001 as an incidence cohort.

Rats were collected from a random selection of houses within the same communities as well as other areas of Belen during November 2001 through May 2002 by using Tomahawk (Tomahawk Live Trap Co., Tomahawk, WI) and back-break traps. Kidneys were preserved at –20°C in 0.1 mol/L Tris-HCl, pH 8.0 for subsequent testing.

Rural Communities

Data on residents of Villa Buen Pastor, Moralillo, and San Carlos were coded; a census was conducted, and samples were obtained from study participants from February 2000 to August 2000. A total of 650 serum samples were collected from 316 randomly selected participants ≤65 years of age. Participants were afebrile and apparently healthy.

San Juan de Miraflores

A population of 38,721 was coded and a census conducted in April 1997. During the same period, serum samples were collected from 150 randomly selected participants ≤65 years of age. Participants were afebrile and apparently healthy. Rats from a random selection of houses were trapped live with Tomahawk traps.

Laboratory Assays

A combined immunoglobulin (Ig) M + IgG assay (INDX IVD Leptospira Microwell ELISA, PanBio INDx, Inc., Baltimore, MD) was used to obtain evidence of leptospiral exposure from serum samples. The assay uses *Leptospira biflexa* serovar patoc I as antigen to detect genus-specific antibodies. Leptospiral exposure was defined as positivity on an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instruc-

Figure 2. A typical view of a rural village area near Iquitos. Near Villa Buen Pastor, located 21 km along the major (unfinished) road that leads from Iquitos to Nauta, substantial secondary growth of forest is evident after removal of primary forest for human agricultural and living activities. One must walk approximately 1–2 km from the road to get to the village and a further 1–2 km from Villa Buen Pastor to Moralillo, another village studied in this report.

tions (optical density at 1/100 dilution of sample greater than or equal to weak positive control). ELISAs were performed twice with identical results.

DNA was extracted from rat kidneys by using a published procedure based on a phenolchloroform-isoamyl alcohol-extraction method (21). The presence of *Leptospira* DNA was assessed by a polymerase chain reaction-based assay (PCR) using G1/G2 primers (22).

The chi-square test was used, or the Fisher exact test, when mandated by sparse data, to compare groups for categorical outcomes. For nonparametric data, either the Student *t* test or the Mann-Whitney U test was used. Associations between seropositivity to *Leptospira* spp. and study variables were analyzed by both univariate and stratified analysis by using odds ratios (ORs). Because the study population included sets of persons for each household, data were presumed to violate the standard logistic regression assumption of independent response probabilities across observations. To avoid potential underestimation of standard errors (23), we estimated ORs by using the logistic-binomial random effects model for distinguishable data from the EGRET software package (Cytel Software Corporation, Cambridge, MA). This model includes a random effects parameter, based on the variability of average outcome probabilities across households, which measure a residual household effect on the probability of having leptospirosis. ORs were adjusted for the effect of household. Confidence intervals (CI) are 95%; p values <0.05 were considered significant and two-sided. Other statistical analysis was performed with Stata v. 7.0 (Stat Corp., College Station, TX).

This study was approved by the Dirección de Salud, Iquitos, Peru; the Ethical Committee of Asociación Benéfica Prisma, Lima, Peru; and the Johns Hopkins Bloomberg School of Public Health Committee on Human Research, Baltimore, Maryland. Written informed consent was obtained from each participant. Guidelines for human experimentation according to institutional, U.S. federal, and Peruvian standards were followed.

Results

Demographic Description of Study Populations

In Belen, 650 people from 386 households participated in the study (Table 1); this figure represented 18% of the population and 60% of the households. The median number of persons per household was 5.8 (range 1–21). In the rural communities, 316 participants (26% of local population) were enrolled. The median number of persons per household was 6.4 (range 1–24). In Las Pampas de San Juan de Miraflores, 150 (0.4% of the local population) people were sampled. The median number of people per household in Las Pampas was 5.4 (range 1–14).

Prevalence of Leptospiral Seropositivity

In Belen, 182 (28.0%) of 650 people were positive for anti-*Leptospira* antibodies (Table 2). No sex differences in seropositivity rates were evident. Age groups in Belen did not differ statistically (children versus adults; p = 0.122), but a significant trend for seropositivity with age was evident (Figure 3, p = 0.018).

In the rural peri-Iquitos communities, the rate of seropositivity was lower than in Belen (16.5% vs. 28.0%, p < 0.001, Table 2). No sex difference in seropositivity

Table 1. Site description and demographic characteristics of the study population

Feature	Pampas San Juan	Rural communities[a]	Belen
Study period	April 1997	Feb–Jun 2000	Feb–Oct 2001
Population	38,721	1,197	3,704
No. households selected (%)	7,744 (20)	132 (56)	386 (60)
No. participants tested (%)	150 (0.4)	316 (26)	650 (18)
Sample population			
Female (%)	57 (38)	152 (48)	356 (55)
Age >18 y (%)	64 (43)	170 (54)	533 (82)
Mean (SD) age, y	17.5 (14.5)	24.9 (17.9)	30 (15.2)
Median	11	22	29
Mean (SD) no. persons per household	5.7 (2.2)	2.4 (1.6)	1.7 (0.9)
Range	3–14	1–9	1–5

[a]Combined populations of the villages of San Carlos, Moralillo, and Buen Pastor in the peri-Iquitos area.

Table 2. Leptospirosis seroprevalence among residents by study site

	Pampas San Juan % (pos/total)[a]	Rural communities[b] % (pos/total)	Belen % (pos/total)	Comparison among all sites	Comparison of Belen vs. rural community
Overall[c]	0.7 (1/150)	16.5 (52/316)	28.0 (182/650)	p < 0.001	p < 0.001
Sex					
F	0.0 (0/57)	15.8 (24/152)	29.6 (105/356)	p < 0.001	p = 0.001
M	1.1 (1/93)	17.1 (28/164)	26.4 (77/292)	p < 0.001	p = 0.024
M vs. F comparison	p = 0.999	p = 0.758	p = 0.428		

[a]Leptospirosis prevalence (number of positive cas es/total of persons tested).
[b]San Carlos, Moralillo, and Buen Pastor.
[c]Optical density ≥0.500.

(17.1% vs. 15.8%; p = 0.758) was evident. Although 6- to 11-year-old children had a higher prevalence than 12- to 17-year-old children, overall seroprevalence increased with age, from 4.0% in those ≤5 years of age to 21.2% in persons 18–65 years (Figure 3, p = 0.012). In Las Pampas de San Juan de Miraflores, 1 of 150 participants was seropositive (Table 2), a finding that yielded an estimated prevalence of 0.7%.

Incidence of Leptospiral Seroconversion

In Belen, 195 participants (ages 18–65 years) tested in the first 2 months of the prevalence study were retested ≈6 months later; 161 were initially negative. On retesting, 20 (12.4%; 11 women, 9 men) seroconverted from negative to positive (IgM + IgG). Median time between sampling was 157 days, the approximate interval between flooding seasons. The estimated incidence rate of seroconversion was 288/1,000 people per year. Of 34 persons who had a positive first sample and were retested, 18 (53%) seroreverted to negative, a finding that demonstrates that IgM + IgG ELISA–detectable antibodies are short-lived, consistent with results of previous studies (4,24–26).

Univariate analysis showed that history of fever in the previous 6 months was associated with seroconversion (OR = 3.29, 95% CI 1.19 to 9.12, p = 0.022), a finding that remained significant after controlling for age and sex (OR = 3.32, 95% CI = 1.18 to 9.29, p = 0.023). Severe leptospirosis did not develop in any patient.

Risk Factors for Leptospiral Seropositivity

Univariate analysis demonstrated several potentially modifiable risk factors (Table 3), including river bathing, owning a television, living close to the river, and living in San José, one area of Belen where sanitation is particularly poor. Particular animals present in the home or workplace were not found to be significant risk factors. Educational level was associated with protection when a trend analysis was used (p = 0.032). By multivariate analysis, risk factors were not wearing shoes in the field and living in San José.

Prevalence of Leptospiral Infection in Rats

Of 234 rats (151 [64.0%] *Rattus rattus*, 83 [36%] *R. norvegicus*) trapped in Belen, 25 (10.6%) were positive for *Leptospira* by PCR (Table 4). *R. norvegicus* were more frequently infected than *R. rattus* (21.7% vs. 4.6%, p < 0.001). While PCR positivity increased with rat age (infantile rats 5.9%, juveniles 8.8%, adults 12.0%), this apparent trend was not significant (p = 0.328). Of rats in San Juan de Miraflores (all *R. norvegicus*), 2 of 41 (4.9%) were PCR-positive for *Leptospira* (1/25 males, 1/16 females). Both infections were confirmed by culture. Isolates were not further identified.

Discussion

This study produced two major findings. First, human exposure to *Leptospira* in sites with contrasting ecologic features depended more on environmental context than the prevalence of infection in peridomestic zoonotic sources. Second, in Belen, a flood-prone urban slum area of Iquitos where exposure to *Leptospira* was common, seroconversion was associated with symptomatic, but not severe, disease.

The evidence for human leptospiral infection in Iquitos is different from what is typically reported from the tropics (9). In areas where *Leptospira* would be predicted to be common, epidemics are usually described in the context of outbreaks caused by events such as the flooding associated with the Nicaraguan epidemic of 1995 (9), not in the leptospirosis-endemic setting such as we describe here. Previously described risk factors elucidated in other tropical contexts were investigated in Belen (Table 3). Most had

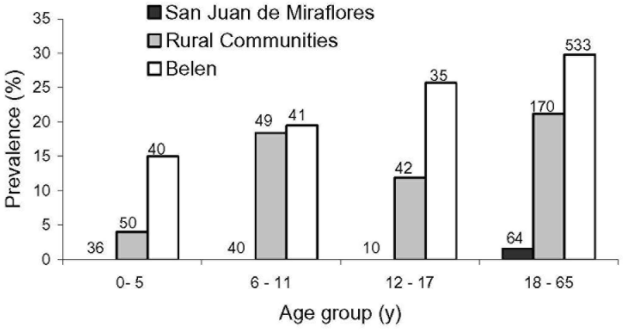

Figure 3. Prevalence of antileptospiral immunoglobulin (Ig) M/IgG antibodies by age group. Number above each bar is the sample size for the specified age group and site. The trend of increasing prevalence by age is significant for Belen and the rural communities (p = 0.018 and p = 0.012, respectively).

Table 3. Risk factors associated with leptospiral seropositivity, Belen [a]

	Prevalence		Incidence (n = 158)	
Risk factor	OR (95% CI)	p	RR (95% CI)	p
Living in San José	1.90 (1.27 to 2.83)	0.002	1.88 (0.83 to 4.23)	0.191
Bathing in the river	1.75 (1.09 to 2.82)	0.021	2.70 (0.83 to 8.78)	0.132
Living near the river	1.58 (1.07 to 2.32)	0.022	1.94 (0.84 to 4.47)	0.149
Owning a television	1.55 (1.01 to 2.38)	0.043	1.56 (0.66 to 3.69)	0.438
Fisherman (male)	1.57 (0.86 to 2.87)	0.141	1.94 (0.73 to 5.18)	0.250
Not wearing shoes in the field	1.63 (0.85 to 3.13)	0.139	2.29 (0.93 to 5.59)	0.135
Drinking primarily river water	1.28 (0.82 to 2.01)	0.272	0.91 (0.35 to 2.40)	0.999
Rats in the workplace	1.14 (0.52 to 2.48)	0.740	0.79 (0.12 to 5.18)	0.999
Rats in the home	1.36 (0.00 to 3.61)	0.538	NA	NA
Dogs in the workplace	1.12 (0.66 to 1.90)	0.684	1.72 (0.69 to 4.31)	0.317
Dogs in the home	0.67 (0.43 to 1.02)	0.063	1.06 (0.46 to 2.44)	0.891
Pigs in the home	0.77 (0.38 to 1.53)	0.452	0.44 (0.06 to 3.06)	0.699
Education (minimal vs. secondary)	1.37 (0.90 to 2.05)	0.139	1.59 (0.56 to 4.51)	0.368
Education (secondary vs. superior)	2.63 (0.58 to 11.96)	0.207	NA	NA
Education (minimal vs. superior)	3.60 (0.81 to 15.99)	0.092	NA	NA
Positive household member	1.18 (0.75 to 1.85)	0.468		
Multivariate logistic regression for independent risk factors				
Living in San José	2.17 (1.39 to 3.37)	0.001		
Not wearing shoes in the field	2.17 (1.1 to 4.05)	0.015		

[a]OR, odds ratios; RR, relative risk; CI, confidence interval. CIs and ORs were calculated for adults only (>18 years of age). NA, not applicable.

no pertinence in Belen because of their ubiquity in this tropical, urban slum. Only two factors were significant in multivariate analysis: not wearing shoes when working in the field and living in the San José area of Belen.

Two risk factors for leptospiral seropositivity of particular interest were education and living close to the river, specifically in San José. Prevalence of antileptospiral antibodies decreased with increased education, most likely associated with a greater degree of personal hygiene. Living in San José was a significant positive risk factor in the prevalence study by both univariate and multivariate analysis and was a risk factor in the incidence cohort as well. Rats also had higher rates of infection in San José than elsewhere in Belen, a finding that suggests that, within the overall highly disease-endemic area of Belen, smaller microhabitats may have even higher rates of leptospirosis transmission.

The overall high prevalence and incidence of leptospirosis transmission found in the absence of a disease outbreak, as well as similar rates of seropositivity in both sexes, indicate the high level of risk for infection in this area. In most epidemiologic studies of leptospirosis, infection rates differ for men and women, related to gender-specific activities (16,27–31) and associated leptospiral serovars (32). In the Iquitos study populations, the prevalence and incidence were very similar between the sexes, although the occupations of men and women were very different. Furthermore, the relationship between seropositivity and age shows the same trend as found in studies of other, largely native, inhabitants of *Leptospira*-endemic

Table 4. Characteristics of rats in Belen and Las Pampas de San Juan de Miraflores [a]

Risk factor	Pampas San Juan % (pos/total)[b]	Belen % (PCR positive/total)[b]	Comparison of sites p value
No. rats tested	4.9 (2/41)	10.6 (25/235)	p = 0.392
Sex[c]			
F	6.3 (1/16)	11.6 (15/129)	p = 0.999
M	4.0 (1/25)	8.5 (8/94)	p = 0.683
M vs. F comparison	p = 0.999	p = 0.594	
Age groups			
Adult	3.9 (2/41)	12.0 (18/150)	p = 0.255
Young (prepubescent)	0	8.8 (6/68)	p = NA
Infant	0	5.9 (1/17)	p = NA
Trend	p = NA	p = 0.328	
Species			
Rattus norvegicus	4.9 (2/41)	21.7 (18/83)	p = 0.019
R. rattus	0	4.6 (7/151)	p = NA
Comparison of rat species	p = NA	p < 0.001	

[a]PCR, polymerase chain reaction; NA, not applicable.
[b]Leptospirosis prevalence (number of positive rats/total of rats tested).
[c]Sex was not known for 12 infants.

regions (33,34). These data support the hypothesis that continuous exposure throughout life may result in an age-dependent increase in leptospiral seropositivity.

Limitations of this study must be considered. First, the different components of the study took place during different periods, rather than concurrently. Since the study sites in the Iquitos region are climatologically different from those in Lima and no disease outbreaks were observed in either location, our results likely reflect a reasonable estimate of endemic leptospiral transmission. Second, the high seropositivity rate in the absence of observed disease in Iquitos could relate to potential cross-reactivity of currently used ELISA antigens rather than true differences. However, the reproducible finding of 0.7% seroprevalence in a desert community where very little leptospiral infection might be predicted (San Juan de Miraflores), compared to 28.0% in Belen, suggests that the ELISA we used was specific, consistent with previous studies that used similar testing procedures (9,16,24,35,36). In addition, we found that 53% of persons with positive ELISA results seroreverted to negative within 6 months. This finding is consistent with known short-lived serologic responses to leptospiral lipopolysaccharide antigens (4). Third, several hypotheses might explain the observed differences between study sites. For example, the intrinsic pathogenicity of *Leptospira* in the study region may differ. Whether the immunogenetics of the human immune response to *Leptospira* differs between populations is unknown. The prevalence in rats may also have been underestimated. The G1/G2 primer set used to detect this genus in rat kidneys detects most, but not all, leptospires. Our overall findings are also consistent with previous studies (33,34), in which high prevalence rates of antileptospiral antibodies have been found despite the absence of observed severe disease. Thus, protective immunity against severe disease from repeated infection may develop in persons in areas with ubiquitous leptospirosis transmission.

The high rate of exposure to *Leptospira* in Iquitos, and the likely ubiquity of these organisms in the environment, suggests that implementing control measures to prevent leptospirosis in this setting might be difficult. The seeming absence (or at least very low rates) of severe leptospirosis in this region suggests the possibility that protective immunity may develop in this population. Larger prospective, population-based studies will be necessary to test these possibilities.

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Mr. Johnson is a graduate student in the Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland. His research focus is on integrating laboratory and field-based approaches to the study of environmental and ecologic determinants of infectious disease transmission.

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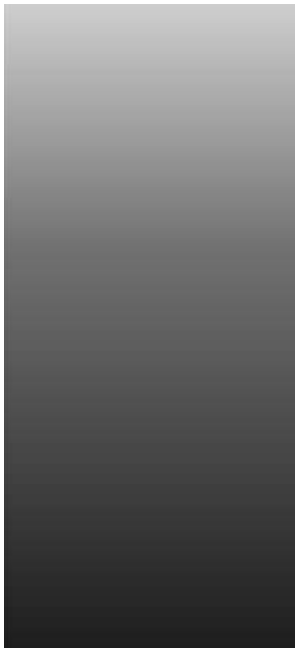
Address for correspondence: Joseph M. Vinetz, Associate Professor of Medicine, Division of Infectious Diseases, Department of Medicine, University of California San Diego School of Medicine, Cellular and Molecular Medicine East-Room 2052, 9500 Gilman Drive, 0640 La Jolla, CA 92093-0640, USA; fax: 858-534-6020; email: jvinetz@ucsd.edu



Swab Materials and *Bacillus anthracis* Spore Recovery from Nonporous Surfaces

Laura Rose,* Bette Jensen,* Alicia Peterson,* Shailen N. Banerjee,* and Matthew J. Arduino*

Four swab materials were evaluated for their efficiency in recovery of *Bacillus anthracis* spores from steel coupons. Cotton, macrofoam, polyester, and rayon swabs were used to sample coupons inoculated with a spore suspension of known concentration. Three methods of processing for the removal of spores from the swabs (vortexing, sonication, or minimal agitation) and two swab preparations (premoistened and dry) were evaluated. Results indicated that premoistened swabs were more efficient at recovering spores than dry swabs (14.3% vs. 4.4%). Vortexing swabs for 2 min during processing resulted in superior extraction of spores when compared to sonicating them for 12 min or subjecting them to minimal agitation. Premoistened macrofoam and cotton swabs that were vortexed during processing recovered the greatest proportions of spores with a mean recovery of 43.6% (standard deviation [SD] 11.1%) and 41.7% (SD 14.6%), respectively. Premoistened and vortexed polyester and rayon swabs were less efficient, at 9.9% (SD 3.8%) and 11.5% (SD 7.9%), respectively.

The Centers for Disease Control and Prevention (CDC), along with its partners in public health, law enforcement, environmental protection, defense, and the U.S. Postal Service, has been investigating a series of bioterrorism-related anthrax deaths and illnesses that occurred from October to December 2001. As of January 2002, 22 cases of confirmed or suspected cutaneous or inhalation anthrax were identified (1). Twenty of these cases were associated, or were likely to have been associated with, materials containing *Bacillus anthracis* spores that were delivered through the U.S. Postal Service. The source of the infection remains unknown for the other two cases. During the investigation, thousands of swabs, wipes, and high-efficiency particulate air (HEPA) filter sock samples were collected. A review of the sampling data in one publication suggests that HEPA socks and wipes were superior to swabs for recovery of *B. anthracis* spores (2). The above-mentioned study was conducted within the contaminated Brentwood Mail Processing and Distribution Center in Washington D.C. The comparisons were considered semiquantitative in that sampling sites were chosen to be directly adjacent and the distributions of spores were assumed to be similar, but the initial inoculum was unknown.

Originally, the swab-rinse method was developed to assess bacterial contamination of food utensils (3–7). This method was modified by the National Aeronautics and Space Agency (NASA) for environmental sampling of spacecraft and equipment (8–11). Historically, the number of organisms recovered from swabs used for environmental sampling has shown a poor correlation with the number of microbial contamination on surfaces (3,12–14). Several factors can contribute to this poor correlation, including differences in materials used (e.g., cotton, polyester, rayon, calcium alginate) (3,13,15–17), the organisms targeted for culture (3,16,17), variations in surface (10), and differences in the personnel who are collecting and processing samples (3,13,18,19).

In this study, the recovery efficiencies of four swab materials, both dry and premoistened, were compared, and different methods for swab processing were assessed for the recovery of known quantities of *B. anthracis* spores from a nonporous stainless steel surface.

Materials and Methods

Spore Preparation

The veterinary vaccine strain of *B. anthracis* Sterne 34F2 (Colorado Serum, Denver, CO) was grown in Leighton–Doi liquid medium (20) for 7 days at 36°C. The cells were checked for sporulation by microscopic examination of a slide preparation stained with malachite green (Fisher Scientific, Springfield, NJ), then harvested by centrifugation at 5,000 x *g* for 15 min and washed 3 times in sterile, ultrapure reverse osmosis (RO) water. The spores were purified by centrifugation through 58% Hypaque 76 (NYCOMED, Inc., Princeton, NJ) at 7000 x *g*, followed by

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

three additional washes in sterile RO water. The spores were pelleted by centrifugation one final time, then resuspended in 50% ethanol. This stock spore suspension was stored at 4°C.

Swab Description

Four types of swabs were evaluated in this study: cotton (Baxter Healthcare Corp., Deerfield, IL cat #A5002-5), polyester (Falcon, Becton Dickinson Microbiology Systems, Sparks, MD, cat #220690), rayon (Cole Parmer, Vernon Hills, IL, cat #14001-55), and macrofoam (VWR, Suwanee, GA, cat #10812-046). Surface characteristics were visualized by environmental scanning electron microscopy (SEM).

Direct Inoculation

The stock spore suspension was added to Butterfield Buffer (BB) (3 mmol/L KH_2PO_4, pH 7.2; Becton Dickinson Microbiology Systems) to attain 0.5 McFarland standard containing 10^6 CFU of spores/mL with a Microscan turbidity meter (Dade Behring, West Sacramento, CA). This suspension was diluted 1:10 in BB, and the swabs were inoculated directly with 100 µL of this dilution to compare the ability of each material to retain spores. Swabs were placed immediately into tubes containing 5 mL phosphate-buffered saline (pH 7.2) containing 0.04% Tween 80 (PBST) and vortexed at high speed for 2 min in 10-s bursts. Serial dilutions were performed (10^{-1}–10^{-5}) in BB, and 100 µL from each tube was spread onto each of three plates of Trypticase soy agar containing 5% sheep blood (TSAB, Becton Dickinson Microbiology Systems). Plates were incubated at 36°C overnight, and colonies were counted the next day.

Preparation of Coupons

Stainless steel coupons (2 x 2 inches) were cut from a sheet of S-180 grade, T-304 stainless steel (Stewart Stainless Supply, Inc., Suwanee, GA) and were used as test surfaces. This grade of stainless steel is commonly used in food service settings (J. Willingham, Stewart Stainless Supply, Inc., pers. comm.). The stainless steel was previously characterized for roughness by using a profilometer (Tencor AS500 profilometer, KAL-Tencor, San Jose, CA) and for contact angle (hydrophobicity) with a goniometer (Ramé-Hart, model number 100-00, Ramé-Hart, Inc, Mountain Lakes, NJ) (21). Surface characteristics had been visualized previously by environmental SEM (Phillips SL30 ESEM, FEI Co., Hillsboro, OR) (21).

Each coupon was washed with nonbactericidal detergent (Versa-Clean, Fisher Scientific, Pittsburgh, PA); rinsed with ultrapure, RO water, air dried, placed into 10x100-mm glass petri dishes, and sterilized in an autoclave. A spore preparation was adjusted to a 0.5 McFarland standard with a Microscan turbidity meter, resulting in a 1 x 10^6 CFU spores/mL suspension. This suspension was diluted 1:10 in 95% ethanol and vortexed at high speed for 1 min. A 0.5-mL aliquot was placed on the coupon with a repeat pipettor, then evenly spread over the surfaces of each of the stainless steel coupons with the side of a sterile disposable pipette tip. The lids of the petri dishes were closed, and the dishes with test coupons were placed in a biological safety cabinet and allowed to dry overnight. The coupons were then sampled with swabs.

Sampling

For each material, 70 spore-laden coupons were used: 10 controls, 30 sampled with dry swabs, and 30 sampled with swabs premoistened with PBST. If premoistened, swabs were dipped in a tube containing PBST, then pressed against the side of the tube to express excess liquid. Swabs were swiped across each coupon methodically in a horizontal, then vertical, and then diagonal direction several times. During sampling, care was taken to sample up to, but not over, the edge of the coupon. The swabs were rolled to expose unused sides as they were moved across the surface of the coupon.

After sampling, swabs were placed into tubes containing 5 mL of PBST. From the 60 swabs of each material that were used for sampling, 10 premoistened and 10 dry swabs were subjected to minimal agitation, 10 premoistened and 10 dry swabs were vortexed for 2 min in 10-s bursts, and 10 premoistened and 10 dry swabs were placed into a Branson 42 kHz (100 W) ultrasonic bath (Branson Instruments, Danbury, CT) and sonicated for 12 min. Serial dilutions were performed (10^{-1}–10^{-5}) in BB, and 100 µL from each dilution tube was spread on TSAB plates in duplicate. Plates were incubated at 36°C overnight, and colonies were counted the next day.

Ten control coupons were processed as follows: each coupon was aseptically transferred to a 600-mL beaker and covered with 20 mL of PBST, sonicated for 12 min, and then scraped with a sterile cell scraper (Fisher Scientific, cat # 07-200-365) for 1 min to remove the spores. Two mL from the 600-mL beaker was plated directly onto TSAB plates (500 µL to each of four plates). Serial dilutions were performed (10^{-1}–10^{-3}) in BB, and 100 µL from each dilution tube was spread on TSAB plates in duplicate. Plates were incubated at 36°C overnight, colonies were counted the next day, and the number of CFUs recorded.

Analysis and Statistics

Ten coupons were used for each swab material, swab preparation, and processing protocol to be evaluated. This procedure allowed us to identify significant differences in the sample means (CFUs) of ≥12% with 80% power. Mean CFUs were determined for each dilution, and the total

number of organisms per coupon was calculated by multiplying by the dilution factors. Percent recovery efficiencies (%RE) were calculated by using the following equation: $\%RE = (\sum [N_{SW}/N_0]/n) \times 100$, where N_0 is the number of CFUs from the control surfaces, N_{SW} is the number of CFUs from the swab material, and n is the sample size. Effects of swab preparation and processing protocol (combined as recovery method) and swab materials and their interactions were analyzed with general linear model procedure for analysis of variance of unbalanced data. Pairwise comparison of appropriate treatment means was done by Student *t* test and also by Bonferroni adjustment for multiple comparisons (22).

Results

Directly Inoculated Swabs versus Surface-Sampled Swabs

Scanning electron micrographs of the swab materials used in this study are shown in the Figure. At the scale provided by micrographs, three materials (polyester, rayon, and cotton) appear to have fibers similar in size and density, though the polyester has more spaces closed by irregularly shaped fibers. The macrofoam appears to have a more open structure than the other three materials. When swabs were inoculated directly with the spore suspension, then processed with vortexing, all swab materials tested released significantly higher percentages of spores than were recovered by swabs that sampled spores from the stainless steel surfaces (Table 1). No significant differences were observed between cotton, macrofoam, and rayon in their abilities to release spores ($p > 0.05$) when directly inoculated. Cotton, macrofoam, and rayon released 93.9%, 93.4%, and 91.7% of spores inoculated onto them, respectively. The polyester swab released a significantly lower percentage than the other three materials (83.8%, $p < 0.01$).

Dry versus Moist Swabs

For each material tested, premoistened swabs were more efficient than dry swabs at recovering spores from the stainless steel coupons. Results of unadjusted *t* tests show that recovery of spores from all compared materials (Table 2) is significantly improved by premoistening the swabs, regardless of which processing protocol is used ($p < 0.05$). However, when the multiple comparison adjustment was applied, the efficiency of polyester and rayon swabs do not appear to be significantly improved by premoistening ($p = 1.0$), regardless of processing method. When no extraction was performed during laboratory processing of the swabs, no significant differences were found between spore recovery with dry and premoistened swabs of any material ($p = 1.0$) (Table 2). When sonication was used as the extraction method during laboratory processing of swabs, no differences were seen between spore recovery with dry and premoistened swabs of any material ($p = 1.0$) (Table 2). Spore recovery with vortexed cotton and macrofoam swabs improved significantly ($p < 0.01$) when swabs were premoistened. This combination of materials and processing method provided the highest percentage of spores recovered.

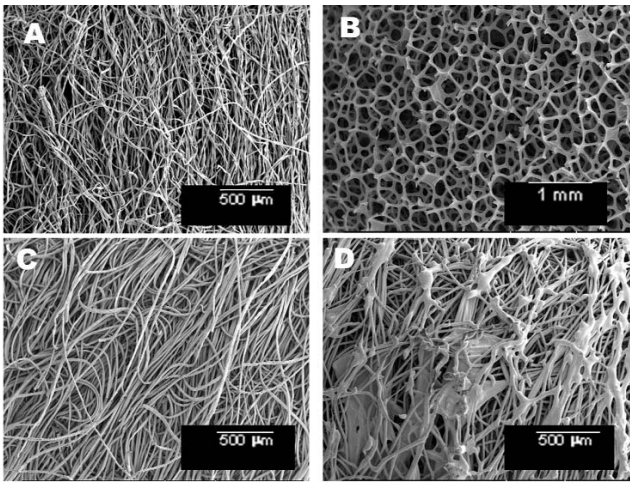


Figure. Environmental scanning electron micrographs of swab material: cotton (A), macrofoam (B), rayon (C), polyester (D).

Extraction versus No Extraction

When premoistened swabs were considered, an extraction method enhanced recovery for all materials when compared to processing the same materials with minimal agitation (no extraction) (Table 3). These improved recovery efficiencies were significant for all materials ($p < 0.01$) when an unadjusted *t* test was used for comparison, but not for polyester or rayon when the multiple comparison

Table 1. Percentage of spores recovered from premoistened, directly inoculated, and vortexed swabs [a]

Swab material	Mean	Median	SD	Range[b]	95% CI[c]
Cotton	93.9	93.1	10.1	72.5–112.9	87.7–100.2
Macrofoam	93.4	96.6	10.8	73.4–107.7	86.6–100.1
Polyester	83.8	81.9	7.2	73.3–98.2	79.4–88.3
Rayon	91.7	92.6	6.3	79.8–100.7	87.8–95.5

[a]N = 10.
[b]Percentages calculated relative to mean of control tests, allowing maximum to be >100%.
[c]CI, confidence interval.

Table 2. Comparison of spore recovery efficiencies by swab preparation, material, and recovery methods

	Mean percentage recovery from 2x2-inch steel coupon (SD), N = 10				
Recovery method	All swabs	Cotton	Macrofoam	Polyester	Rayon
All					
Dry	4.4 (4.7)	5.1 (3.9)	8.4 (6.2)	1.2 (1.0)	3.0 (2.2)
Premoistened	14.3 (14.9)	20.0 (18.1)	22.5 (17.5)	7.7 (5.3)	7.0 (6.8)
p[a]	< 0.01	< 0.01	< 0.01	1.0	1.0
Extraction[b]					
Dry	6.5 (4.4)	7.5 (2.3)	12.3 (3.2)	1.7 (0.8)	4.4 (1.0)
Premoistened	19.7 (15.5)	27.7 (17.7)	30.7 (15.9)	10.6 (4.1)	10.0 (6.4)
p[a]	< 0.01	< 0.01	< 0.01	1.0	1.0
No extraction					
Dry	0.4 (0.7)	0.5 (0.4)	0.7 (1.1)	0.1 (0.2)	0.1 (0.2)
Premoistened	3.5 (3.1)	4.7 (2.2)	6.3 (3.9)	2.0 (1.0)	1.0 (0.8)
p[a]	1.0	1.0	1.0	1.0	1.0
Vortex					
Dry	6.6 (4.2)	8.0 (1.4)	11.9 (3.1)	2.1 (0.9)	4.4 (1.0)
Premoistened	26.7 (18.9)	41.7 (14.6)	43.6 (11.1)	9.9 (3.8)	11.5 (7.9)
p[a]	< 0.01	< 0.01	< 0.01	1.0	1.0
Sonication					
Dry	6.4 (4.8)	6.9 (3.0)	12.7 (3.4)	1.4 (0.5)	4.5 (1.0)
Premoistened	12.7 (5.6)	13.6 (3.2)	17.7 (5.9)	11.2 (4.4)	8.5 (4.4)
p[a]	1.0	1.0	1.0	1.0	1.0

[a]Adjusted for multiple comparisons by Bonferroni correction.
[b]Vortex and sonication combined.

correction was applied (p = 1.0). When only premoistened swabs were considered, the macrofoam yield increased from 6.3% to 30.7% with extraction, and the cotton yield increased from 4.7% to 27.7% with extraction (Table 3).

Comparison of Premoistened, Extracted Materials

If we consider only premoistened, extracted swabs, the macrofoam and cotton were the most efficient of the four materials with percentages of recovered spores of 30.7% and 27.7%, respectively, with no significant difference between them (p = 1.0). Polyester and rayon swabs (10.6% and 10.0%, respectively, Table 3) were significantly less efficient than the cotton and macrofoam swabs (cotton and macrofoam vs. polyester and rayon, unadjusted $p < 0.01$). However, no significant difference was found between the recovery efficiencies of rayon and polyester swabs if swabs were premoistened and extracted (p = 1.0).

Vortex versus Sonication

Of the two extraction methods (Table 3), vortexing premoistened macrofoam and cotton swabs (43.7% and 41.7% recovery, respectively) resulted in a significantly greater recovery than did sonication of each material (17.7% and 13.6%, respectively) ($p < 0.01$). The differences between the two methods were not significant for polyester or rayon (p = 1.0).

Discussion

The swab-rinse method was originally developed by Mannheimer and Ybanez in 1917 to assess the bacterial contamination of eating utensils (5). In 1944, the American Public Health Association included it in its recommended methods for food utensil sanitation monitoring (23). It is still recommended for various applications in the food industry (18). NASA adapted this method for spacecraft applications and developed other methods, such as a wipe-rinse and vacuum probe method, to assess organisms in outgoing spacecraft (8–11,24). NASA recommended that the swab not sample more than a 4-in^2 area and that a 2-min sonication step be included during swab extraction. The American Society for Microbiology's Clinical Microbiology Procedures Handbook also recommends that a 2x2-in area be used in environmental and medical device sampling (16).

The results of this study suggest the superiority of macrofoam swabs that are moistened before sampling and vortexed during processing. The findings of this study are consistent with previous work showing the overall low efficiency of using swabs for surface sampling and the low precision of the method as reflected in the wide range in recovery of spores from steel coupons. Angelotti et al. (3) found that cotton swabs recovered 30.4%–69.9% of *Micrococcus pyogenes* and 30.1%–43.2% of *B. globigii* (currently *B. atrophaeus*) (25) spores. They suggested that the variations in a controlled laboratory setting were minimal when compared to those in field applications, where factors such as variations in sampling area, sampling technique (pressure applied, speed of sampling), distribution of spores on the surface, presence of dust or soil, or physical or chemical properties of the surface could further reduce recovery. They proposed that the low precision of swabs is not only inherent in sampling, but that each step in

extraction can also introduce error that contributes to the low precision (3). Suggested examples of processing variables include inconsistent release of spores from swabs due to variations in vortexing or sonication, pipetting errors, and colony-counting errors. Some have suggested that alginate swabs would be better for recovery of spores, since they dissolve completely in sodium hexametaphosphate and the potential for spores to be retained in the swab would be eliminated. Angelotti et al. (3) and Strong et al. (26), however, found that calcium alginate swabs were less efficient at removing spores from a surface than were cotton swabs, and may inhibit some organisms, including *B. globigii* spores.

Work by Barnes (13) showed that the percentage of *Bacterium* (currently *Escherichia*) *coli* and *Staphylococcus albus* recovered from a smooth drinking glass by a cotton swab varies with inoculum level. For *E. coli*, the percentage recovered was lower when the inoculum was higher (56% at 10^4/glass and 40% at 10^5/glass), but *S. albus* demonstrated a higher percentage recovered with a higher inoculum (38% at 10^4/glass, and 71% at 10^5/glass). Inherent differences likely exist in each organism's ability to adhere to smooth glass. *B. anthracis* spore adherence properties were not explored in this study. Hucker et al. (27) demonstrated that recovery of microorganisms from surfaces by cotton swabs is directly proportional to the ease of wetting the surface. This work reinforces the idea that swabs should be premoistened with a solution containing a surfactant, such as Tween 80, for maximum retrieval of spores.

Sampling efficiency of cotton swabs was investigated by Buttner et al. (28), in which glass petri dishes were inoculated with 10^6 *B. subtilis* subsp. niger (currently *B. atrophaeus*) (25) spores suspended in buffer with 0.05% Tween 20, distributed within a 5-cm^2 area and sampled with cotton swabs. The higher mean recovery (68.6%) in this study may be attributed to the higher spore inoculum contained in a smaller surface area, reduced spore adherence to the more hydrophilic glass surface, or the spores being suspended in the buffer with a surfactant that would also reduce adhesion to the surface.

Our study found that recovery was most efficient when macrofoam or cotton swabs were moistened before sampling and subjected to vortex extraction. Puleo et al. (24) reported that sonication provided a better recovery of *B. subtilis* subsp. niger (currently *B. atrophaeus*) (25) spores

Table 3. Percentage recovery of premoistened swabs

Recovery method	Mean	Median	SD	Range	95% CI[a]	p[b]
All swabs						
All	14.3	9.4	14.9	0.4–63.9	11.2 to 17.4	
Extraction[c]	19.7	14.4	15.5	4.8–63.9	16.3 to 23.1	< 0.01
No extraction	3.5	2.7	3.1	0.4–13.5	2.5 to 4.4	
Vortex	26.7	23.7	18.9	1.4–29.0	20.8 to 32.6	< 0.01
Sonication	12.7	13.0	5.6	4.8–63.9	11.0 to 14.5	
Cotton swabs						
All	20.0	13.3	18.1	2.6–62.5	13.5 to 26.5	
Extraction	27.7	20.0	17.7	7.3–62.5	16.7 to 38.7	< 0.01
No extraction	4.7	4.0	2.2	2.6–9.7	3.3 to 6.1	
Vortex	41.7	43.7	14.6	23.9–62.5	33.7 to 51.8	< 0.01
Sonication	13.6	13.3	3.2	7.3–19.5	11.3 to 15.3	
Macrofoam swabs						
All	22.5	16.7	17.5	1.8–63.9	16.3 to 28.8	
Extraction	30.7	29.7	15.9	7.0–63.9	20.8 to 40.5	< 0.01
No Extraction	6.3	6.5	3.9	1.8–13.5	3.9 to 8.7	
Vortex	43.6	44.9	11.1	30.4–64.0	36.8 to 50.5	< 0.01
Sonication	17.7	16.7	5.9	7.0–29.0	14.0 to 21.3	
Polyester swabs						
All	7.7	6.4	5.3	0.5–16.5	5.8 to 9.6	
Extraction	10.6	11.1	4.1	4.8–16.5	8.1 to 13.1	1.0
No Extraction	2.0	2.0	1.0	0.5–3.4	1.4 to 2.5	
Vortex	9.9	10.0	3.8	4.8–14.4	7.5 to 12.3	1.0
Sonication	11.2	12.3	4.4	4.8–16.5	8.5 to 13.9	
Rayon swabs						
All	7.0	6.0	6.8	0.4–24.0	4.6 to 9.4	
Extraction	10.0	8.1	6.4	1.4–24.1	6.0 to 14.0	1.0
No extraction	1.0	0.9	0.7	0.4–2.9	0.5 to 1.4	
Vortex	11.5	11.5	7.9	1.4–24.1	6.6 to 11.2	1.0
Sonication	8.5	7.5	4.4	2.5–18.9	5.7 to 11.3	

[a]CI, confidence interval.
[b]Adjusted for multiple comparisons by Bonferroni correction.
[c]Vortex and sonication methods combined.

than mechanical agitation from stainless steel coupons. Their study differed in that the mechanical agitation in Puleo's study consisted of placement on a platform shaker at 270 oscillations per min for 10 min, rather than agitation by vortexing, which provides a more vigorous motion, as was done in this study. Since Puleo's experimental methods and equipment differed from those used in this study, a comparison of results may not be valid. His study, however, does illustrate the wide variability of recovery inherent in sampling with swabs. Puleo et al. (29), in a separate study, also established that sonication does not affect spore viability.

When swabs were inoculated directly, approximately 84%–94% of spores were recovered, yet surface sampling in the current study yielded <50% of spore inoculum. If the swabs retain only 6.1%–16.2% of the spores, the differences in recovery efficiencies of spores from directly inoculated swabs and those used to sample spore-inoculated surfaces can be explained only by assuming a substantial number of spores remain on the stainless steel coupon. Unlike powder preparations, spores, when applied with alcohol may become fixed to the surface after evaporation of the alcohol, which may represent a challenge to their recovery; however, the method provides a standard application to enable comparison of the swab materials and processing protocols. In this evaluation, no attempt was made to measure the amount of spores remaining fixed to the coupon surface. Since a perception exists (though no supporting data could be found by the authors) that polymerase chain reaction–based methods for detecting *B. anthracis* in processed samples are hindered by the presence of cotton fibers or impurities associated with cotton swabs, it was important to find that comparable results can be obtained by using macrofoam swabs.

Though no significant differences were seen between premoistened and dry rayon or polyester swabs, regardless of the processing method (Table 2), all of these recovery efficiencies were ≤11.5%, and in many cases, standard deviations were high. Similarly, no significant differences were seen between extracted and nonextracted premoistened rayon and polyester swabs (Table 3). The percentage recovery efficiencies of each of these groups were small, and the standard deviations were large.

All currently available environmental sampling techniques (i.e., wipes, HEPA sock) have inherent advantages and disadvantages. Each method should be evaluated to determine the overall recovery efficiencies of the materials together with the processing protocols. With this information, incident response personnel will be better able to choose the best sampling methods needed for each surface within the contaminated area. Swabbing environmental surfaces may not be the most efficient means of recovering bacterial contamination if quantitation (i.e., estimate of magnitude) is the objective of the sampling; however, in some situations a swab sample may be the best available sampling method. We hope that this brief study will help in the choice of the best material for environmental sampling and aid in interpreting results.

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Ms. Rose is a research microbiologist with the National Center for Infectious Diseases, Division of Healthcare Quality Promotion. Her research interests include environmental sampling, disinfection, and fate of select agents in environmental settings.

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Address for correspondence: Laura Rose, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop C16, Atlanta, GA 30333, USA; fax: 404-639-3822; email: lrose@cdc.gov

Epidemiologic Clues to SARS Origin in China

Rui-Heng Xu,* Jian-Feng He,* Meirion R. Evans,†‡ Guo-Wen Peng,* Hume E Field,§ De-Wen Yu,* Chin-Kei Lee,¶ Hui-Min Luo,* Wei-Sheng Lin,* Peng Lin,* Ling-Hui Li,* Wen-Jia Liang,* Jin-Yan Lin,* and Alan Schnur#[1]

An epidemic of severe acute respiratory syndrome (SARS) began in Foshan municipality, Guangdong Province, China, in November 2002. We studied SARS case reports through April 30, 2003, including data from case investigations and a case series analysis of index cases. A total of 1,454 clinically confirmed cases (and 55 deaths) occurred; the epidemic peak was in the first week of February 2003. Healthcare workers accounted for 24% of cases. Clinical signs and symptoms differed between children (<18 years) and older persons (≥65 years). Several observations support the hypothesis of a wild animal origin for SARS. Cases apparently occurred independently in at least five different municipalities; early case-patients were more likely than later patients to report living near a produce market (odds ratio undefined; lower 95% confidence interval 2.39) but not near a farm; and 9 (39%) of 23 early patients, including 6 who lived or worked in Foshan, were food handlers with probable animal contact.

On March 12, 2003, the World Health Organization (WHO) issued a global alert about cases of atypical pneumonia in Guangdong Province and Hong Kong Special Administrative Region, China, and in Vietnam (1). The disease, now known as severe acute respiratory syndrome (SARS), is caused by coronavirus infection (2,3) and subsequently spread rapidly worldwide. The earliest identified cases of the disease occurred in Guangdong Province in late 2002 (4).

On January 2, 2003, two cases of atypical pneumonia in the city of Heyuan, Guangdong Province, were associated with transmission of infection to several healthcare workers at the hospital (5). Investigation by the Guangdong Provincial Center for Disease Control and Prevention led to the identification of clusters of cases in six other municipalities (Foshan, Jiangmen, Zhongshan, Guangzhou, Shenzhen, Zhaoqing) from November 2002 to mid-January 2003. On February 3, 2003, province-wide mandatory case reporting of atypical pneumonia that used a standard case definition and reporting form was instituted. The provincial health department also introduced a range of public health control measures, including guidelines on epidemiologic investigation of cases and contacts (February 3) and on hospital admission, clinical management, and infection control arrangements for patients (February 9). Subsequently, the department issued guidelines on community prevention and control, including mandatory home quarantine of contacts (March 27); commenced public service announcements about personal protection and seeking prompt medical attention (March 27); and introduced free hospital treatment for patients with SARS (April 30). Border control measures were introduced at all points of entry into the province during mid-April according to WHO recommendation (6). We describe the epidemiology of the SARS epidemic in Guangdong through April 30, 2003, focusing on the observed pattern of spread of disease, the signs and symptoms, and the investigation of early cases.

Methods

Study Population

Guangdong Province has a population of 85.2 million, including 9.9 million in Guangzhou city (7). All public health and most hospital services are under the direction of the Health Bureau of Guangdong Provincial People's Government. The public health function is performed by one provincial Center for Disease Control and several municipal centers, together with a network of district and county centers, each responsible for a population of 500,000–1 million. Nearly all hospitals are operated by the public sector, but patients are charged for medical

*Guangdong Province Center for Disease Control and Prevention, Guangzhou, China; †University of Wales College of Medicine, Cardiff, United Kingdom; ‡National Public Health Service for Wales, Cardiff, United Kingdom; §Animal Research Institute, Brisbane, Australia; ¶Australian National University, Canberra, Australia; and #World Health Organization, Beijing, China

[1]Drs. Evans, Field, and Lee were consultants for the World Health Organization; they assisted in its Beijing office.

treatment. Primary health care in the province is rudimentary, and most patients report directly to hospital emergency rooms.

Data Sources

We analyzed data from two sources: the Guangdong surveillance database and a case investigations database. We also interviewed staff from the Guangdong Provincial Centers for Disease Control, and Foshan Municipal Center for Disease Control to obtain supplementary information on early-onset cases. Information on early cases in the neighboring Guangxi Province was obtained from local investigators by a visiting WHO team, led by one of the authors (CKL). Early cases were defined as those with a date of onset from November 1, 2002, to January 31, 2003, and late cases as those with a date of onset from February 1 to April 30, 2003. Population denominators were obtained from the Guangdong provincial census for 2000 (7).

Surveillance Database

Guangdong Provincial Center for Disease Control coordinated the surveillance database. Early cases were identified during the course of case investigations or after voluntary reporting by clinicians. Such cases were only included in the database if they conformed to the case definition subsequently adopted for surveillance. Since early February 2003, hospitals at all levels in the health system were required to report cases of atypical pneumonia (probable SARS) immediately by telephone to the local center for disease control, which then forwarded reports electronically to the provincial center on the same day. The diagnostic criteria for reporting were: 1) having close contact with a patient or having infected other people, 2) fever (>38°C) and symptoms of respiratory illness, 3) leukocyte count $\leq 10.0 \times 10^9$/L, 4) radiographic evidence of infiltrates consistent with pneumonia or respiratory distress syndrome on chest x-ray, and 5) no response to antimicrobial drug treatment (within 72 hours). Patients were considered to be probable SARS patients if they meet criteria 1–4 or 2–5 but were excluded if an alternative diagnosis could fully explain their illness. The dataset contains patient demographics, including occupation; dates of onset, admission and report; criteria required for the case definition; and details of laboratory specimens that were collected.

Case Investigation Database

Contact tracing staff at the district center level administered a standard questionnaire to all case-patients within 24 hours of reporting. These data form the basis of the case investigation database and comprise patient demographics; clinical features on admission to hospital; contact history (living with, working with, caring for, or visiting the home of a patient) and name, age, and address details of contacts; and exposure risk factors for community cases (non-healthcare workers), including travel history, hospital visits, animal contact, and living conditions. Patients not employed as healthcare workers were classified as community case-patients. Comparisons were made between features of early-onset and late-onset community cases, and primary (no contact history) and secondary community cases. Extra information was collected on early patients by means of informal interviews with center staff, which focused on index patients in each of the seven municipalities initially affected. Data were particularly sought on occupational history and contact networks, and a detailed case series was compiled.

Data Analysis

Data were entered into Excel databases (Microsoft, Redmond, WA). Descriptive analyses were carried out using EpiInfo version 6 (Centers for Disease Control, Atlanta, GA) and SPSS version 10.0 (SPSS Inc., Chicago, IL). The surveillance database was used for analysis by age, sex, occupation, and clinical signs and symptoms. The case investigation database was used for comparing early- and late-onset cases and cases with or without a contact history. Incidence rates were calculated for November 2002 to April 2003. For comparisons of signs and symptoms by age, younger adults were used as the reference group for both children and older persons. Chi-square test or, when appropriate, Fisher exact test was used for comparison of proportions, Mann-Whitney test for comparison of continuous variables, and chi-square test for linear trend for analysis of time trends. We report maximum likelihood estimates for odds ratios (OR) with exact 95% mid-p confidence intervals (CI) and consider $p < 0.05$ to be significant.

Results

Surveillance Database

A total of 1,454 SARS cases were reported in Guangdong Province from November 16, 2002, through April 30, 2003, including 55 deaths, a crude case-fatality rate of 3.8% for all ages, and 12.7% in people ≥65 years. Two children died: a 4-year-old, previously healthy girl with lobar pneumonia of unknown cause and a 10-year-old boy with recent acute hepatitis B.

The initial phase of the epidemic, November–late January, was characterized by sporadic cases followed by a sharp rise in late January and a sharp decline in the first half of February, and thereafter a gradual decline (Figure 1). The epidemic peak occurred at the end of the first week of February with approximately 55 new cases each day. Cases occurred in 15 municipalities in the province but were concentrated in the Pearl River delta area (Figure 2A), and confined almost exclusively to urban areas, particularly the

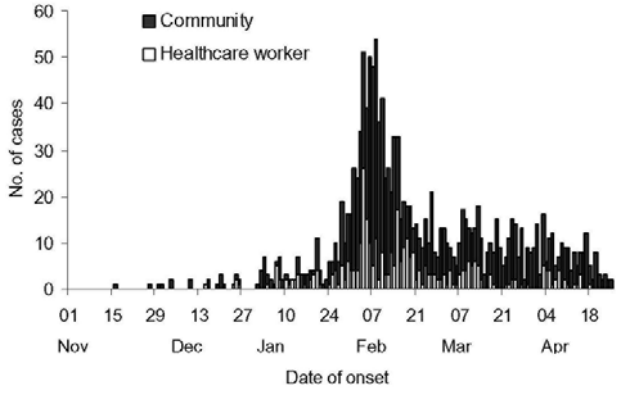

Figure 1. Epidemic curve of cases of severe acute respiratory syndrome by date of onset, November 1, 2002–April 30, 2003, in Guangdong Province, China, showing cases in the community and in healthcare workers.

seven municipalities of Foshan, Guangzhou, Heyuan, Jiangmen, Shenzhen, Zhongshan, and Zhaoqing (located 20–130 km from Guangzhou). The highest incidence occurred in Guangzhou city (12.5 cases per 100,000 people) (Figure 2B), and outbreaks appear to have occurred in different municipalities at varying stages of the epidemic (Figure 3).

Median age of patients was 35.0 years (range 0–92 years), and the highest age-specific incidence was in persons 65–69 years (3.2 per 100,000 people) (Figure 4); 53.2% of cases were female. Five deaths occurred among 343 cases in healthcare workers (24% of 1,429 cases for whom occupation is known); 75.1% of healthcare worker cases were in women. The proportion of healthcare worker patients was highest in the early phase of the epidemic (32% with date of onset in January 2003) and declined as the epidemic progressed (27% in February, 18% in March, and 17% in April) (Table 1). Throughout the epidemic, a high proportion of community case-patients did not report contact history, ranging from 58% in February to 74% in April. This proportion was even higher in children (91% in those <5 years, 81% in those 5–14 years) and in persons ≥65 years (82%).

Analysis of occupation status, excluding healthcare workers and case-patients with known exposure, shows that the proportion of cases in students (0% in January 2003 or before; 7% in February; 14% in March; 18% in April, $p < 0.001$) and housewives (0% in January 2003 or before; 5% in February; 14% in March; 15% in April, $p < 0.001$) increased as the epidemic progressed (Table 2). A high proportion (9/23, 39%) of early cases were food handlers (this category includes persons who handle, kill, and sell food animals, as well as those who prepare and serve food), but none were farmers handling livestock or poultry. Of the nine early cases in food handlers, seven were restaurant chefs working in township restaurants (where a variety of animals were slaughtered on the premises), one was a market produce buyer for a restaurant, and one was a snake seller in a produce market (where a variety of live animals were offered for sale). Six of the food handlers lived or worked in Shunde (1.7 million population), an urban district of Foshan municipality, though none could be directly linked to each other through contact history.

Case Investigation Database

Detailed data from case investigation interviews were available for 662 (45%) of 1,454 patients. Median age was 31.0 years (range 0–86 years), 56% were female, and 44% were male. The signs and symptoms in adults (18–64 years) were compared with those in children (<18 years) and older persons (≥65 years) (Tables 3 and 4). Children were more likely to have a runny nose and dry cough on physical examination but less likely to have chills, malaise, headache, muscle aches, or difficulty breathing. Older persons were more likely to report having sputum and to have a dry or productive cough on physical examination but less likely to complain of chills, malaise, or sore throat. Nearly

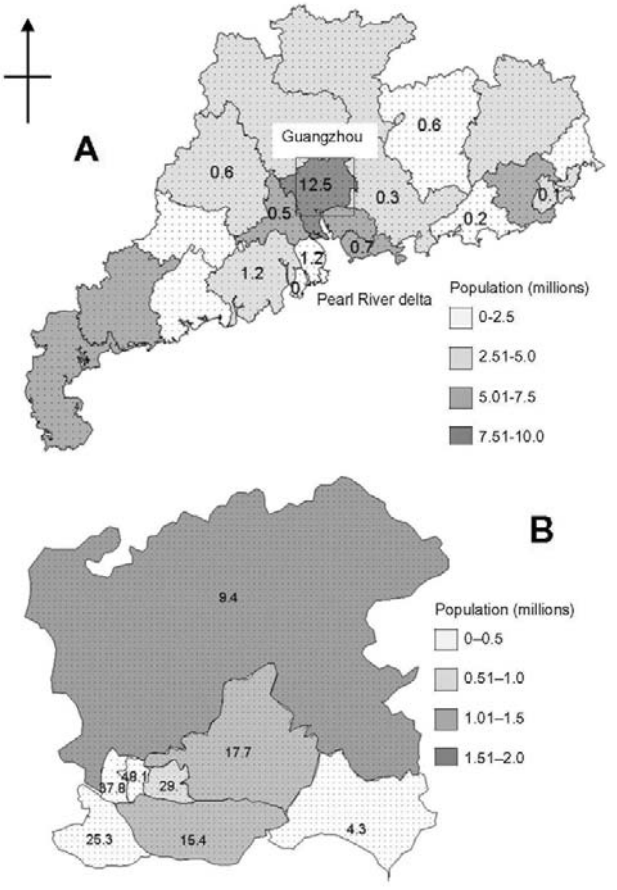

Figure 2. Geographic distribution of population in: (A) urban districts of Guangzhou city, (B) Guangdong Province and district-specific incidence of severe acute respiratory syndrome (per 100,000 population).

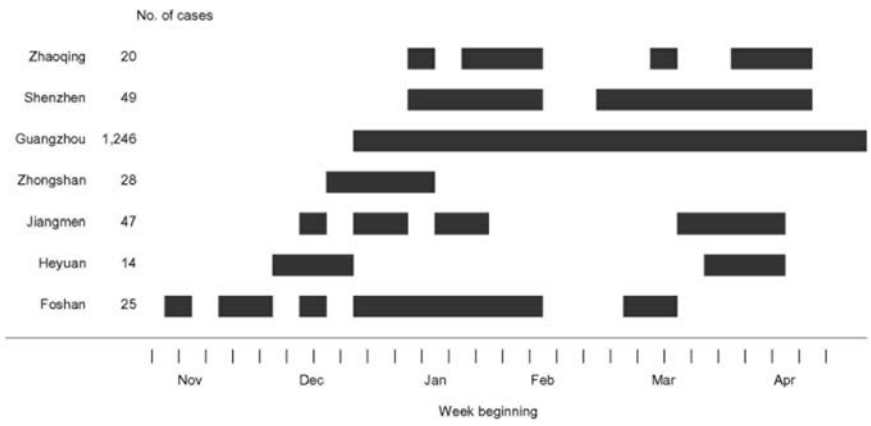

Figure 3. Timeline of cases of severe acute respiratory syndrome by week of onset, November 1, 2002–April 30, 2003, in the seven predominantly affected municipalities of Guangdong Province, China.

all patients had a high body temperature (median 39.0°C, range 36.8–42.0°C) lasting in most patients for 1 to 4 days (median 4.0 days; range 1–9 days). Median leukocyte count was 5.8 x 10^9/L (range 1.0–63.4 x 10^9/L), and 13.9% of patients had leukopenia (<3.5 x 10^9/L). Older persons had a higher median leukocyte count than younger adults (6.6 x 10^9/L for those ≥65 years, 5.6 x 10^9/L for those 18–64 years, p = 0.056), and fewer had leukopenia.

Comparison of case categories indicate that community case-patients with a contact history were more likely to have visited a hospital in the previous 2 weeks than patients without a contact history (OR 6.83, 95% CI 2.89 to 16.73) (Table 5). Patients without a contact history were no more likely to report a history of travel or animal contact. Early-onset patients were more likely to live within walking distance of a produce market (an agricultural market where live animals are sold, killed, and butchered in situ, also known as a "wet market") than late-onset patients (OR undefined, lower 95% CI 2.39). Living near a poultry or livestock farm or having other types of animal contact, including domestic pets or livestock, poultry, or specific wild animals or birds, was not associated with a high risk for SARS.

Case Series of Index Patients

The index patients in each of the seven earliest affected municipalities all had a date of onset before January 31, 2003 (Table 6). In five municipalities (Foshan, Jiangmen, Zhongshan, Guangzhou, Shenzhen), outbreaks appear to have occurred independently, but the outbreak in Heyuan may be linked to that in Shenzhen and the outbreak in Zhaoqing to that in Guangzhou. Index patients from the eight other municipalities involved in the epidemic had a date of onset after March 1, 2003, and a travel history to an affected area, so these were excluded from the analysis.

Patient 1 had the earliest case, identified by retrospective case searching. He lived with his wife and four children in Foshan city and became ill on November 16, 2002. He had not traveled outside Foshan in the 2 weeks before his illness and had no contact history, but he had prepared food including chicken, domestic cat, and snake. He was part of a cluster of five patients, including his wife (42 years old, onset December 1), a 50-year-old aunt who visited him in the hospital on November 20 (onset November 27), and the aunt's 50-year-old husband (onset November 30) and 22-year-old daughter (onset December 4) (online Appendix, Cluster A; available from: http://www.cdc.gov/ncidod/EID/vol10no6/03-0852_app.htm). None of patient l's four children were ill. Subsequent clusters in Foshan included a food handler who infected a family member and two healthcare workers (online Appendix, Cluster B) and a food handler who infected several healthcare workers (online Appendix, Cluster C).

Patient 2 lived in Heyuan but worked as a restaurant chef in Shenzhen. His work was mainly stir-frying and did not involve killing animals. His animal contact history is unknown. He returned to Heyuan after becoming ill, was admitted to the local hospital and transferred to Guangzhou 2 days later. He infected a work colleague (41-year-old man, onset December 16), six healthcare workers in Heyuan (onset December 24–28), and a physician who

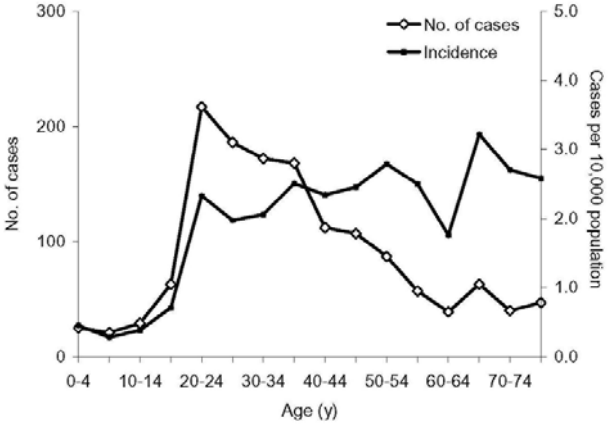

Figure 4. Number of patients with severe acute respiratory syndrome by age, and age-specific incidence (per 10,000 population), November 1, 2002–April 30, 2003, Guangdong Province, China.

Table 1. Month of onset of SARS in community case-patients and in healthcare workers, Guangdong, China, November 2002 –April 2003[a]

Month of onset	Community case-patients contact history		Healthcare worker (%)	Total[b]
	Yes (%)	No (%)		
Nov 2002	0 (0)	3 (100)	0 (0)	3 (100)
Dec 2002	2 (11)	12 (63)	5 (26)	19 (100)
Jan 2003	31 (18)	87 (50)	55 (32)	173 (100)
Feb 2003	104 (14)	419 (58)	195 (27)	718 (100)
Mar 2003	63 (20)	197 (62)	59 (18)	319 (100)
Apr 2003	16 (9)	129 (74)	29 (17)	174 (100)
Total	216 (15)	847 (60)	343 (24)	1,406 (100)

[a]SARS, severe acute respiratory syndrome.
[b]Information was not available on contact history for 48 cases.

accompanied him in the ambulance from Heyuan to Guangzhou (28-year-old man, onset December 25). Patient 3, from Jiangmen, had no contact history, no history of animal contact, and no known forward transmission. Patient 4 worked as a chef in a Zhongshan township restaurant, where he prepared steamed dishes and had contact with snakes, civet cats, foxes, and rats. He infected his 30-year-old wife (onset January 3), a 39-year-old male friend who visited him in the hospital (onset January 5), and a physician (35-year-old man, onset January 4). Patient 4 was one of two patients responsible for infecting at least three healthcare workers (onset January 4–7). Patient 5, from Guangzhou, had no history of animal contact other than with a pet guinea pig that died a month before his symptoms began. He infected a hospital intern (onset January 14) and six other healthcare workers (onset January 14–22) at Guangdong Traditional Chinese Medicine Hospital. Patient 6, from Shenzhen, had visited Hong Kong on January 14, the day before symptom onset. However, he had no contact history or contact with animals. He infected a work colleague (43-year-old man, onset January 29) and died 14 days after becoming ill. Patient 7, from Zhaoqing, was the only female index case. She traveled to Guangzhou 2 weeks before becoming ill, although she could not recollect contact with anyone with symptoms of SARS. She worked at a market but did not sell animals. She infected her 16-year-old son (onset February 3) and a physician (25-year-old woman, onset January 31).

The index patient in the neighboring province of Guangxi was a 26-year-old man, who infected several family members. He worked as a driver for a wild animal dealer and returned home to Guangxi after becoming ill on January 8, 2003. The dealer supplied Guangdong markets with wild animals from Guangxi, other Chinese provinces, and Vietnam.

Discussion

The epidemic of atypical pneumonia in Guangdong Province that we describe bears all the hallmarks of SARS (8–11). It demonstrates the typical time course of the epidemic, the preponderance of cases in urban areas, and the epidemiologic and clinical features of the disease. Since the SARS epidemic began in Guangdong, we have sought epidemiologic clues about the origin of the disease. Approximately 75% of emerging infectious diseases are zoonotic (12), and evidence is accumulating that an animal origin for SARS is probable. However, phylogenetic analysis and sequence comparisons of the coronavirus that causes SARS (SARS-CoV) indicate that the virus is not closely related to any of the previously characterized human or animal coronaviruses (13). The reservoir is still unknown, but SARS-CoV has been isolated from Himalayan palm civets (*Paguma larvata*), and evidence of infection has been found in a raccoon dog (*Nyctereutes procyonoides*), a Chinese ferret-badger (*Melogale moschata*), and humans working at a live animal market in

Table 2. SARS cases (%) by month of onset and occupational status, Guangdong, China, November 2002–April 2003[a]

Occupational status[b]	Jan 2003 or before no. (%)	Feb 2003 (%)	Mar 2003 (%)	Apr 2003 (%)	Total (%)
Retired	2 (9)	44 (10)	46 (23)	32 (16)	124 (15)
Worker	2 (9)	40 (9)	28 (14)	22 (11)	92 (11)
Student	0 (0)	29 (7)	28 (14)	34 (18)	91 (11)
Civil servant	3 (13)	43 (10)	26 (13)	19 (10)	91 (11)
Housewife	0 (0)	20 (5)	28 (14)	30 (15)	78 (9)
Food industry worker	9 (39)	20 (5)	4 (2)	19 (10)	52 (6)
Farmer	1 (4)	10 (2)	4 (2)	4 (2)	19 (2)
Teacher	1 (4)	7 (2)	6 (3)	4 (2)	18 (2)
Child	0 (0)	9 (2)	4 (2)	4 (2)	17 (2)
Other	2 (9)	49 (11)	14 (7)	18 (9)	83 (10)
Unknown	3 (13)	157 (37)	14 (7)	8 (4)	182 (21)
Total	23 (100)	428 (100)	202 (100)	194 (100)	847 (100)

[a]SARS, severe acute respiratory syndrome.
[b]Excluding healthcare workers or case-patients with known exposure.

Table 3. Prevalence (%) of symptoms on admission to hospital, SARS patients, Guangdong, China, November 2002–April 2003[a]

Symptoms	All (n = 662)	Adults (18–64 y) (n = 534)	Children (<18 y) (n = 51)	OR (95% CI)	p	Older persons (≥ 65 y) (n = 66)	OR (95% CI)	p
Fever	97.4	97.4	98.0	1.4 (0.2 to 29.3)		98.5	1.8 (0.3 to 37.9)	
Chills	51.8	56.0	31.4	0.4 (0.2 to 0.7)	0.002	37.9	0.5 (0.3 to 0.8)	0.008
Malaise	42.3	45.7	23.5	0.4 (0.2 to 0.7)	0.004	31.8	0.5 (0.3 to 1.0)	
Headache	40.0	42.1	25.5	0.5 (0.2 to 0.9)	0.03	43.1	0.7 (0.4 to 1.3)	
Muscle ache	30.8	35.0	5.9	0.1 (0.0 to 0.3)	<0.001	16.7	0.4 (0.2 to 0.7)	0.004
Cough	69.8	60.0	82.4	2.2 (1.1 to 4.9)	0.05	80.3	1.9 (1.1 to 3.8)	0.05
Sputum	38.2	36.9	39.2	1.7 (0.9 to 3.3)		51.5	1.8 (1.1 to 3.1)	0.03
Sore throat	16.3	16.7	25.5	1.1 (0.6 to 2.0)		6.1	0.3 (0.1 to 0.8)	0.04
Runny nose	7.4	6.7	15.7	2.6 (1.1 to 5.7)	0.04	7.6	1.1 (0.4 to 2.8)	
Breathing difficulty	26.7	27.0	11.8	0.4 (0.1 to 0.8)	0.03	43.3	1.8 (1 to 3.0)	0.05
Nausea	8.8	9.4	5.9	0.6 (0.1 to 1.8)		7.6	0.8 (0.3 to 2.0)	
Vomiting	6.2	6.0	7.8	1.3 (0.4 to 3.7)		7.6	1.3 (0.4 to 3.3)	
Diarrhea	8.6	9.0	7.8	0.9 (0.3 to 2.3)		7.6	0.8 (0.3 to 2.2)	

[a]SARS, severe acute respiratory syndrome; OR, odds ratio, using adults as the reference group; CI, confidence interval.

Shenzen municipality (14). Seroprevalence of immunoglobulin (Ig) G antibody to SARS-CoV is substantially higher among traders of live animals (13.0%) in Guangzhou municipality than among healthy controls (1.2%), and the highest prevalence of antibody is among those who traded primarily masked palm civets (72.7%) (15). The pattern of the Guangdong epidemic is consistent with the classical process of emergence from an animal reservoir: the initial introduction of the virus into a nonimmune human population followed by the establishment and rapid dissemination of infection (16). The traditional practice of using wildlife for food and medicine, still observed by some persons in southern China, offers an effective bridge from a natural animal host to humans. Several observations support this hypothesis. Two of the seven index patients were restaurant chefs; food handlers (who handle, kill, or butcher animals) were overrepresented among early-onset cases with no contact history (including the first reported death, in a snake seller); and patients with early onset were more likely than patients with late onset to live near an agricultural produce market (where live wild animals are generally offered for sale). However, none of the early patients were commercial farmers nor was living near a farm associated with increased risk, findings that suggest a wild animal rather than a livestock or poultry source. Although trade in wildlife is now illegal in China, a range of mammalian, avian, and reptile species can still be found in some produce markets, and a black market in these species probably exists. Many such animals come from outside Guangdong Province, often through Guangxi Province to the west, and may originate in Vietnam or other parts of Southeast Asia. The observation that the index patient in Guangxi Province was a wild animal trader who supplied Guangdong markets offers some circumstantial evidence for such a link.

Our data have several limitations. First, surveillance for SARS was only established in February 2003; thus, information on earlier cases was collected retrospectively and will be influenced by reporting bias. Second, our analysis is based on cases that are not laboratory confirmed. Thus, the diagnosis relies on a clinical case definition and the sensitivity and specificity are unknown. Third,

Table 4. Prevalence (%) of physical signs, chest x-ray findings, and blood count abnormalities on admission to hospital, SARS patients, Guangdong, China, November 2002–April 2003[a]

Variable	All (n = 662)	Adults (18–64 y) (n = 534)	Children (<18 y) (n = 51)	OR[b] (95% CI)	p	Older persons (≥65 y) (n = 66)	OR[b] (95% CI)	p
Physical signs								
Temperature (>38°C)	91.3	90.9	97.8	0.9 (0.3 to 3.9)		89.7	0.5 (0.1 to 1.2)	
Rigors	15.9	16.9	10.9	0.5 (0.2 to 1.3)		10.6	0.6 (0.2 to 1.2)	
Lethargy	10.3	11.8	2.0	0.2 (0.0 to 0.8)		4.5	0.4 (0.1 to 1.1)	
Myalgia	6.6	8.1	2.0	0.2 (0.0 to 1.2)		0.0	0.0 (0.0 to 0.6)	0.009
Cough	50.0	46.6	64.7	2.1 (1.9 to 3.9)	0.02	68.2	2.5 (1.4 to 4.3)	0.002
Sputum	10.4	8.6	13.7	1.7 (0.7 to 3.8)		24.2	3.4 (1.8 to 6.4)	<0.001
Dyspnea	6.0	5.8	2.0	0.3 (0.0 to 1.8)		12.1	2.2 (0.9 to 4.5)	
Clinical test results								
Abnormal chest x-ray	87.2	87.5	84.3	0.8 (0.4 to 1.8)		86.4	0.9 (0.4 to 2.0)	
Leukopenia ($<3.5x10^9$/L)	13.9	14.2	16.3	1.2 (0.5 to 2.7)		8.3	0.6 (0.2 to 1.5)	

[a]OR, odds ratio; CI, confidence interval.
[b]Using adults as the reference group.

Table 5. Case-case comparisons of community SARS patients, Guangdong, China, November 2002–April 2003, according to contact history and onset date[a]

Exposure (in previous 2 weeks)	No contact history (n = 406)		Contact history (n = 103)		OR (95% CI)	Early onset[b] (n = 19)[c]		Late onset[b] (n = 387)[c]		OR (95% CI)
	Yes	No	Yes	No		Yes	No	Yes	No	
Visited hospital	17	70	22	13	6.83 (2.89 to 6.73)	0	10	45	169	0.00 (0.00 to 1.36)
Visited by a friend	4	71	1	17	1.04 (0.04 to 8.93)	0	1	4	70	0.00 (0.00 to 337)
Regular hand washing	122	15	44	3	1.80 (0.53 to 8.10)	4	1	118	14	0.48 (0.06 to 12.55)
Travel history	45	179	13	62	0.83 (0.41 to 1.63)	0	10	45	169	0.00 (0.00 to 1.36)
Animal contact	37	262	13	56	1.64 (0.80 to 3.25)	1	3	36	259	2.39 (0.09 to 23.02)
Visited produce market	9	79	1	19	0.41 (0.02 to 2.75)	0	1	9	69	0.00 (0.00 to 148)
Lives near produce market	89	169	19	43	0.84 (0.45 to 1.52)	5	0	84	169	Undef. (2.39 to Undef.)
Lives near poultry or livestock farm	6	252	3	59	2.13 (0.42 to 8.81)	0	19	6	247	0.00 (0.00 to 40.15)

[a]SARS, severe acute respiratory syndrome; CI, confidence interval; OR, odds ratio.
[b]Defined as November 1, 2002–January 31, 2003 for early onset; February 1, 2003 –April 30, 2003 for late onset.
[c]Cases with no contact history and for whom case investigation data are available.

case investigation data were only available on approximately half of all cases because of poor transfer of data (regardless, all categories of cases and all reporting districts were similarly affected). Finally, information on several earlier cases was incomplete or may be unreliable (because of fear of prosecution associated with the trade in wild animals), and some persons are no longer traceable.

The data also highlight several unanswered questions. The SARS epidemic started in Guangdong, but how it began, why it peaked so suddenly, why Guangzhou was so badly affected and other cities spared, and what caused the gradual decline are all unclear. The decline in the epidemic is probably a result of hospital and community infection control measures introduced in early February, including strict isolation of patients, use of protective equipment by healthcare workers, prohibition of hospital visitors, and guidelines on epidemiologic investigation. Such measures may also explain why later cases did not trigger such extensive chains of transmission. The concentration of cases in urban areas may be due to factors associated with access to healthcare or to incomplete or poor surveillance in rural areas. However, in Guangdong Province, emphasis was placed on reporting from less developed prefectures and rural areas, including supervisory visits and review of hospital records. Many larger cities in Guangdong, as well as rural areas, were also apparently spared by the epidemic.

Although the possibility that SARS may pre-date the earliest known case cannot be excluded, the temporal and spatial clustering of index cases described in the case series suggests that the initial source of the Guangdong epidemic was either a single point source (with the links between cities not identified) or several point sources in the Pearl River basin. Outbreaks in cities affected later in the epidemic can all be traced to an imported case. These later cases are probably due to horizontal transmission rather than to further contact with the initial source. The reason for the sudden rise in the incidence of cases at the beginning of February is unclear, although the rise coincides with the admission of a highly infectious index patient who transmitted SARS to healthcare workers at three different hospitals in Guangzhou city and to a large number of family members (5,17). The absence of such a trigger may explain the much smaller outbreaks in other cities in the province. The case-fatality rate in Guangdong was also lower than documented elsewhere (11,18). The most likely explanation for this lower rate is the younger age structure of the population in mainland China compared to that of Hong Kong, Taiwan, or Canada. Children have slightly different initial signs and symptoms than adults perhaps because symptoms are milder (19), children are less able to describe their symptoms, or the case definition is less specific in this age group. Older persons are more likely to have a

Table 6. Case series of index cases by municipality in SARS epidemic, Guangdong, China, November 2002–April 2003[a]

Case no.	City	Sex	Age	Occupation	Date of onset	Animal contact	Secondary transmission
Case 1	Foshan	M	45	Administrator and village leader	Nov 16, 2002	Yes	Yes
Case 2	Heyuan	M	34	Restaurant chef	Dec 10, 2002	Unknown	Yes
Case 3	Jiangmen	M	26	Factory worker	Dec 21, 2002	No	No
Case 4	Zhongshan	M	30	Restaurant chef	Dec 26, 2002	Yes	Yes
Case 5	Guangzhou	M	49	Office worker	Jan 2, 2003	No	Yes
Case 6	Shenzhen	M	46	Office worker	Jan 15, 2003	No	Yes
Case 7	Zhaoqing	F	39	Market vendor	Jan 17, 2003	Probably	Yes

[a]SARS, severe acute respiratory syndrome; M, male; F, female.

productive cough and difficulty breathing than younger adults, which suggests either a misdiagnosis or an underlying chest disease.

The high proportion of community case-patients with no apparent contact history, especially in Guangzhou city, may be due to inadequate tracing, poor reporting of the results of contact investigation, asymptomatic transmission, or the nonspecificity of the clinical case definition. Similar findings have been observed in Beijing (20). Little evidence as yet exists for asymptomatic infection with SARS, but seroprevalence studies will help determine whether this occurs. Laboratory testing of stored clinical specimens may also clarify the specificity of the case definition, particularly if positivity rates vary during different stages of the epidemic or in cases with no contact history. An alternative explanation for the absence of a contact history is the continuing existence of an environmental source; however, this explanation is not borne out by case investigation data. Clarifying this factor is important not only to help understand the transmission dynamics of SARS but also to allay concerns about the risk for epidemic spread in the community if SARS is reintroduced. Resolving this issue will be vital to prospects for preventing the spread of SARS beyond China (21).

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Dr. Xu Rui-Heng is an epidemiologist and deputy director of the Guangdong Province Center for Disease Control and Prevention. He coordinates the provincial communicable disease surveillance network and has research interests in the epidemiology of communicable diseases.

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Address for correspondence: Meirion Evans, Communicable Disease Surveillance Centre, National Public Health Service for Wales, Abton House, Cardiff CF14 3QX, UK; fax: +44 2920 521987; email: meirion.evans@nphs.wales.nhs.uk

Mycobacterium ulcerans Treatment Costs, Australia

Christina Drummond* and James R.G. Butler†

Mycobacterium ulcerans gives rise to severe skin ulceration that can be associated with considerable illness. The cost of diagnosis, treatment, and lost income has never been assessed in Australia. A survey of 26 confirmed cases of the disease in Victoria was undertaken. Data were collected on demographic details, diagnostic tests, treatment, time off work, and travel to obtain treatment. All costs are reported in Australian dollars in 1997–98 prices. The cost varies considerably with disease severity. For mild cases, the average direct cost is $6,803, and for severe cases $27,681. Hospitalization accounts for 61% to 90% of costs, and indirect costs amount to 24% of the total per case. *M. ulcerans* can be an expensive disease to diagnose and treat. Costs can be reduced by early diagnosis and definitive treatment. Research is needed to find cost-effective therapies for this disease.

Mycobacterium ulcerans causes disfiguring ulcers with substantial illness (1). These distinctive ulcers were first described in Australia in 1948 and given the name Bairnsdale ulcer because of their focal distribution. In Australia, in addition to the Bairnsdale area in Gippsland, Victoria, cases have been reported from foci in Queensland and the Northern Territory (1,2).

Until 1982 to 1983, when three cases were detected near Western Port Bay, cases in Victoria were confined to the Bairnsdale area. Since 1983, over 50 cases have been recorded in Victoria outside this area, and the disease appears to be spreading progressively westward. An outbreak on Phillip Island in 1993 and 1994 resulted in 27 reported cases (3,4). From 1990 to 1997, 22 cases within approximately 70 km of Melbourne were reported, 19 of these on the Mornington Peninsula with 12 in the Frankston/Langwarrin area (Figure). For the first time, cases were detected west of Melbourne in 1998, and the number occurring in that area is increasing (Victorian Mycobacterium Reference Laboratory and Department of Human Services).

*Monash Medical Centre, Melbourne, Victoria, Australia; and †Australian National University, Canberrra, Australian Capital Territory, Australia

Treatment involves hospitalization for debridement and often skin grafting, which frequently has to be repeated (5–7). Patients are frequently absent from work for long periods, which results in considerable cost to the community. In Australia, the cost of the diagnosis, treatment, and lost income has never been accurately assessed. The objective of this study was to assess the direct and indirect cost of *M. ulcerans* infection occurring outside previously known disease-endemic areas of Victoria, Australia.

Methods

A survey of 26 cases of the disease in Victoria was undertaken. A case-patient was defined as a person with a clinical lesion with histologic, culture, or polymerase chain reaction evidence of *M. ulcerans* infection. Eligible patients with *M. ulcerans* were contacted and sent a questionnaire requesting demographic data, treatment information, time off work, and distance traveled to obtain treatment. One or more phone calls were made by the primary investigator to clarify and expand the data and seek evidence. Calls often lasted for 1 hour. Pathology reports, patient diaries, and information from physician files were used, where possible, to confirm information. The eligibility criteria were based on age (6 months of age to 100 years) and date of onset of the disease (January 1, 1991–August 31, 1998). Cases occurring in the disease-endemic areas of Gippsland and Phillip Island were excluded.

Two sources were used to find cases. The first source was the Victorian Mycobacterium Reference Laboratory. It receives specimens either directly, when mycobacteria cultures are requested, or as cultures from other laboratories to confirm mycobacterium and determine the species. The second source included records kept by a well-known Victoria pathologist with a longstanding interest in the disease; other pathologists frequently refer specimens from patients with suspected cases to this physician.

Treating physicians were contacted and approval sought from them before attempts were made to contact their patients. All known case-patients were contacted, and the questionnaire was completed by telephone interview. When a telephone number was incorrect, all leads were

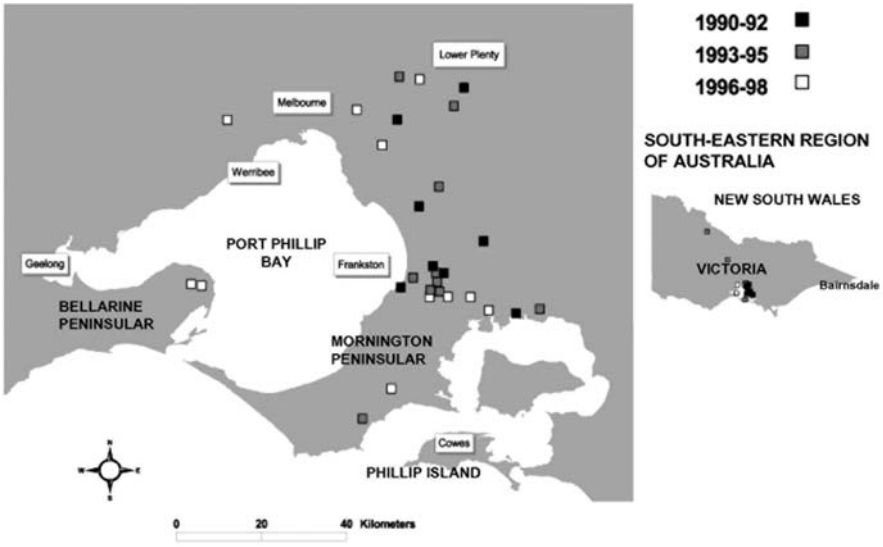

Figure. Cases of *Mycobacterium ulcerans* occurring in areas of Victoria (southeastern Australia), where disease is nonendemic, January 1990–August 1998.

followed as far as possible to locate the patient. Private and public hospitals might have served as a third source for case finding, but they are not obligated to report cases of *M. ulcerans*, and their record-keeping systems are such that abstracting cases would be very difficult.

In total, 32 persons with *M. ulcerans* who met the case definition during the study period were located. Of these, 26 (81%) could be contacted. All agreed to complete the questionnaire (100% response rate).

Patients were divided into three categories on the basis of their clinical history. Patients with a mild disease had a biopsy and excision only. Patients with moderate disease had one lesion only, which was treated with biopsy and excision, with only one skin graft required. Patients with severe disease had multiple lesions, multiple debridements, or multiple skin grafts.

Medical, hospital, and other services used to diagnose and treat each patient, the time lost from his or her usual occupation, along with the cost of transportation to care facilities for patients traveling over 50 km each way were recorded. Physicians who treated the patients, hospital records, old prescriptions, and the like were used to verify cases.

Cost Data

All cost data are expressed in Australian dollars using Australian financial year 1997–1998 prices. The unit cost of a visit to a local physician (general practitioner) and to a specialist (apart from inpatient care) was estimated by using the average fee charged across a range of relevant Medicare Benefits Schedule (MBS) item numbers for the year 1997–98 (Table 1). For specialists, the average was calculated across both first and subsequent visits. Patients treated as outpatients had a biopsy of the lesion with microscopy and culture for mycobacteria. MBS fees for the biopsy (MBS item no. 30071) and for the microscopy and culture (MBS item no. 69207) were taken as the unit costs of these tests.

The cost of blood tests required for monitoring a patient who was receiving special medications was estimated by using the MBS fee for liver function tests (MBS item no. 66211). Most patients receiving special medications would have these tests at least monthly.

Hospital costs were calculated according to whether the patient was treated in a public or a private hospital. For both hospital types, the per diem cost for the first 4 days of hospitalization was based on the national average cost per day for DRG 505 (other skin graft ± debridement) in 1996–97. The per diem cost for any days of hospitalization in excess of 4 was then taken as the overall average cost per day. This per diem cost is the same for both public and private hospitals (8).

The cost of antimicrobial agents was estimated by using the cost paid by the patient (if known) or the costs paid by a hospital pharmacy to purchase the drug. These costs were for bulk purchases of the various medications. When the name of the antimicrobial agent was unknown, the cost of a course of antimicrobial agents was estimated at $5 per course. Medications specific for mycobacteria included clarithromycin 500 mg twice daily, rifampicin 600 mg daily, and ethambutol 400 mg three times/day. Hospitals' costs for these drugs were used to estimate the cost of each patient's course of treatment.

A visiting nurse attended the homes of some patients. The Royal District Nursing Service estimates that the average cost of a home visit was $44.10/hour (The Service, pers. comm.). When this figure is used, an average visit (including travel time) for dressing changes was estimated at 40 minutes. Thus, a rate of $29/visit was used as the estimated cost of a visit. A number of patients had heat treatment with

Table 1. Cost of diagnosing and treating *Mycobacterium ulcerans* cases (Australian dollars, 1997–98 prices)[a]

Item	Unit	Cost
General practitioner visits	Visit	$24.68
Specialist visits	Visit	$57.98
Hospitalization	Cost per day for DRG 505 (other skin graft ± debridement): public hospital	$776
	Cost per day for DRG 505 (other skin graft ± debridement): private hospital	$935
	Hospitalization over 4 days	$598/day
Dressings	Bandages[b]	$18/week
	Dressings[b]	$20/week
Medications	Agent unknown	$5/course
	Clarithromycin 500 mg twice daily	$3.89/dose
	Rifampicin 600mg daily	$0.36/dose
	Ethambutol 400 mg three times/day	$0.71/dose
Home nursing	Visits	$29/visit
Laboratory costs	Biopsy (MBS item no. 30071)	$38.80
	Microscopy and culture (MBS item no. 69207)	$28.00
	Liver function tests (MBS item no. 66211)	$16.80
Heat treatment	Day[b]	$3.00/day
Lost income	Day[b]	Average weekly earnings of $766.80
Transport	Kilometer[b]	$0.50/km

[a]DRG, diagnosis-related group; MBS, Medicare Benefits Schedule.
[b]If known, the actual cost was used; otherwise, it was estimated from the unit cost.

various devices at variable costs. If known, the actual cost was included. Some patients used electrical devices such as electric blankets continuously and for prolonged periods. If costs were not known, a standard rate was used.

Dressings and bandages used by patients were priced at a commercial pharmacy, and the likely cost to the patients was calculated on the basis of their reported use. When details of the amount used were not obtainable, a standard rate for bandages and dressings was used. The cost of treatment not routinely incurred was estimated, where possible, for individual patients. These items included aids such as crutches, extra investigations such as ultrasound, occupational therapy, and physiotherapy.

The value of time lost from work because of illness was calculated by using an estimate of average weekly earnings (9). No cost was included for travel time per se or the time lost by children from school or kindergarten. The time lost from work by parents as a result of a child's illness or hospitalization was also not included. No cost was included for the time lost from normal duties by retired persons or those engaged in home duties at the time of their illness. An estimate for travel expenses was included only for patients whose treatment required substantial travel (>50 km each way).

Results

Direct Costs

The average direct cost of treating patients with this disease was $14,608 (Table 2). This cost differed most

Table 2. Average direct cost for diagnosing and treating a patient with *Mycobacterium ulcerans*

	Cost in Australian dollars, 1997–1998 prices (% of column total)[a]			
	Mild case	Moderate case	Severe case	All cases
No. of cases	12	8	6	26
Medical practitioner costs				
General practitioner visits	274	253	82	223
Specialist visits	377	739	628	546
Total visits	651 (10)	992 (6)	710 (3)	769 (5)
Other costs				
Hospitalization	4,139 (61)	12,607 (76)	24,840 (90)	11,522 (79)
Dressings	1,134 (17)	1,206 (7)	1,230 (4)	1,178 (8)
Medications	357 (5)	959 (6)	449 (2)	561 (4)
Home nursing	458 (7)	680 (4)	211 (1)	468 (3)
Laboratory costs	49 (0.7)	57 (0.3)	31 (0.1)	47 (0.3)
Heat treatment	15 (0.2)	24 (0.1)	210 (0.8)	63 (0.4)
Total direct costs	6,803 (100)	16,525 (100)	27,681 (100)	14,608 (100)

[a]Any differences between totals and sums of components are due to rounding.

noticeably between patients who required, or did not require, skin grafting. The average cost increased from $6,803 for patients not requiring a skin graft to $27,681 for those requiring multiple procedures.

Hospitalization was by far the most expensive component of the direct costs. This cost ranged from an average of $4,139 (61% of total direct cost) for mild cases to an average of $24,840 (90%) for severe cases. The second greatest expense in all categories of the disease was for dressings, approximately $1,200 for all categories of disease severity. This cost was much less than the cost of hospitalization in all categories but it was equivalent to approximately 5% of the cost of hospitalization in the severe disease category. In this category, the cost of dressings was more than double that of medications.

The third greatest expense in all categories was medical care. As the disease severity increased, the cost of medical care shifted from general practitioners to specialist care. The average cost of specialist visits ranged from $377 for mild cases to $739 for patients who received one skin graft, while average general practitioner costs fell from $274 for mild cases to $82 for severe cases. For patients with mild cases, the cost of outpatient specialist visits accounted for 58% of the cost of medical practitioner care. For patients with severe disease, this cost increased to 88%.

For the moderate and severe categories, medications were the next greatest expense, which included regular antimicrobial agents and drugs targeted for mycobacteria. These latter medications accounted for most of the cost of medications. Although approximately 50% of patients in each category were treated with these expensive drugs, those in the moderate category incurred the greatest expense as they had the longest courses of these medications. The cost per case in the moderate category was twice that in the severe category.

Only nine patients received home nursing care. One of these received 108 visits and another 96 visits. More cases in the moderate disease category received home nursing care than those in the other categories (five of the eight cases compared to two of the six in the severe category). Home nursing service in the moderate category cost an average of $680.

Heat treatment and laboratory costs were the least expensive components of the treatment costs. Heat treatment was mainly confined to the severe disease category. Laboratory costs were similar across all categories.

Indirect Costs and Transport Costs

Time missed from regular occupation and income losses were estimated for nine patients who had regular jobs at the onset of the disease (six mild, one moderate and two severe cases) (Table 3). Of the 17 for whom income losses were not estimated, 9 were children and 5 were retired persons. Three were housewives who had difficulty estimating the amount of lost performance. A monetary value is difficult to determine for this occupation, and thus no estimate was included for their time or financial loss (Table 3). The average income loss for the nine patients increased as the severity of the disease increased, from $12,451 for those with mild cases to $16,431 and $19,170 for those with moderate and severe cases, respectively. Income losses for these nine patients were 0–$40,000. Average income losses (including for unemployed patients) are seen in Table 4.

A small number of case-patients (n = 3) incurred major costs as a result of transportation from the country to city centers for treatment. The greatest cost estimate for this transport was $2,900 for one patient with severe disease. A patient with a mild case incurred transport costs of $1,000 and a patient with a moderate case, $46. Averaged across all patients within each severity grouping, the average transport cost per patient was $83, $6, and $483 for the mild, moderate and severe groups, respectively (Table 4).

Discussion

M. ulcerans is costly to treat. This study found that the average cost of diagnosing and treating a case was $14,608. This figure is approximately seven times the average health expenditure per person in Australia in 1997–98 of $2,557 (10). Hospitalization costs form most of the overall costs, accounting for 90% of the total cost for severe cases and 79% for all cases. Indirect costs accounted for 25% of the overall (direct, indirect, and transportation) cost but considerably more for individual patients. For those with mild disease, income losses accounted for 47% of the overall cost.

These cost assessments are conservative for several reasons. First, most patients were treated in public hospitals as public patients; in Australia, such treatment is usually less costly than treating a private patient. Thus these costs could have been substantially higher if more cases were treated privately in Australia and could vary substantially in other populations. Second, income losses would have been much greater if more patients had been in the income-

Table 3.Time missed from work or school as a result of *Mycobacterium ulcerans* infection, January 1, 1991–August 31,1998

	Categorization of case (n = 23)			
Days missed	Mild (n = 11)	Moderate (n = 8)	Severe (n = 4)	All
Days missed from work or school (median)[a]	2	21	159	28
Range	0–365	0–150	0–548	0–548

[a]Some patients were retired, and some illness occurred during school holidays.

Table 4. Average aggregated cost per case of *Mycobacterium ulcerans* cases, January 1991–August 1998 (26 cases)

	Cost in Australian dollars, 1997–98 prices (percentage of column total)[a]			
Costs	Mild cases	Moderate case	Severe case	All cases
Total direct costs	6,803 (52)	16,525 (89)	27,681 (80)	14,608 (74)
Lost income	6,226 (47)	2,054 (11)	6,390 (18)	4,980 (25)
Transport	83 (0.6)	6 (0.0)	483 (1.4)	152 (0.8)
Total costs	13,112 (100)	18,585 (100)	34,554 (100)	19,740 (100)

[a]Any differences between totals and sums of components are due to rounding.

generating age group. Third, the cost of impaired productivity of parents caring for their children and accompanying them for appointments and hospitalizations was not included in this study, but this cost is likely to have been considerable.

The patients who had one skin graft incurred the highest cost for medications because they were treated with longer courses of drugs specific for mycobacteria. Three of these patients were treated for ≥6 months. These patients were given a trial of medication in an attempt to avert the need for surgery or as an adjunct to surgery in an attempt to prevent recurrence of the disease.

The marked decrease in general practitioner costs in severe cases reflects the referral pattern for this disease. Some patients with severe disease had previous contact with a specialist (usually a surgeon) as a result of treatment for a previous lesion, or they required more extensive surgery and skin grafting, which general practitioners would not normally perform. As patients with severe cases received more treatment as inpatients, and the cost of surgeon visits while in the hospital were included in the hospital charges, the cost of specialist treatment incurred by the patient does not clearly reflect the amount of care they received.

Variation around the mean cost within each category of disease severity was marked. Some of this variation was due to the lack of standardized, accepted treatment regimes. In addition, delays until definitive treatment varied greatly between patients, sometimes because of difficulties in making the correct diagnosis. This wide range probably accounted for the failure of indirect costs to increase in direct proportion to disease severity.

A limitation of this study was its reliance upon a patient questionnaire to collect data on diagnosis, treatment services received, and time off work. Because of the considerable amount of time that had lapsed since their illness, some patients had difficulty accurately recalling details of the events in their disease history. The number of visits to physicians and the duration of particular events such as hospitalization or time missed from work were most difficult to recall precisely. Some patients provided very accurate information because of diaries or prescriptions that they had kept during their illness or because of records from hospitals and the physicians who treated them.

Validating some information provided by patients was difficult. The date of onset, time before seeking medical help, and date of cure were frequently not documented and may be difficult to determine accurately. Where possible, attempts were made to validate dates and number of physician visits or duration of hospitalization, but insufficient resources were available to validate all data provided by patients. A prospective study, performed at the time of an outbreak of this disease, would be useful to more accurately determine the cost of treatment.

The early diagnosis and implementation of effective, definitive treatment would greatly reduce both the illness and economic impact from *M. ulcerans* infection. Education of medical practitioners and the public is required. If community awareness of this disease is increased, patients will seek treatment earlier. Educating medical practitioners about the clinical features of this disease and its recent geographic spread are essential for early diagnosis and appropriate treatment to be promptly implemented. Since this disease is very appropriate for nursing assistance at home, hospitalization costs can be drastically reduced by implementing this method of treatment after surgery. Early and definitive treatment would also substantially reduce the time missed from work and the associated cost.

The role of medication directed against mycobacteria, heat therapy, and other therapies reported as helpful by individual patients requires investigation. An alternative to the extensive surgery currently required by most patients would greatly reduce the illness and cost of this disease. Recently, much progress has been made in detecting these bacteria in the environment and in rapidly diagnosing lesions (11,12).

M. ulcerans is of increasing public health concern worldwide. In African countries, treatment costs for one case far exceed the governmental health spending per capita (13). The increasing numbers of cases in many countries, the increasing number of countries affected, and the substantial disability and loss of income that result from this disease underscore the need for continuing research into rapid diagnostic methods and cost-effective treatments.

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Dr. Drummond is a specialist in infectious diseases and public health. Her research interests are in communicable diseases and their control and providing health care to disadvantaged populations. At the time of this research, she was a visiting medical officer, Department of Human Services, Melbourne.

Dr. Butler is a senior fellow in health economics and deputy director at the National Centre for Epidemiology and Population Health, Australian National University. He is also president of the Australian Health Economics Society. As a health economist, his interests include healthcare financing and economic evaluation. He has recently undertaken cost-effectiveness analyses of pneumococcal conjugate vaccine and meningococcal C conjugate vaccine.

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Address for correspondence: Christina Drummond, Monash Medical Centre, Clayton Rd, Clayton, 3168, Victoria, Australia; fax: 61-(3)-9594-4533; email: drcdrummond@optusnet.com.au

Distribution of Bovine Spongiform Encephalopathy in Greater Kudu (*Tragelaphus strepsiceros*)

Andrew A. Cunningham,* James K. Kirkwood,*[1] Michael Dawson,†[2] Yvonne I. Spencer,† Robert B. Green,† and Gerald A.H. Wells†

Of all the species exposed naturally to the bovine spongiform encephalopathy (BSE) agent, the greater kudu (*Tragelaphus strepsiceros*), a nondomesticated bovine from Africa, appears to be the most susceptible to the disease. We present the results of mouse bioassay studies to show that, contrary to findings in cattle with BSE in which the tissue distribution of infectivity is the most limited recorded for any of the transmissible spongiform encephalopathies (TSE), infectivity in greater kudu with BSE is distributed in as wide a range of tissues as occurs in any TSE. BSE agent was also detected in skin, conjunctiva, and salivary gland, tissues in which infectivity has not previously been reported in any naturally occurring TSE. The distribution of infectivity in greater kudu with BSE suggests possible routes for transmission of the disease and highlights the need for further research into the distribution of TSE infectious agents in other host species.

To date, 13 species of zoo animal have been confirmed as having died with a novel scrapie-like spongiform encephalopathy (SE) concurrent with the bovine spongiform encephalopathy (BSE) epidemic (Table 1). The disease is thought, in some, if not all, of these species, to be caused by infection with the BSE agent. In addition, natural infection with BSE has been reported in five species of primate in French zoos (11), but these results are considered equivocal for the confirmation of a spongiform encephalopathy (12, G.A.H. Wells, unpub. data). BSE was diagnosed in six of eight greater kudu (*Tragelaphus strepsiceros*), a member of the family Bovidae, subfamily Bovinae, that died at the London Zoo from 1989 through 1992 (2,13,14). The epidemiology of this disease in greater kudu is consistent with either a particularly high susceptibility to infection, the occurrence of direct animal-to-animal transmission of the disease, or with a combination of these factors (2,14,15). To investigate further the biology of BSE in greater kudu, the distribution of the infectious agent in greater kudu with BSE was determined by using the mouse bioassay method.

Materials and Methods

Tissues

Tissues from four greater kudu that died with spongiform encephalopathy were tested for infectivity by bioassay in C57Bl-J6 mice (Table 2). The epidemiologic, clinical, and pathologic findings of the disease in the kudu have been described in detail previously (2,14–16), and a summary of the relevant details is given in Table 3. Tissues for bioassay were collected principally from kudu A1212; each sample was collected in a sterile container by using new disposable instruments and gloves to prevent cross contamination between tissues. As TSE infectivity has been demonstrated previously by bioassay in tissues preserved in formalin and paraffin wax (13,17,18), tissue samples obtained opportunistically after routine postmortem examinations of three additional kudu (A664, A666, and A1221) were also tested for infectivity by using mouse bioassay. Samples collected from kudu A666, A1212, and A1221 were stored in separate, sterile containers and were either frozen at –20°C or fixed in neutral buffered 10% formalin. Non-neural tissues from kudu A664 were fixed in neutral buffered 10% formalin in a common container. The brain from this animal was the last organ removed at necropsy and was fixed in 10% formol saline in a separate container.

Previously, infectivity had been detected in formalin-fixed brain tissue from kudu A664 by bioassay using five

*Institute of Zoology, Regent's Park, London, United Kingdom; and †Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom

[1]Present affiliation is Universities Federation for Animal Welfare, Wheathampstead, Hertfordshire, UK.

[2]Present affiliation is National Scrapie Plan Administration Centre, Worcester, UK.

Table 1. Species of zoo animal with confirmed novel spongiform encephalopathy acquired contemporaneously with epidemic of bovine spongiform encephalopathy in domestic cattle[a]

Species	No. of cases	Reference
Bovidae		
Nyala, *Tragelaphus angasi*	1	1
Greater kudu, *Tragelaphus strepsiceros*	6	2
Gemsbok, *Oryx gazella*	1	1
Arabian oryx, *Oryx leucoryx*	1	3
Scimitar-horned oryx, *Oryx dammah*	1	2
Eland, *Taurotragus oryx*	6	2,4
American bison, *Bison bison*	1	5
Felidae		
Cheetah, *Acinonyx jubatus*	10[b]	2,5–8
Puma, *Felis concolor*	3	2,9
Ocelot, *Felis pardalis*	3	4,5
Tiger, *Panthera tigris*	3	5
Lion, *Panthera leo*	4	4,5
Asian golden cat, *Catopuma temminckii*	1[c]	10

[a]Animals were born and cases occurred in Great Britain unless stated otherwise.
[b] The initial case of transmissible spongiform encephalopathy in a cheetah occurred in Australia, one case in the Republic of Ireland, and three cases in France; all animals were born in Britain except the most recently reported case in France.
[c]Reported from Australia, born in Germany, and kept for a period in the Netherlands (10).

inbred mouse strains, with similar incubation periods and lesion profiles to those demonstrated for the BSE agent from domestic cattle with BSE (13). Bioassay of brain from kudu A664 was not repeated in the current study, but a positive control sample of fresh brain tissue from clinically affected kudu A1212 was tested.

Bioassay

The tissues injected into mice for BSE-bioassay are listed in Table 2. Most of the tissue homogenates were prepared from thawed samples of fresh tissues frozen at –20°C. Tissue homogenates prepared from formalin-fixed tissues were rinsed overnight in running water to leach out the fixative, while formalin-fixed, paraffin-embedded tissues were dewaxed in chloroform (two changes) and washed in several changes of absolute alcohol before being rehydrated by immersion in a series of aqueous solutions of descending concentrations of alcohol, through to 100% water. Material for each tissue homogenate was dissected from the center of each tissue sample by using single-use disposable instruments and rigorous sterile procedures. Each sample was homogenized in 10% physiologic saline to make a 10% wt/vol suspension, which was then passed through a gauze filter. To tissue homogenates containing distal ileum or feces, ampicillin was added at the rate of 1.25 mg/mL.

For each tissue homogenate, 20 C57Bl-J6 mice (4–7 weeks old) were each injected by the intracranial route (0.02 mL) and by the intraperitoneal route (0.10 mL). Single tissue or pooled tissue samples were prepared and injected into C57Bl-J6 mice for a standard qualitative assay of infectivity (13,19).

Mice injected with different tissue or tissue-pool homogenates were housed in separate cages. Injected mice were coded, and detailed clinical monitoring of the mice was carried out by using a standard protocol. The clinical endpoint was determined when mice either showed clear signs of neurologic disease (20) or other deterioration of health. Surviving mice were killed 950 days after injection. Postmortem confirmation of disease in mice was routinely carried out by histopathologic examination of the brain for morphologic changes of spongiform encephalopathy.

After the histopathologic assessment of mice, immunohistochemical examination (IHC) for evidence of spongiform encephalopathy disease-specific PrP (PrP^{Sc}) was performed on the brains of all mice in selected tissue groups. The groups were mice in which either a low number were positive, testing was inconclusive on histopathologic assessment, or the results indicated a novel or anomalous distribution of the agent in kudu compared to that in other TSE. Additional groups of interest (skeletal muscle, endometrium, and mammary gland), which were negative on the histopathologic examination of mouse brains, were also examined by IHC. Immunohistochemical detection of PrP^{Sc} was introduced to the standard protocol to improve specificity and sensitivity of detecting BSE transmission to mice (21,22) and interpret inconclusive histopathologic results (23). For control purposes, the brains from mice that had been injected with pathologically affected cranial thoracic spinal cord from kudu A1212 were also immunostained. Brains from normal mice that were not injected with infected tissues were similarly examined to provide negative controls.

The immunohistochemical method used was essentially that applied previously to cattle central nervous system tissues (24) and adapted for use in mouse brain tissue. Anti-bovine PrP^{Sc} serum (971) was used at 1/8,000 and 1/16,000 dilutions in an avidin-biotin-peroxidase (ABC) complex technique. Transmission was defined by histopathologic evidence of spongiform encephalopathy, or, where applied, immunohistochemical presence of disease-specific PrP (PrP^{Sc}) in the brains of the mice.

Results

The results of the bioassay of tissues are given in Table 2. Based on the histopathologic examination of mouse brains, 15 of the 32 tissue homogenates were positive. The nine histopathologically positive groups examined immunohistochemically were confirmed positive by this method with

Table 2. Bioassay results for greater kudu tissues injected into C57Bl-J6 mice

Kudu identification no.	Tissue[a] injected	Positive mice/total[b]	Mean survival period post injection (days) ± SD[c]	Survival period range (d)[c]
A1212	Rostral cerebrum	13/20	595±84	428–745
	Cranial thoracic spinal cord	19/20[d]	557±121	413–821
	Lumbar spinal cord	15/19	521±69	432–634
	Spleen	3/11[d]	819±41	773–851
	Retropharyngeal lymph node	6/11[d]	784±77	691–921
	Popliteal lymph node	0/20[d]	N/A[e]	N/A
	Visceral lymph nodes (pool)	14/18	622±114	448–860
	Submandibular lymph node	0/20[d]	N/A	N/A
	Distal ileum	11/20	547±98	426–718
	Lung	1/14[d]	N/A	746
	Kidney	0/18	N/A	N/A
	Caruncular endometrium	0/20[d]	N/A	N/A
	Ovary	0/20	N/A	N/A
	Mammary gland	0/20[d]	N/A	N/A
	Submandibular salivary gland	1/17[d]	N/A	599
	Conjunctiva	1/16[d]	N/A	659
	Nasal mucosa	0/20	N/A	N/A
	Skeletal muscle (biceps brachii + vastus lateralis)	0/19[d]	N/A	N/A
	Skin (flank)	2/18[d]	713±22	697–728
	Feces	0/11	N/A	N/A
	Serum	0/19	N/A	N/A
A664	Spleen (P)	1/8[d]	N/A	929
	Visceral lymph nodes (pool) (P)	6/15[d]	843±113	649–952
	Lung (P)	0/20	N/A	N/A
	Kidney (P)	0/20	N/A	N/A
	Skeletal muscle (P)	0/19	N/A	N/A
A1221	Brainstem	10/18	634±62	541–762
	Kidney	0/20	N/A	N/A
	Skeletal muscle (vastus lateralis) (F)	0/19	N/A	N/A
A1221 + A666 (pool)	Spleen (pool)	0/20	N/A	N/A
	Visceral lymph nodes (pool)	10/18	700±108	455–851
	Lung (pool) (F)	0/19	N/A	N/A

[a]Tissues not prepared fresh are suffixed: (F), fixed, (P), paraffin wax embedded.
[b]Number of mice positive/number of mice surviving when the first mouse was confirmed positive by histopathologic or immunochemical examination. The denominator for negative groups is the number of mice examined.
[c]Survival periods of positive mice determined positive either by histopathologic or immunohistochemical examination results.
[d]Mice examined by PrP^{Sc} immunohistochemical examinations.
[e]N/A, not applicable.

marginal improved sensitivity of detection (2–3 more mice positive) in only three groups. For nine of the positive tissue homogenates, prepared from fresh central nervous, lymphoreticular, or distal ileum tissue, the proportion of positive mice (≥40%) indicated moderate or high levels of spongiform encephalopathy infectivity. The remaining six positive groups (Table 2) had low proportions of positive mice (6%–27%), indicative of relatively low titres or only traces of infectivity. Low numbers (1–2) of histopathologically inconclusive mice in five tissue homogenate groups, which included two groups (A1212, popliteal and submandibular lymph nodes) that contained no histopathologically positive mice, were resolved almost exclusively as negative when examined using immunohistochemistry. The one exception was an inconclusive mouse in A1212 retropharyngeal lymph node group, which proved immunohistochemically positive.

Discussion

Fifteen of the 32 kudu tissue homogenates transmitted BSE to mice. The positive result for brain tissue from kudu A1221 confirms the diagnosis of subclinical spongiform encephalopathy in this animal (15) and is the first to demonstrate transmission from a subclinical natural case of spongiform encephalopathy in a bovine species. Also, this report is the first of infectivity in the ileum from a field case of spongiform encephalopathy other than scrapie in sheep.

Apparently low titers or only traces of infectivity were detected in spleen, lung, submandibular salivary gland, conjunctiva, and skin. In bioassays of TSE infectivity, the possibility that trace levels of infectivity in tissues may represent postmortem or laboratory contamination of uninfected tissues with infected material has to be considered. Such an explanation is unlikely in the present study for the

Table 3. Details of spongiform encephalopathy–positive greater kudu used for mouse inoculation studies

Kudu ref no.	Age at death (mo)	Sex	Brief history	Basis of diagnosis
A664	30	F	Born at London Zoo. Died after progressive neurologic disease of approximately 72 hours. Examined postmortem on the same day.	Histopathologic examination of brain and experimental transmission to mice
A666	37	M	Born at London Zoo. Killed after progressive neurologic disease of approximately 24 hours. Examined postmortem on the same day.	Histopathologic, SAF, and PrP^{Sc} immunocytochemical examinations of the brain and spinal cord[a]
A1221	18	M	Born at London Zoo. Killed for management reasons as a clinically healthy animal and immediately examined postmortem.	Histopathologic, SAF, and PrP^{Sc} immunocytochemical examinations of the brain and spinal cord
A1212	39	F	Born in Britain, moved to London Zoo at 12 months of age. Killed following progressive neurologic disease lasting approximately 1 month and immediately examined postmortem.	Histopathologic and SAF examinations of the brain

[a]SAF, scrapie-associated fibrils; PrP^{Sc}, transmissible spongiform encephalopathy disease-specific form of the PrP protein.

following reasons. Many of the tissues which contained only traces of infectivity were taken from kudu A1212, an animal from which tissues were collected by using rigorous sterile procedures to prevent cross-contamination, and no pattern between the order of tissue sampling and the bioassay results from this animal suggests a sequence of tissue contamination. A low incidence of disease in the mice or failure to detect infectivity from tissues previously fixed or processed to paraffin wax may be attributable to a reduced titer of infectivity, which can occur as a result of such treatments. The wide range of survival periods for positive mice in these assays (Table 2) is similar to those seen when brain tissue from confirmed cases of BSE in domestic cattle was injected into C57Bl-J6 mice of the same source as used in the current study (G.A.H. Wells and M. Dawson, unpub. data). These results contrast with those from a previous study (13) in which 20 of 21 C57Bl mice were positive for spongiform encephalopathy after injection of formalin-fixed brain from the index case of TSE in greater kudu (kudu A664), with a mean incubation period of 465±14 days (M. Bruce, pers. comm.). We conclude that the low incidence of positive mice in certain tissue groups is due to a lower titer of infectious agent within these tissues when compared with the CNS or ileum.

The distribution of BSE infectivity in tissues of greater kudu contrasts with that in tissues of BSE-infected cattle (24,25) but is more like the distribution found in genetically susceptible sheep infected with scrapie or experimental BSE (26–28). In field cases of cattle with BSE, infectivity has been found only in the CNS (25), but in cattle experimentally challenged orally with the agent of BSE, ileum and bone marrow have also been shown to contain infectivity (21,24,29). In classical studies of scrapie in sheep and goats, infectivity was detected in the nervous and lymphoreticular systems, placenta, adrenal gland, nasal mucosa, lung, pancreas, liver, bone marrow, thymus, and alimentary tract, although most of the non-neural, non-lymphoreticular peripheral tissues contained only low titers of agent (30,31).

The finding of infectivity in kudu skin, conjunctiva, and submandibular salivary gland was unexpected as these tissues have not been previously shown to be infective in scrapie, BSE, or any naturally occurring TSE. Nonetheless, infectivity has been found previously in salivary glands of mice after injection of infected tissues with a high titer of scrapie agent (32) and of mink injected with the transmissible mink encephalopathy agent (33). In experimentally-induced transmissible mink encephalopathy, low concentrations of agent occurred in liver, kidney, intestine, and salivary gland only after replication in the CNS and in some lymphoreticular tissues (33). The inconsistent observation of low levels or traces of infection in certain non-neural and non-lymphoreticular tissues is in general a feature of both natural and experimental TSE. Given the relative paucity of data on the tissue distribution of infectivity in TSEs, the finding of infection in any given tissue should probably not be regarded as surprising. The infectivity of a certain tissues subsequent to CNS involvement may be a rare event incidental to the pathogenesis of the disease.

We have previously indicated that the epidemiology of BSE in the small kudu herd at London Zoo was consistent with either a particularly high susceptibility to infection, the occurrence of direct animal-to-animal transmission of the disease, or with a combination of these factors (2,14,15). The presence of infectivity in tissues, such as the skin and salivary gland, suggests possible routes by which direct transmission could occur. Eklund et al. (32), for example, suggested infection of the salivary gland as an explanation for contact infection of scrapie between mice.

Given the extended survival period range with BSE in the C57Bl-J6 mice used in the current study compared to the incubation periods in C57Bl mice used previously (13) and the relative insensitivity of the mouse model (24), these results may be an underestimate of the extent of infectivity

in the kudu tissues assayed. A recently reported rapid immunoassay shown to be capable of detecting PrP^{BSE} in the brainstems of cattle with a sensitivity similar to that of the infectivity levels determined by end-point titration in Tg(BoPrP) mice (34) possibly offers prospects for more sensitive detection of disease-related PrP as a proxy for infectivity bioassay. An important area for further research, therefore, is to investigate whether our results represent true qualitative differences in the biology of BSE in the greater kudu and the domestic cow or possibly indicate similarities, unapparent only because of the variables inherent in the sensitivities of current bioassay methods.

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Dr. Cunningham, a veterinary pathologist, is a senior research fellow and head of Wildlife Epidemiology at the Institute of Zoology, Zoological Society of London. His research interests include emerging infectious diseases of wildlife and disease threats to biodiversity conservation.

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Address for correspondence: Andrew Cunningham, Institute of Zoology, Regent's Park, London NW1 4RY, UK; fax: +44-0-20-7586-1457; email: A.Cunningham@ ioz.ac.uk

Nursing Home Residents and *Enterobacteriaceae* Resistant to Third-Generation Cephalosporins

Carolyn Sandoval,* Stephen D. Walter,* Allison McGeer,† Andrew E. Simor,‡ Suzanne F. Bradley,§ Lorraine M. Moss,* and Mark B. Loeb*

Limited data identify the risk factors for infection with *Enterobacteriaceae* resistant to third-generation cephalosporins among residents of long-term-care facilities. Using a nested case-control study design, nursing home residents with clinical isolates of *Enterobacteriaceae* resistant to third-generation cephalosporins were compared to residents with isolates of *Enterobacteriaceae* susceptible to third-generation cephalosporins. Data were collected on antimicrobial drug exposure 10 weeks before detection of the isolates, facility-level demographics, hygiene facilities, and staffing levels. Logistic regression models were built to adjust for confounding variables. Twenty-seven case-residents were identified and compared to 85 controls. Exposure to any cephalosporin (adjusted odds ratio [OR] 4.0, 95% confidence interval [CI] 1.2 to13.6) and log percentage of residents using gastrostomy tubes within the nursing home (adjusted OR 3.9, 95% CI 1.3 to 12.0) were associated with having a clinical isolate resistant to third-generation cephalosporins.

Antimicrobial drug resistance is a concern in nursing homes, facilities where most residents are elderly, frail, and on multiple medications. Gram-negative bacteria resistant to third-generation cephalosporins have emerged as a challenge both in the acute and long-term-care setting (1–9). These organisms, such as those that produce extended-spectrum β-lactamase (ESBL) or contain the AmpC β-lactamase, can spread rapidly, especially in close living quarters (1,2).

Identifying modifiable risk factors for acquiring these organisms, such as antimicrobial drug use, can clarify strategies to reduce spread (1,2). Little is known about whether prior exposure to antimicrobial drugs is a risk factor for *Enterobacteriaceae* resistant to third-generation cephalosporins in the long-term care setting. Wiener et al. reported that prior exposure to ciprofloxacin or trimethoprim-sulfamethoxazole was an independent predictor of colonization with *Escherichia coli* resistant to ceftazidime among nursing home residents (2). Molecular analysis of isolates showed that a particular resistance-conferring plasmid appeared frequently, which supports the growing concern that long-term facilities may act as a reservoir for antimicrobial drug–resistant organisms. We previously conducted a prospective, cohort study that examined risk factors for antimicrobial drug resistance in 50 nursing homes in Canada and the United States (10). Using these data, we performed a nested case-control analysis to assess whether prior exposure to antimicrobial drugs was a risk factor for infection with *Enterobacteriaceae* that are resistant to third-generation cephalosporins. Staffing characteristics, facilities for hand hygiene, and nursing home resident characteristics were examined as potential risk factors as well.

Materials and Methods

Study Design

The methods of the original study from which these data were acquired have been previously published (10). Briefly, 50 nursing homes with ≥100 beds in four provinces (Ontario, Manitoba, Alberta, Saskatchewan) and four states (Minnesota, Michigan, North Dakota, Montana) were enrolled in the study. During a 12-month period, residents treated with systemic antimicrobial drugs were identified, and antimicrobial prescriptions were recorded, including name, dose, and duration. Infection control practitioners from each facility recorded the name and antimicrobial-susceptibility patterns of all clinical bacterial

*McMaster University, Hamilton, Ontario, Canada; †Mount Sinai Hospital, Toronto, Ontario, Canada; ‡Sunnybrook and Women's College Health Science Centre, Toronto, Ontario, Canada; §Veterans' Affairs Health Systems, and University of Michigan Medical School, Ann Arbor, Michigan, USA

cultures obtained from study residents. Private laboratories or hospital laboratories performed laboratory testing. Susceptibility testing was performed by using methods recommended by the National Committee for Clinical Laboratory Standards (NCCLS). Complete information on all clinical bacterial cultures sent for testing from each home was obtained. These clinical isolates were obtained for diagnostic purposes among residents in the nursing homes. To minimize biased sampling, only cultures sent for signs of suspected clinical infection were included. None of the homes at the time of study collected bacterial specimens from residents for surveillance purposes only. *Enterobacteriaceae* were identified by conventional methods. A survey was performed before the study to ensure that laboratories adhered to NCCLS methods. Infection control practitioners from each facility recorded the name and antimicrobial-susceptibility patterns of all clinical bacterial cultures obtained from study residents. Data recorded for each nursing home included the following: number of resident beds; staffing levels (registered and nonregistered nursing staff hours, healthcare aide hours, infection control practitioner hours, physicians hours, and access to an infectious disease specialist); infrastructure for hygiene facilities (number of showers and tubs per facility and sinks per 100 resident beds); percentage of residents with intravenous catheters, urinary catheters, and gastrostomy tubes; percentage of residents that were either wheelchair or bed bound; and information on recent acute-care stay (new admissions and returns).

Case Definition

The study population was drawn from the fixed cohort of nursing home residents used in the original study. Only those residents with clinical isolates of *Enterobacteriaceae* resistant or susceptible to third-generation cephalosporins were eligible to be case-residents and controls in this analysis. Case-residents were defined as nursing home residents in whom a clinical *Enterobacteriaceae* isolate resistant to third-generation cephalosporins was detected. For each case-resident, we randomly selected three control-residents from those residents in whom clinical isolates of *Enterobacteriaceae* sensitive to third-generation cephalosporins were obtained. Since repeat isolates may not represent independent events, we assessed only the first clinical isolate obtained from each resident (case-residents and control-residents).

Data were collected at the individual-resident level regarding prior antimicrobial drug use and antimicrobial drug resistance and susceptibility of bacterial isolates. Total antimicrobial drug use, use of any cephalosporins, use of third-generation cephalosporins, use of fluoroquinolone, and use of trimethoprim-sulfamethoxazole were recorded. Covariates were defined as those variables collected at the level of the nursing home, including staffing and patient and facility characteristics.

Statistical Analysis

We sought to compare antimicrobial drug exposures in nursing home residents from whom *Enterobacteriaceae* resistant to third-generation cephalosporins were isolated to antimicrobial drug exposures in nursing home residents from whom susceptible *Enterobacteriaceae* cultures were obtained. Antimicrobial exposures and nursing home covariates were examined as possible predictors. Log transformations were used when distributions of variables were skewed. All possible predictors were tested for collinearity. Antimicrobial drug exposure was measured in the 10 weeks before resistant bacteria were detected in residents and was compared to antimicrobial drug exposure during a 10-week interval in residents with susceptible organisms. In the absence of any evidence defining an optimal period for assessment of antimicrobial drug exposures, 10 weeks was selected by consensus opinion of five infectious disease specialists with research expertise in the field of antimicrobial drug resistance. All antimicrobial exposures were considered as binary. This strategy was used because all microbial variables, despite log transformation, were highly skewed. Odds ratios were calculated, representing the risk of nursing home residents having antimicrobial drug–resistant clinical isolates compared to antimicrobial drug–susceptible organisms. To assess for the possible effect of clustering for facility-level variables, univariate analyses were also performed with logistic regression with random factors (logistic-binomial model) (Egret 2.031, Cytel Software Corp., Cambridge, MA) and compared with a logistic regression model in which the factors are fixed (SPSS 10.0, SPSS Inc., Chicago, IL). A multivariable model was constructed in which variables with a p value <0.25 and variables representing the various antimicrobial drugs were selected for inclusion, and the final multivariable model was constructed by using a backwards, stepwise approach. All data entry was performed with SAS version 6.0 and 7.0 (SAS Institute, Cary, NC). Analysis was performed by using SPSS 10.0 and Egret 2.031. The Hosmer and Lemeshow test was performed to evaluate the overall fit of the model (11). Ethics approval for this study was obtained through McMaster University's ethics review board.

Results

Twenty-nine case-residents were identified, and 87 control-residents were initially selected from 26 nursing homes. Because of organizational changes in one nursing home during the course of the study, no covariate data could be obtained. Since such group-level data could not

be imputed, all participants selected from this particular home were dropped from the analysis. This included two case-residents and two control-residents. Ignoring the missing group-level variables and including these residents in the analysis on the basis of their individual-level variables made no difference in the estimates subsequently reported (data not shown). The distribution of types of *Enterobacteriaceae* among case-residents and control-residents is shown in Table 1. A greater proportion of *Citrobacter* and *Enterobacter* species were identified in samples from case-residents compared to samples from control-residents (p = 0.01 for each), and a greater proportion of *E. coli* isolates were identified in samples from control-residents compared to samples from case-residents (p = 0.01). Most clinical specimens were isolated from urine samples, and a greater proportion of urine isolates were detected in specimens from control-residents as compared to case-residents (p = 0.05) (Table 2).

Univariate analyses of individual-level exposures and facility-level exposures are shown in Tables 3 and 4. The following variables were considered in the multivariable model: prior exposure to any antimicrobial drug, to a cephalosporin, to a third-generation cephalosporin, to a fluoroquinolone, or to trimethoprim-sulfamethoxazole; the log number of primary care physician hours per 100 resident beds; whether an infectious disease physician was on staff; the number of new admissions from acute care hospitals within the last year per 100 resident beds; and the log percentage of residents in the nursing home with a gastrostomy tube.

The only variables that remained significant after multivariable modeling were prior use of any cephalosporin (odds ratio [OR] 4.0, 95% confidence interval [CI] 1.2 to 13.6, p = 0.029), and log percentage of residents with a gastrostomy tube (OR 3.9, 95% CI 1.3 to 12.0, p = 0.016). The p value obtained for the Hosmer and Lemeshow test was 0.138, which suggests that the overall fit of this model is reasonable.

Discussion

Enterobacteriaceae infections resistant to cephalosporins are of concern in long-term care facilities and in the acute-care setting (1–9). Patients with infections resistant to third-generation cephalosporins have been reported to have had longer hospital stays, higher death rates, and greater hospital costs than patients whose infections are susceptible to third-generation cephalosporins (4). A survey of infection control practitioners in Ontario showed that no standard approach exists to dealing with ESBL-producing *E. coli* and *Klebsiella* spp. in long-term-care facilities (12). Reservoirs of resistant *Enterobacteriaceae* species will continue to emerge in this setting despite implementation of control measures.

Table 1. Distribution of *Enterobacteriaceae* organisms isolated from case-patients and controls

Genus	Case-patients (%)	Controls (%)
Proteus spp.	7 (26)	17 (20)
Citrobacter spp.	6 (22)	4 (5)
Enterobacter spp.	5 (18)	2 (2)
Escherichia coli	4 (15)	47 (55)
Klebsiella spp.	3 (11)	11 (13)
Morganella spp.	1 (4)	4 (5)
Serratia marcescens	1 (4)	0 (0)
Total	27	85

Our findings show that recent exposure to any cephalosporin is associated with the isolation of third-generation cephalosporin–resistant *Enterobacteriaceae* in nursing home residents. Knowledge of previous exposure may help physicians anticipate this particular pattern of resistance.

Few studies have assessed risk factors for *Enterobacteriaceae* resistance to cephalosporins in long-term-care facilities. Weiner et al. described an outbreak of ceftazidime-resistant *E. coli* infections in Chicago nursing homes (2). Those researchers conducted a case-control study and found that ciprofloxacin or trimethoprim-sulfamethoxazole exposure was associated with ceftazidime-resistant *E. coli* in nursing home residents. Cephalosporin treatment may have been given after fluoroquinolone resistance was detected, and this treatment may be linked to cephalosporin resistance found in their study. Muder et al. evaluated modifiable risk factors for antimicrobial drug–resistant *Enterobacteriaceae* infection among patients from a long-term Veterans Affairs facility in Pittsburgh (13). In this case-control study, patient debility, age, coexisting conditions, and prior antimicrobial therapy were examined as risk factors associated with multidrug-resistant *Enterobacteriaceae* infections. Case-patients were identified as having an *Enterobacteriaceae* infection resistant to two of the following groups of antimicrobial drugs: piperacillin, third-generation cephalosporins, or gentamicin. Only a pressure ulcer [OR 12.2, 95% CI 3.3 to 44.2] and prior ampicillin therapy [OR 13.7, 95% CI 2.2 to 84.0] were associated with resistant *Enterobacteriaceae* infection. In contrast, we did not assess individual-level covariates. No association was found between prior cephalosporin therapy and a multiple-resistant infection,

Table 2. Distribution of sites from which *Enterobacteriaceae* were isolated

Site	Case-patients (%)	Controls (%)
Urine	20 (74)	77 (91)
Wound	3 (11)	2 (2)
Eye	3 (11)	2 (2)
Skin	1 (4)	3 (4)
Sputum	0 (0)	1 (1)
Total	27	85

Table 3. Univariate logistic regression analyses of individual-level variables with clinical isolate of *Enterobacteriaceae* resistant to third-generation cephalosporins as the dependent variable

Variables[a]	Case-patients (%)	Controls (%)	OR (95% CI)[b]	p value
Any antimicrobial drug	13 (48)	27 (32)	2.0 (0.8 to 4.8)	0.125
Any cephalosporin	8 (30)	6 (7)	5.5 (0.1 to 0.6)	0.004
Third-generation cephalosporin	2 (7)	2 (2)	3.3 (0.04 to 2.2)	0.242
Fluoroquinolone	3 (11)	7 (8)	1.4 (0.1 to 3.0)	0.649
Trimethoprim/sulfamethoxazole	4 (15)	5 (6)	2.8 (0.1 to 1.4)	0.150

[a]10 weeks before date of confirmed *Enterobacteriaceae* infection.
[b]OR, odds ratio; CI, confidence interval.

but whether prior cephalosporin therapy and the acquisition of cephalosporin-resistant infections are linked is unknown. A similar study performed previously by this group identified exposure to ciprofloxacin as a risk factor for the acquisition of ciprofloxacin-resistant gram-negative bacteria (14). Our findings confirm that patient debility and prior antimicrobial drug therapy are associated with acquisition of resistant bacteria.

In the acute-care setting, similar risk factors for the acquisition of broad-spectrum cephalosporin-resistant *Enterobacteriaceae* have been recognized (15–19). The risk of acquiring multidrug-resistant bacterial infection has been reported as increasing in hospitalized patients with prior third-generation cephalosporin therapy (6,18,19). A hospital-based case-control study performed in Argentina examined risk factors for ceftazidime-resistant *K. pneumoniae*. Prior antimicrobial drug use was associated with acquisition of this type of resistant infection [adjusted OR 6.21, 95% CI 1.20 to 32.01]. Other risk factors found were prior use of ciprofloxacin, nosocomial infection, and hospitalization stay >6 days (15). Bisson et al. also looked at risk factors for acquisition of ESBL-producing *Klebsiella* spp. and *E. coli* in which patients with this resistance pattern were compared to patients with no culture exhibiting this resistance pattern (16). Only length of hospitalization stay was associated with colonization with ESBL-producing *E. coli* and *Klebsiella* spp. [adjusted OR 1.11, 95% CI, 1.02 to 1.21]. Prior receipt of antimicrobial therapy was not associated with colonization in these patients, but none were exposed to third-generation cephalosporins. Since our study was set in long-term-care facilities and because individual measurements were not done, length of stay was not assessed. Lin et al. performed a case-control study evaluating risk factors for ESBL-producing *K. pneumoniae* among hospital patients in Taiwan (17). Prior use of ceftazidime was found to be associated with ESBL-producing *K. pneumoniae* infection compared to non–ESBL-producing *K. pneumoniae* infection. A case-control study by Bonomo et al. did not find an association between fecal colonization of cefotaxime-resistant gram-negative bacilli and antimicrobial drug use 4 weeks before admission to the hospital (20).

Comparison of antimicrobial drug-sensitive organisms to antimicrobial drug-resistant organisms cannot infer absolute risk for exposure to that antimicrobial drug (21,22). However, Lipsitch has discussed several advantages of calculating conditional OR (OR_c) versus simple OR (OR_s) when interpreting associations between antimicrobial drug

Table 4. Univariate logistic regression analyses of nursing home–level variables with clinical isolate of *Enterobacteriaceae* resistant to third-generation cephalosporins as the dependent variable[a]

Variables	OR (95% CI)	p value
>200 beds (no.)	0.85 (0.35 to 2.05)	0.718
Nursing units per 100 beds (log no.)	4.09 (0.25 to 66.45)	0.322
RN FTE h (log no.)	1.21 (0.18 to 8.31)	0.846
NA FTE h (log no.)	1.91 (0.44 to 8.43)	0.391
Healthcare aide FTE h (log no.)	0.99 (0.95 to 1.02)	0.356
ICP FTE h (log no.)	0.91 (0.19 to 4.43)	0.910
Primary care physician FTE h (log no.)	0.28 (0.08 to 0.91)	0.034
Infectious disease specialist on staff	0.40 (0.16 to 1.03)	0.057
Tubs/showers per 100 beds/facility (log no.)	2.25 (0.48 to 10.48)	0.303
Sinks per 100 beds (no.)	1.00 (0.99 to 1.01)	0.965
New admissions from to acute care prior y/100 beds (no.)	1.00 (0.99 to 1.01)	0.205
Residents readmitted acute care hospitals prior y/100 beds (no.)	1.01 (0.99 to 1.03)	0.481
Residents with urinary catheter (%)	1.01 (0.90 to 1.13)	0.923
Residents with intravenous catheter (%)	1.64 (0.68 to 3.9)	0.272
Residents with gastrostomy tube (log %)	5.02 (1.72 to 14.70)	0.003
>50% residents unable to ambulate (no.)	1.34 (0.54 to 3.31)	0.533

[a]OR, odds ratio; CI, confidence interval; FTE, full -time equivalent; RN, registered nurse; NA, nursing aide; ICP, infection control practitioner.

use and resistant organisms (23). OR_s determines a patient's individual risk of acquiring a resistant infection after receiving treatment compared to not getting an infection after receiving treatment. OR_c determines the risk of a resistant infection compared to the risk of a nonresistant infection, which allows for insight into whether a suspect infection in a patient is more likely to be resistant to a particular antimicrobial drug compared to someone who has not been treated (23). Widespread treatment with antimicrobial drugs promotes the eradication of antimicrobial-susceptible organisms but also confers a selective advantage for developing antimicrobial-resistant organisms. Measuring OR_c may even be more useful for clinicians than knowing the absolute risk because it gives information about the community-level risk of having a resistant organism compared to a susceptible organism in persons with a particular recent antimicrobial drug history (23). Making this comparison is useful for clinicians who are evaluating the odds of a patient's being infected with a resistant organism.

In addition to antimicrobial drug exposure, the percentage of residents with gastrostomy tubes in the home was also found to predict *Enterobacteriaceae* infection resistant to third-generation cephalosporins. In this analysis, percentage of gastrostomy tubes among home residents served as a proxy for generalized debility within each nursing home, and our findings suggest that more debilitated patients may be more predisposed to acquiring resistant organisms. Debilitated patients are most likely to harbor resistant organisms (24). Since we do not know the strain of resistant bacteria applicable to each patient, we cannot infer with certainty that a gastrostomy tube in a nursing home resident is a risk factor for this type of resistant infection. However, Weiner et al. identified gastrostomy tube use at the patient-level as a risk factor for acquiring resistant *Enterobacteriaceae* (2). Infections directly associated with gastrostomy tube contamination, resulting in antimicrobial treatment or hospitalization (25,26), have also been reported,. However, since gastrostomy tube sites were not systematically tested in this study, this finding cannot be inferred.

Strengths of this study are that residents were drawn from a large number of nursing homes in both Canada and the United States, exposures at both the individual and aggregate levels were examined, and a wide range of variables were considered, including staffing and infrastructure for hand hygiene. Our study has some limitations, including the following: the date of actual acquisition of resistant bacteria is not known; we only examined clinical isolates, obtained for diagnostic purposes, which may have led to a sampling bias; and individual level coexisting conditions were not measured. Given the large size of the original study, measuring coexisting conditions was not feasible. Another limitation is that facilities with higher levels of resistant bacteria among residents may obtain more clinical specimens (or vice versa), which may have led to bias in the sample used. Cross-infection within a facility could not be assessed because of lack of molecular typing of isolates. Admissions and readmissions to acute-care facilities within the last year were not identified as risk factors for infection with *Enterobacteriaceae* resistant to third-generation cephalosporins. The 12-month period may have been too long, but a significant association may have been identified with a 3-month period. In conclusion, prior exposure to cephalosporins and an increased use of gastrostomy tubes among nursing home residents predicts for infection with *Enterobacteriaceae* resistance to third-generation cephalosporins.

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Address for correspondence: Mark Loeb, Henderson General Hospital, 711 Concession St., Hamilton, Ontario, Canada L8V 1C3; fax: 905-575-2553; email: loebm@mcmaster.ca

Quinolone-resistant *Campylobacter* Infections in Denmark: Risk Factors and Clinical Consequences[1]

Jørgen Engberg,*† Jakob Neimann,‡ Eva Møller Nielsen,§[2] Frank Møller Aarestrup,§ and Vivian Fussing*[3]

We integrated data on quinolone and macrolide susceptibility patterns with epidemiologic and typing data from *Campylobacter jejuni* and *C. coli* infections in two Danish counties. The mean duration of illness was longer for 86 patients with quinolone-resistant *C. jejuni* infections (median 13.2 days) than for 381 patients with quinolone-sensitive *C. jejuni* infections (median 10.3 days, p = 0.001). Foreign travel, eating fresh poultry other than chicken and turkey, and swimming were associated with increased risk for quinolone-resistant *C. jejuni* infection. Eating fresh chicken (of presumably Danish origin) was associated with a decreased risk. Typing data showed an association between strains from retail food products and broiler chickens and quinolone-sensitive domestically acquired *C. jejuni* infections. An association between treatment with a fluoroquinolone before stool-specimen collection and having a quinolone-resistant *C. jejuni* infection was not observed.

Campylobacter is a leading cause of bacterial gastroenteritis in industrialized and developing countries worldwide (1). Most *Campylobacter* infections need not be treated with antimicrobial agents. However, in a subset of patients *Campylobacter* may cause severe complications and increased risk for death and therefore requires treatment. A recent Danish study has shown that patients with *Campylobacter* infections have higher acute- and long-term death rates than controls after coexisting conditions were taken into account (2). The drug of choice is a macrolide (e.g., erythromycin or a newer agent) for treatment of enteric *Campylobacter* infections after the microbiologic diagnosis. However, for the empiric treatment of adults with suspected bacterial gastroenteritis, the drug of choice typically includes a fluoroquinolone (e.g., ciprofloxacin) because of their activity against almost all enteric bacterial pathogens. Antimicrobial drug resistance in *Campylobacter* infections, in particular to quinolones, has increased dramatically in many countries during the 1990s as reviewed by Engberg et al. (3). According to a recent published report by World Health Organization (4), the sources of antimicrobial drug–resistant *Campylobacter* strains and the clinical impact of such strains need to be determined.

We conducted a 1-year prospective study to address the prevalence of macrolide and quinolone resistance in human *Campylobacter* isolates. Human isolates were compared with isolates from retail food products and broiler chickens. A systematic approach integrating standardized epidemiologic, antimicrobial susceptibility, and typing data was used. We also conducted a case-comparison study to identify risk factors associated with acquiring quinolone-resistant *C. jejuni* infections.

Materials and Methods

Surveillance and Susceptibility Testing of *Campylobacter* Isolates

The study included all culture-positive *Campylobacter* infections from May 1, 2001, through June 10, 2002, from two counties with a catchment area of approximately 1.1 million persons (approximately one fifth of the Danish population). The county of Copenhagen, a metropolitan residential area, has a population of 619,000, and the county of Funen, an island with both urban and rural areas, has

*Statens Serum Institut, Copenhagen, Denmark; †Herlev University Hospital, Herlev, Denmark; ‡Danish Institute for Food and Veterinary Research, Søborg, Denmark; and §Danish Institute for Food and Veterinary Research, Copenhagen, Denmark

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[2]Current affiliation is Statens Serum Institut, Copenhagen, Denmark.

[3]Current affiliation is Danish Toxicology Centre, Hørsholm, Denmark.

a population of 472,000. Because of inconsistencies in the patient enrolment from the county of Funen during May and June 2002, patients from this county who were infected after April 31, 2002, were excluded. Epidemiologic data were captured on self-completed standardized patient questionnaires forwarded by the Danish Zoonosis Centre. Patients were interviewed about clinical symptoms, travel history, and exposures to food, water, and animals in the 7 days before illness onset. Completed questionnaires were returned to the Danish Zoonosis Centre and linked with microbiologic data.

All isolates included in the study were tested for resistance to nalidixic acid and erythromycin. All human isolates from the county of Copenhagen and isolates obtained from retail food products and broiler chickens were screened by a disk-diffusion test using Oxoid disks on 5% blood agar plates. On the basis of zone sizes, this method grouped the isolates in two well-separated populations of susceptible and resistant isolates with both antimicrobial drugs. The few isolates that fell between these populations were retested by using the standardized tablet diffusion and E-test procedures described previously (5), with the modifications that resistance to nalidixic acid was defined as an MIC >64 mg/L for the MIC method and a zone size ≤27 mm for the tablet method. All human isolates from the county of Funen were tested by the standardized tablet diffusion test with both antimicrobial drugs. Finally, all isolates found to be resistant and sensitive to nalidixic acid from our case-comparison study were retested by both the standardized tablet diffusion and E-test procedure.

Case-Comparison Study

In the second half of the study period (from December 1, 2001, to June 10, 2002), characteristics of patients with quinolone-resistant and quinolone-sensitive *C. jejuni* infections were compared. Each patient with a resistant isolate was matched with two randomly selected patients with sensitive isolates. Patients were matched on date of specimen collection.

Patients answered, either by phone or by mail, a short additional questionnaire, which included questions about use of fluoroquinolones the month before onset of illness, use of fluoroquinolones after onset of illness but before specimen collection, use of antimicrobial drugs after specimen collection, and other clinical information. When patients could not answer questions about exposure to fluoroquinolones before fecal sampling, the information was gathered from their healthcare providers.

Food and Animal Isolates

As part of a national surveillance program, food samples from retail outlet stores were analyzed for *Campylobacter* at the regional food safety authorities, according to accredited methods of the Nordic Committee on Food Analysis (6). The samples were taken from whole poultry and different cuts of poultry (frozen and fresh), including chicken and turkey. Samples of pork and beef products were also analyzed. Imported as well as domestic food products were sampled.

As part of a national surveillance program for *Campylobacter* in broiler chickens, chickens were sampled at slaughter and analyzed for *Campylobacter*. In this study, isolates from broiler chicken farms located in Funen County were included (one isolate per flock). Copenhagen County does not have any broiler chicken farms.

Serotyping and Molecular Subtyping of *Campylobacter* Isolates

One isolate from the primary isolation on modified charcoal cefoperazone deoxycholate agar (mCCDA) from each patient, as well as one isolate from each retail food sample and broiler chicken fecal sample were characterized at Statens Serum Institut and the Danish Veterinary Institute. Speciation, serotyping, and RiboPrinting (automated ribotyping) were undertaken as previously described (7,8), with the following modifications for the RiboPrinting method: 1-μL eye needle was filled with bacterial culture and dissolved in 100 μL sample buffer. Ten microliters of 10 g/L lysozyme was added, and the solution was left at 37°C for 10 min. From this solution, 30 μL was transferred to a sample carrier for heat treatment. The RiboPrinter was run according to the SEC protocol at 37°C for 2 h.

Statistical Analysis

Conditional logistic regression was applied to calculate a matched odds ratio for the exposure variables. Variables, which reached a significance level of ≤0.15 in the univariate analysis of the case comparison study, were selected for the multiple logistic regression analysis. Stepwise conditional logistic regression with a backward elimination procedure was conducted to obtain a reduced model. Variables with a p value ≤0.05 were kept in the model. All excluded variables were retested in the final model. The statistical software SAS Release v.8.00 (SAS Institute Inc., Cary, NC) and Epi Info version 6.04d (Centers for Disease Control and Prevention, Atlanta, GA) were used to analyze the data.

Results

Surveillance and Resistance

Of 975 culture-confirmed *Campylobacter* infections in the study, 177 (18.2%) were infected with a quinolone-resistant isolate, whereas 3 (0.3%) isolates were erythromycin-resistant. Linked microbiologic and epidemiologic data were obtained from 678 (69.5%) patients. In total, 152 (22.4%) patients had been outside Denmark within 1

week before illness, whereas 526 (77.6%) were domestically acquired infections. The three erythromycin-resistant isolates were all *C. coli*, two of them were also quinolone-resistant, and these were both isolated from travelers returning to Denmark from Spain and Portugal, respectively.

Quinolone resistance was significantly associated with the origin of infection: 76 (50.0%) of 152 infections among travelers returning to Denmark were quinolone-resistant whereas 52 (9.9%) of 526 of domestically infected patients were infected with a quinolone-resistant strain ($p < 0.001$) (Table 1). For both *C. coli* and *C. jejuni*, a significantly higher proportion of quinolone-resistant infections was found among patients who had been abroad in the week before onset of illness than among patients with domestically acquired infections (risk ratio [RR] 9.3, 95% confidence interval [CI] 1.4 to 63.8, $p = 0.004$ and RR 4.9, CI 3.6 to 6.7, $p < 0.001$). A higher proportion of *C. coli* than *C. jejuni* infections were acquired abroad (48.3% compared with 21.3% of *C. jejuni*).

Foreign travel was associated with different prevalences of quinolone resistance, depending on destination (Table 2). No travelers returning from other Scandinavian countries hosted quinolone-resistant *Campylobacter* isolates, whereas travel to a number of regions and subregions, including southern Europe and Southeast Asia, was significantly associated with a high proportion of quinolone-resistant infections.

C. jejuni infections and *C. coli* infections did not differ in severity, as assessed by frequency of diarrhea, blood in stool, abdominal pain, fever, vomiting, mean duration of illness, or admission to hospitals. However, the mean duration of illness was longer for the 86 patients with quinolone-resistant *C. jejuni* infections and a known duration of illness (median 13.2 days) than for the 381 patients with quinolone-sensitive *C. jejuni* infections and a known duration of illness (median 10.3 days, $p = 0.001$). The association with extended length of illness was independent of foreign travel. For domestically acquired infections, the mean duration of illness was 12.4 and 10.4 days for quinolone-resistant and quinolone-sensitive infections, respectively. For comparison, the mean duration of illness for travel-associated infections was 13.9 and 10.3 days for quinolone-resistant and quinolone-sensitive infections, respectively. For *C. coli*, no difference in mean duration of illness was observed between quinolone-resistant and quinolone-sensitive infections.

Case-Comparison Study

From December 1, 2001, to June 10, 2002, 42 patients were infected by quinolone-resistant *C. jejuni* isolates, and these patients were matched with 84 patients with quinolone-sensitive isolates. No patients were connected on epidemiologic grounds of a recognized outbreak. The patients with quinolone-resistant isolates had a mean age of 33 years (interquartile range 20–45 years), and a male-to-female ratio of 1:1.2. For comparison, patients with quinolone-resistant isolates in the larger study, which were not included in the case-comparison study, had a mean age of 31 years (interquartile range 20–45 years), and a male-to-female ratio of 1:1.3. No strains changed susceptibility category after being retested. However, one case-patient was shown to be co-infected with two *C. jejuni* strains with identical serotype and RiboGroup, but with different susceptibility patterns, i.e., one strain was clearly sensitive (MIC of quinolone = 2 mg/L), whereas the MIC of quinolone for the other one was 128 to >256 mg/L on multiple repeated testings. Subsequent nucleotide sequence analysis indicated a normal consensus sequence in the former and a Thr-86 to Ala-86 mutation and three silent mutations in *gyr*A in the latter. The patient had not been exposed to fluoroquinolones before stool specimen collection.

Risk factors for a quinolone-resistant *C. jejuni* infection identified in the univariate analysis and in the multiple logistic regression analysis are presented in Table 3. According to the multiple logistic regression analysis, the only exposures independently associated with an increased risk for quinolone-resistant *C. jejuni* infection were foreign travel (OR = 16.81), eating fresh poultry other than chicken and turkey (OR = 19.10), and swimming (OR = 5.01). Eating fresh chicken (of presumably Danish origin) was

Table 1. Quinolone resistance by history of recent foreign travel and comparison with *Campylobacter* isolates from food products and broiler chickens

		Human (n = 678)							
		Travel (n = 152)		Domestic (n = 526)		Food (n = 180)		Broiler chickens (n = 49)	
Species	Total	n	% resistant[a]	n	% resistant[a]	n	% resistant[a]	n	% resistant[a]
C. jejuni	1,118	137	48.2	506	9.9	153	8.5	39	5.2
C. coli	79	15	66.7	14	7.1	27	29.6	10	0
C. lari	1	0	–	1	100	0	–	0	–
C. spp[b]	6	0	–	5	20	0	–	0	–
Total	1,204	152	50.0	526	9.9	180	13.7	49	5.2

[a]Quinlone-resistant isolates.
[a]Speciation not performed.

Table 2. Prevalence of quinolone resistance in *Campylobacter* isolates according to destination of foreign travel within 7 days before onset of illness[a]

Origin[b]	No. of patients	Susceptible	Resistant	% resistant	RR	95% CI	p value
Domestic (Denmark)	526	474	52	9.9	–	–	–
Southern Europe	43	15	28	65.1	6.59	4.70 to 9.24	<0.001
Northern Europe	17	17	0	0	–	–	–
Western Europe	18	10	8	44.4	4.50	2.52 to 8.01	<0.001
Central/East Europe	9	8	1	11.0	1.12	0.17 to 7.26	1.00
East Mediterranean Europe[c]	13	6	7	53.8	5.45	3.09 to 9.59	<0.001
South Asia	12	5	7	58.3	5.90	3.43 to 10.16	<0.001
Southeast Asia	13	2	11	84.6	8.56	6.05 to 12.11	<0.001
Middle East[d]	5	2	3	60.0	6.07	2.84 to 12.99	0.009
Africa	5	3	2	40.0	4.05	1.34 to 12.21	0.08
Other regions/subregions[e]	17	9	8	47.1	4.76	2.70 to 8.39	<0.001
No travel information	297	249	48	16.2	1.63	1.13 to 2.36	0.011

[a]Relative risk (RR), p value, and 95% confidence interval (CI) were calculated for the different regions/subregions with domestically acquired infections as reference.
[b]Country grouping according to the World Tourism Organization (9).
[c]Solely Turkey.
[d]Solely Egypt.
[e]Other regions/subregions each with less than five visits (% quinolone resistance): Australasia 1 (0); Caribbean 1 (0); North America 1 (0), South America 2 (100); North Asia 2 (50); unknown destination 1 (0); multiple subregions/regions 9 (56).

associated with a decreased risk (OR = 0.04). Age group did not affect the findings (younger or older than 15 years of age) in either the univariate or the multiple logistic regression analysis.

The case-comparison study identified 12 quinolone-resistant cases that were domestically acquired. However, to determine the sources of infection for the domestically acquired quinolone-resistant infections, an unmatched subanalysis on domestically acquired infections (quinolone-resistant versus quinolone-sensitive) was performed. Infections treated with fluoroquinolones before specimen collection were excluded. In this model, the parameter estimates did not change substantially from the primary model, but because of the lower sample size, the confidence intervals increased, and only eating fresh poultry other than chicken and turkey had p value <0.05. In 10 (11.9%) of 84 domestically acquired infections, patients reported eating fresh poultry other than chicken and turkey compared with 4 (9.5%) of 42 infections in persons with travel-related infections.

Overall, we found information on antimicrobial drugs for 122 of 126 patients. Forty patients (32.8%) were treat-

Table 3. Risk factors for infection with quinolone-resistant *Campylobacter jejuni* as compared with those for quinolone-sensitive *C. jejuni*[a]

	Patients with resistant isolates (n = 42)		Patients with sensitive isolates (n = 84)		Univariate analysis			Multivariate analysis		
Exposures	No.	(%)	No.	(%)	mOR	95% CI	p value	mOR	95% CI	p value
Travel abroad within last 7 days	30	(71.4)	12	(14.3)	12.12	4.23 to 34.73	<0.0001	16.81	3.44 to 82.20	0.001
Fluoroquinolone treatment after illness onset but before stool sample or 4 weeks before symptom onset	8	(19.1)	5	(6.0)	4.44	1.15 to 17.09	0.031	–	–	–
Beef (not cold cuts)	27	(64.3)	73	(86.9)	0.31	0.13 to 0.73	0.008	–	–	–
Fresh chicken	14	(33.3)	58	(69.6)	0.17	0.06 to 0.45	0.0004	0.04	0.004 to 0.39	0.005
Fresh poultry other than chicken and turkey	7	(16.7)	7	(8.3)	2.40	0.73 to 7.86	0.148	19.10	2.18 to 167.30	0.008
Sausages	8	(19.1)	33	(39.3)	0.32	0.12 to 0.88	0.027	–	–	–
Handling of raw meat	9	(21.4)	43	(51.2)	0.14	0.04 to 0.48	0.002	–	–	–
Public water supply	19	(45.2)	66	(78.6)	0.17	0.06 to 0.46	0.001	–	–	–
Swimming (pool, ocean, lake, or other places)	20	(47.6)	16	(19.1)	3.22	1.48 to 7.00	0.003	5.01	1.14 to 21.99	0.033
Animal contact	14	(33.3)	45	(53.6)	0.44	0.20 to 0.94	0.032	–	–	–

[a]Matched odds ratio–univariate and multivariate analysis. mOR, matched odds ratio; CI, confidence interval.

ed with antimicrobial agents for their campylobacteriosis; of these, 33 patients (27%) received a fluoroquinolone, 6 patients (4.9%) received a macrolide, and 1 patient (1%) received both a fluoroquinolone and a macrolide for the *C. jejuni* infection.

Campylobacter Isolates from Retail Food Products and Broiler Chickens

The human isolates were included in a database and compared with 180 *Campylobacter* isolates obtained from retail food products (chicken [n = 139], turkey [n = 39], and pork [n = 2]) and 49 isolates from broiler chicken fecal samples obtained from the same geographic area and time period as the human isolates. Most (63%) food isolates were from Danish-bred food animals; the remaining isolates were from imported food from France (n = 48), Italy (n = 7), and the United Kingdom (n = 9). The origin of three chicken isolates was unknown. Of 180 isolates obtained from food products of both domestic and foreign origin, 153 (85%) isolates and 27 (15%) isolates were *C. jejuni* and *C. coli*, respectively (Table 1). Thirteen (8.5%) of 153 *C. jejuni* isolates and 8 (29.6%) of 27 *C. coli* isolates were resistant to nalidixic acid. Three (2.0%) of 153 *C. jejuni* isolates and 5 (18.5%) of 27 *C. coli* isolates were resistant to erythromycin. Two isolates (one *C. jejuni* and one *C. coli*) from domestic chicken products were resistant to both antimicrobial agents. A subanalysis of resistance status by origin of 139 retail chicken products (domestic versus imported) showed that 7 (8.0%) of 87 *C. jejuni* isolates and three (60%) of five *C. coli* isolates from domestic raised chicken products were resistant to nalidixic acid. Of isolates from imported chicken products, 5 (14.7%) (3 isolates from France and 2 isolates from the United Kingdom) of 34 *C. jejuni* isolates and 1 (10%) (from France) of 10 *C. coli* isolates were resistant to nalidixic acid.

Of 49 isolates from broiler chicken fecal samples, 39 (79.6%) were *C. jejuni* and 10 (20.4%) were *C. coli* (Table 1). Two isolates (4.1%) (both *C. jejuni*) were nalidixic acid–resistant; one was also erythromycin-resistant. Five (10.2%) isolates (four *C. jejuni*, one *C. coli*) were erythromycin-resistant.

Serotyping and Molecular Subtyping of Isolates

We found 133 combinations of serotypes and Ribo-Groups (hereafter subtypes) among 496 typed isolates (10 isolates were not tested or nontypeable) from domestically acquired *C. jejuni* infections (Table 4). Eighteen (13.5%) subtypes were identified exclusively among quinolone-resistant isolates, 102 (76.7%), exclusively among quinolone-sensitive isolates, and 13 (9.8%) among both resistant and sensitive isolates.

Five of 11 subtypes of quinolone-resistant *C. jejuni* found among isolates from retail food products, broiler chickens, or both were also found among quinolone-resistant domestically acquired *C. jejuni* isolates from humans, and 34 of 88 subtypes of quinolone-sensitive *C. jejuni* found among isolates from retail food products, broiler chickens, or both were also found among quinolone-sensitive domestically acquired *C. jejuni* isolates from humans.

Patients with domestically acquired quinolone-sensitive *C. jejuni* infections were more likely to have a *C. jejuni* subtype that was also identified among retail food products and broiler chickens than were patients with domestically acquired quinolone-resistant infections (270 of 444 vs. 15 of 51, RR = 2.07, CI 1.34 to 3.18, $p < 0.001$).

Discussion

Several studies have proposed a causal relation between the veterinary use of fluoroquinolones in food production and the increase in quinolone-resistant *Campylobacter* infections in humans (10–14). However, it has been argued that use of fluoroquinolones in human medicine may be driving the increasing quinolone resistance among human *Campylobacter* isolates (15,16). Our study provides additional epidemiologic and microbiologic data to this discussion.

Our case-comparison study identified three factors to be independently associated with increased risk of attracting a quinolone-resistant *C. jejuni* infection: foreign travel, eating fresh poultry other than chicken and turkey, and swimming. Eating fresh chicken was associated with a decreased risk.

A travel association for quinolone-resistant *Campylobacter* infection has been reported from numerous countries in recent years (14,17–20). A limitation of most studies, apart from the Minnesota study (14), is that the epidemiologic information did not include a question on current or recent treatment with fluoroquinolones before stool-specimen collection. Therefore, uncontrolled confounding might have occurred. In our study, treatment with a fluoroquinolone before stool-specimen collection and having a quinolone-resistant *C. jejuni* infection, though statistically significant in the univariate analysis, was no longer significant in the multivariate analysis (Table 3). This finding suggests that quinolone use in humans is not the major selective force for quinolone resistance among *Campylobacter* spp. that cause human infections. However, use of fluoroquinolones in human medicine may still, to some degree, contribute to quinolone resistance in *Campylobacter.* This finding was instructively illustrated in our study: by an error, two strains were recovered from one *Campylobacter* episode, a fluoroquinolone-sensitive strain obtained from the patient's stool sample on December 7, 2001, and one resistant strain (MIC > 256 mg/L) from the same patient's stool sample on December 14, 2001. The second stool specimen was obtained after at least 3 days' treatment with

Table 4. Number of *Campylobacter jejuni* subtypes by quinolone susceptibility from domestically acquired infections, retail food products, and broiler chickens

Origin[a]	Total no. subtypes	Quinolone-resistant (%)	Quinolone-sensitive (%)	Quinolone-resistant and quinolone-sensitive (%)
Humans (n = 496)	133	18 (13.5)	102 (76.7)	13 (9.8)
Retail food products (n = 172)	81	9 (11.1)	70 (86.4)	2 (2.5)
Broiler chickens (n = 46)	20	2 (10.0)	18 (90.0)	0
Total	234	29 (12.4)	190 (81.2)	15 (6.4)

[a]Ten, 8, and 3 isolates from humans, retail food products, and broiler chickens, respectively, were not tested or nontypeable.

ciprofloxacin. The isolates had the same serotype and RiboGroup, and subsequent sequence analysis showed a Thr-86 to Ile-86 mutation in *gyr*A, the most common identified mutation in quinolone-resistant *C. jejuni* field strains. Treatment with quinolones has previously been shown to be associated with isolation of a resistant strain (15,21–23), but this case is, to our knowledge, the first documented clinical case in which the exact mutation is presented by a comparison of pre- and posttreatment *gyr*A genes. In the study by Smith et al. in Minnesota (14), human exposure to a fluoroquinolone before stool specimen collection was identified as a risk factor for quinolone-resistant *C. jejuni* infection, but their study also showed that treatment with a fluoroquinolone before stool culture accounted for a maximum of 15% of resistant isolates in Minnesota during 1996 and 1998. Therefore, fluoroquinolone use in humans can (and did in a small extent in this study) result in emergence of quinolone resistance in the treated patient, but the treated patient is unlikely to be a source of quinolone-resistant *Campylobacter* for other people, because person-to-person transmission of *Campylobacter* is not considered epidemiologically important.

In our study, eating fresh poultry other than chicken and turkey was rare, but a significant risk factor for both quinolone-resistant infections in general, and for domestically acquired quinolone-resistant infections. The type of fresh poultry other than chicken and turkey was not specified in the questionnaire but could have been duck, goose, or ostrich.

Swimming was also associated with an increased risk for quinolone-resistant infections. The exposure was frequently reported by travel-related infections (20 [48%] of 42), compared with domestically acquired infections (16 [19%] of 84). Patients were questioned about swimming in pool, ocean, lake, or other places combined. Future studies should specify the type of water more specifically.

Eating fresh chicken was associated with a decreased risk for quinolone-resistant infections. The fresh chicken was of presumably Danish origin, as most fresh chicken eaten in Denmark is domestically raised. In addition, as travelers often eat at restaurants, where information about whether a served chicken is fresh or has been frozen is normally not available, patients who reported eating fresh chicken were likely to have consumed it in Denmark. This is supported by the fact that of 56 (67%) of 84 domestically acquired infections, patients reported eating fresh poultry compared with 17 (38%) of 42 patients with travel-related infections. Eating poultry is believed to be the primary means of acquiring human campylobacteriosis, although other sources also exist (1). This corroborates the hypothesis that quinolone-resistant–*C. jejuni* infections could result from the use of quinolones in animals and in food production. The veterinary antimicrobial drug use hypothesis is supported by the findings of this study and studies by our European and American colleagues that a significantly higher proportion of quinolone-resistant *C. jejuni* infections occur among patients who had been abroad, often to destinations with recognized high quinolone-resistance in *Campylobacter* in food animals as well as established high risk of attracting quinolone-resistant human *Campylobacter* infections, than among domestically acquired infections (Tables 1 and 2) (3,11,12,18, 24–27). In Denmark, as part of the Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP), antimicrobial drug susceptibility in *Campylobacter* is monitored systematically in food animals, retail food products, and humans as well as use of antimicrobial drugs, including quinolones at food animal species level. Compared to the practice in many other countries, only small amounts of fluoroquinolones are used in broiler chicken production, and during 2002, use of fluoroquinolones decreased significantly after restrictions imposed by the Danish Veterinary and Food Administration called for reducing fluoroquinolone use (28). According to DANMAP surveillance data for 2002, no resistance among *C. jejuni* to quinolones was found in broiler chickens, and quinolone resistance to *C. jejuni* was found in only 6% of imported and domestic retail chicken meat (28). This finding may explain why eating fresh chicken (of presumably Danish origin) was associated with a decreased risk for quinolone-resistant *C. jejuni* infection in the matched multivariate analysis. Our typing data also support this explanation, because patients with domestically acquired quinolone-sensitive *C. jejuni* infections were more likely to have a *C. jejuni* subtype that was also identified among retail food products and broiler chickens than were patients with domestically acquired quinolone-resistant infections.

A potential limitation of our study is the fact that only one isolate from each retail food sample or broiler fecal sample was characterized. Previous studies have shown that multiple strains of *Campylobacter* may be recovered. Capturing the diversity of strains may have been helpful in accounting for a higher percentage of human strains, as would analysis of additional food and broiler chicken samples.

In many countries, including Denmark, fewer *Campylobacter* infections are identified in the winter months, but among the ones that are, a higher percentage are associated with foreign travel. In the case-comparison study, 30 (71%) of 42 quinolone-resistant *C. jejuni* infections were associated with foreign travel versus 66 (57%) of 116 quinolone-resistant *C. jejuni* infections in the larger study (not seasonal). A limitation of our study is therefore its time frame (December–June). The patients with resistant isolates in the case-comparison study were demographically (age and sex) comparable to the group of quinolone-resistant infections from the larger study, which were not included in the case-comparison study.

In the Minnesota study (14), a clinical implication of fluoroquinolone-resistance among *C. jejuni* infections was identified: the duration of diarrhea among patients treated with a fluoroquinolone was significantly longer if the patient had a fluoroquinolone-resistant infection (median 10 days) versus a fluoroquinolone-susceptible infection (median 7 days). We also found significantly longer duration of illness among patients with quinolone-resistant *C. jejuni* infections (median 13.2 days) compared to that of patients with quinolone-sensitive infections (median 10.3 days). However, as history of antimicrobial treatment was only obtained from the case-comparison proportion of our study, stratifying by treatment to determine whether the negative impact on public health was caused by true treatment failures is not possible.

We found three macrolide-resistant strains; all were *C. coli* isolated from travelers returning to Denmark. Our finding is in line with current surveillance data on the level of macrolide resistance in Danish broiler chickens, cattle, and chicken meat (28). Resistance to macrolides has also been reported at continued low level in a number of other countries and should remain the first drug of choice for verified campylobacteriosis (3,29).

In conclusion, the current study found evidence of prolonged duration of illness associated with quinolone resistance and supports the conclusions drawn by the U.S. Food and Drug Administration: human quinolone-resistant *Campylobacter* infections have increased, and this increase has a negative impact on public health. This study also suggests that in a country with restricted fluoroquinolone use in poultry production, chicken is not a source of domestically acquired quinolone-resistant *Campylobacter* infections, and that in countries with less restrictive use, poultry is an important source of such infections. The use of fluoroquinolones for food production animals should be discontinued or minimized to preserve fluoroquinolone sensitivity in *Campylobacter*.

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Dr. Engberg is a physician at Statens Serum Institut, the national institute for prevention and control of infectious diseases and congenital disorders in Denmark. His research interests focus on epidemiology, antimicrobial susceptibility, and molecular typing aspects of *Campylobacter*.

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Address for correspondence: Jørgen Engberg, Department of Bacteriology, Mycology and Parasitology, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark; fax: +45-3268-3873; email: eng@ssi.dk

Yellow Fever Outbreak, Imatong, Southern Sudan

Clayton O. Onyango,* Victor O. Ofula,* Rosemary C. Sang,* Samson L. Konongoi,* Abdourahmane Sow,† Kevin M. De Cock,‡ Peter M. Tukei,§ Fredrick A. Okoth,§ Robert Swanepoel,¶ Felicity J. Burt,¶ Norman C. Waters,# and Rodney L. Coldren#

In May 2003, the World Health Organization received reports about a possible outbreak of a hemorrhagic disease of unknown cause in the Imatong Mountains of southern Sudan. Laboratory investigations were conducted on 28 serum samples collected from patients in the Imatong region. Serum samples from 13 patients were positive for immunoglobulin M antibody to flavivirus, and serum samples from 5 patients were positive by reverse transcription–polymerase chain reaction with both the genus *Flavivirus*-reactive primers and yellow fever virus–specific primers. Nucleotide sequencing of the amplicons obtained with the genus *Flavivirus* oligonucleotide primers confirmed yellow fever virus as the etiologic agent. Isolation attempts in newborn mice and Vero cells from the samples yielded virus isolates from five patients. Rapid and accurate laboratory diagnosis enabled an interagency emergency task force to initiate a targeted vaccination campaign to control the outbreak.

Yellow fever virus (YFV), an arthropod-borne virus in the genus *Flavivirus* of the family *Flaviviridae*, is the etiologic agent of yellow fever, a viral hemorrhagic fever that occurs in South American countries and much of sub-Saharan Africa (1,2). Flaviviruses are single-stranded, positive-sense RNA viruses with a genome organization of 5′-C, prM, M, E, NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5-3′ (3,4). YFV is transmitted by the bite of an infected female mosquito, usually *Aedes* species in Africa (5). YFV transmission has two epidemiologic patterns, the sylvatic (jungle) and urban cycles. The primary transmission cycle (sylvatic) involves nonhuman primates and tree hole–breeding mosquitoes. The urban cycle is defined by human involvement; humans are exposed to infected mosquitoes, which leads to infection (5). YFV, considered one of the reemerging human infections, is an important public health problem with a case-fatality rate that can exceed 50% in symptomatic patients (6).

YFV has been successfully isolated from acute-phase serum and liver samples by injection of suckling mice and cell cultures (7), and recent infections can be confirmed serologically by demonstrating immunoglobulin (Ig) M antibody to YFV in serum samples with a specific IgM-capture enzyme-linked immunosorbent assay (MAC-ELISA) (8,9). Viral antigen can be detected in liver tissue by using immunohistochemistry techniques, and viral nucleic acid can be detected in serum or tissue samples collected during the acute phase of illness by reverse transcription–polymerase chain reaction (RT-PCR) (10).

A safe and effective YFV vaccine, 17D, has been available since 1937. However, as it is not used universally, probably because of cost or inefficient networks for vaccination, the disease continues to occur in Africa and South America (1). Periodic outbreaks of yellow fever in East Africa have been reported since 1940 (11). The largest outbreak, estimated at 300,000 cases, occurred from 1960 to 1962 in Ethiopia (12). In 1966, yellow fever reappeared in Arba-Minch, Ethiopia, east of Lake Abaya, in an area unaffected by the outbreak of 1960. During the 1966 outbreak, 2,200 cases with 450 deaths were reported (13). Seropositivity of 14% had been recorded in northern Kenya (14,15), yet no outbreaks of yellow fever were reported until 1992, when an epidemic emerged in northwest Kenya, with at least 54 cases and 29 deaths (16,17).

On May 5, 2003, Norwegian Church Aid reported to the World Health Organization (WHO) office for southern Sudan a suspected disease outbreak in Imatong, a mountain range located in the southern part of Eastern Equatoria, southern Sudan. The range extends northwards to the town of Torit at 3,000 m above sea level. The mountain is covered by tropical rain forest with rich flora and fauna. Though the civil war has had a negative effect on the ecosystem, the Lango people continue to live on the eastern slopes of this mountain range. The total population

*World Health Organization (WHO) Collaborating Center for Arbovirus and Viral Hemorrhagic Fever Reference and Research, Nairobi, Kenya; †WHO South Sudan, Warwick Center, Nairobi, Kenya; ‡Centers for Disease Control and Prevention, Atlanta, Georgia, USA; §Kenya Medical Research Institute, Nairobi, Kenya; ¶National Institute for Communicable Diseases, Johannesburg, Republic of South Africa; and #U.S. Army Medical Research Unit-Kenya, Nairobi, Kenya

in Imatong is estimated to be 24,387 people (WHO southern Sudan, 2003, unpub. data). Their primary means of livelihood is agriculture, although they also keep some livestock. According to the report by WHO's Early Warning and Response Network (EWARN), which was established in southern Sudan in 1999 to monitor disease outbreaks, and the Norwegian Church Aid, the disease has clinical signs and symptoms of a viral illness and has caused the deaths of seven adults ≥30 years of age. On the basis of clinical symptoms, a viral hemorrhagic fever was suspected. On May 12, 2003, a team led by WHO arrived in southern Sudan to investigate and verify the cause of the outbreak. We report on laboratory investigations performed and the identified causative agent.

Materials and Methods

Collecting Human Serum

A case definition was established as follows: an illness in a patient of any age with high fever, severe headache, neck and back pain, possibly accompanied by vomiting, abdominal pain, diarrhea, hematemesis, bloody diarrhea, jaundice, and epistaxis. A total of 28 serum samples were collected: 18 from patients with acute disease who met the working case definition, 8 from patients with possible disease with headache and joint pain (patients 010, 013, 018, 020, 021, 022, 025, and 027), and 2 from convalescent patients (patients 020 and 028). Patient details, including their locations, are shown in the Table. Blood was collected in sterile evacuated clot activator Vacutainer tubes. Thick and thin smears were made on microscope slides for 17 patients as indicated in the Table, and the serum samples were then separated in the admission facility and stored in a liquid nitrogen dry shipper for transportation to the WHO Collaborating Center for Arbovirus and Viral Hemorrhagic Fever Reference and Research (CCAVHFRR) Laboratory at the Kenya Medical Research Institute (KEMRI) in Nairobi, where they were frozen at –70°C before testing.

Serologic Testing

All the serum samples were heat-inactivated at 56°C for 30 min and tested for antibodies specific to flaviviruses by MAC-ELISA (8,9). Briefly, the plates were coated overnight at 4°C with anti-human IgM capture antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD), the plates were washed, and aliquots of test sera (positive and negative controls) were added in separate wells at a dilution of 1:400. After a 1-h incubation, the plates were washed, and each serum and control sample was reacted with sucrose-acetone extracted yellow fever antigen and control antigen at optimal dilution. Positive samples were detected with a commercially available monoclonal antibody against flaviviruses (6B6C-1) conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA) according to the manufacturer's protocol and 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) substrate (Kirkegaard and Perry Laboratories). Absorbance was read at 405 nm. A presumptive diagnosis was made if IgM antibody in the test sample had a higher optical density than the ratio between the positive and negative control antigens.

RT-PCR

All 28 serum samples were subjected to RT-PCR by using a universal pair of primers for members of the genus *Flavivirus*. Viral RNA was extracted directly from the samples by using the QIAamp viral RNA isolation kit (Qiagen, GmbH, Hilden, Germany). RT-PCR specific for amplification of a 267-bp region of NS5 gene of flaviviruses was performed with 50 pmol of oligonucleotide primers FU1 (TAC AAC ATG ATG GGA AAG AGA GAG AA) and CFD2 (GTG TCC CAG CCG GCG GTG TCA TCA GC) (18) and the Titan One Tube RT-PCR system (Roche Diagnostics GmbH, Mannheim, Germany), according to manufacturer's instructions. The RT-PCR reactions were performed on a Perkin Elmer GeneAmp 9700 Thermocycler (Applied Biosystems, Warrington, England). The following cycling conditions were used: 50°C for 30 min followed by denaturation at 94°C for 3 min, 40 cycles of 94°C for 30 s, annealing at 55°C for 30 s, extension at 68°C for 30 s, and a final extension at 72°C for 7 min. PCR products were resolved by using a 1.5% agarose gel. A confirmatory RT-PCR was performed to amplify a 670-bp region of the YFV prM, M, and E genes with 50 pmol of oligonucleotide primers CAG (CTGTCCCAATCTCAGTCC) and YF7 (AATGCTTCCTTTCCCAAAT) (19). The following cycling conditions were used: 50°C for 30 min followed by denaturation at 94°C for 3 min, 40 cycles of 94°C for 30 s, annealing at 50°C for 30 s, extension at 68°C for 30 s, and a final extension at 72°C for 7 min. PCR products were then resolved on a 1.5% agarose gel. All the positive samples were exposed to a second RNA extraction and RT-PCR to rule out the possibility of contamination. Positive amplicons obtained with the *Flavivirus*-reactive primers were purified by excising the 267-bp fragment from the agarose gel and purifying the fragments with Qiagen gel purification kit (Qiagen) for nucleotide sequence determination.

Nucleotide Sequencing

Gel-purified PCR products were quantitated, and the partial nucleotide sequences of amplicons with a minimum of 60 ng DNA/μL were determined by using Big Dye terminator sequencing ready reaction kits with AmpliTaq DNA polymerase FS (Applied Biosystems**,** Foster City,

Table. History, location and results obtained from patients during yellow fever outbreak, Imatong, southern Sudan, May 2003[a]

Case no	Village	Sex	Age (y)	Days after onset	Flavivirus IgM	RT-PCR[a]	Virus isolation mice/cells	Blood smear MPS[c]
001[d]	Lofi	Male	48	8	–	+	+/+	nd
002[e]	Locomo	Male	60	4	–	–	nd[e]/–	–
003[e]	Tarafafa	Female	unknown	1	–	+	+/+	+
004[e]	Itohom	Female	12	Unknown	+	–	nd/–	+
005[e]	Tarafafa	Female	16	6	+	–	nd/–	+
006[e]	Locomo	Female	7	1	–	+	+/+	nd
007[e]	unknown	Male	unknown	Unknown	+	–	nd/–	+
008[e]	Lofi	Female	40	1	–	+	+/+	–
009[d]	Imatong	Female	35	Unknown	–	–	nd/–	nd
010[e]	Locomo	Female	15	Unknown	+	–	nd/–	+
011[e]	Locomo	Male	45	Unknown	+	–	nd/–	+
012[e]	Lofulang	Male	25	7	+	–	nd/–	nd
013[e]	Locomo	Female	8	1	–	+	+/+	nd
014[e]	Locomo	Male	60	Unknown	+	–	nd/–	–
015[e]	Ogolok	Male	42	Unknown	–	–	nd/–	–
016[e]	Locomo	Male	55	7	+	–	nd/–	–
017[e]	Tarafafa	Female	9	3	–	–	nd/–	–
018[e]	Lotodo	Female	3	Unknown	–	–	nd/–	–
019[d]	Imatong	Male	14	Unknown	–	–	nd/–	nd
020[d]	Imatong	Female	30	20	–	–	nd/–	+
021[f]	Lofi	Female	10	Unknown	+	–	nd/–	–
022[f]	Locomo	Male	25	Unknown	+	–	nd/–	nd
023[f]	Tarafafa	Male	60	Unknown	+	–	nd/–	nd
024[e]	unknown	Male	23	Unknown	–	–	nd/–	–
025[f]	Locomo	Female	40	Unknown	+	–	nd/–	nd
026[e]	Lofong	Female	16	Unknown	–	–	nd/–	–
027[e]	Locomo	Female	4	Unknown	+	–	nd/–	nd
028[f]	Itede	Female	23	21	–	–	nd/–	nd

[a]Ig,immunoglobin; RT-PCR, reverse transcriptase-polymerase chain reaction; MPS, mucopolysacchariodisis; nd, not done.
[b]Specimens were tested using primers reactive with genus *Flavivirus* and with a second set of primers specific to yellow fever virus. Nucleotide sequences were also determined.
[c]Malaria parasite smear.
[d]Fatal case.
[e]No outcome for cases due to inaccessibility.
[f]Recovered.

CA). A cycle sequencing reaction was performed with each of the primers CFD2 and FU1 in a final volume of 20 µL with 30 ng of PCR product, 3.5 pmol of primer, and 4 µL of BigDye Terminators premix, according to the manufacturer's protocol. Briefly, the tubes were heated to 96°C for 2 min, and the reaction mixture underwent 25 cycles of 30 s at 96°C, 30 s at 50°C, and 4 min at 60°C. Excess Big Dye Terminators were removed by precipitation with absolute ethanol. The partial nucleotide sequence was obtained with an Applied Biosystems 377 sequencer, according to manufacturer's instructions.

A search performed on Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD) suggested that the sequence obtained was amplified from the yellow fever genome. This finding was confirmed by aligning the sequence data with data from other known flaviviruses retrieved from GenBank.

Virus Isolation

Aliquots of each of the serum samples were injected intracerebrally into litters of eight newborn (24–48 hours after birth) Swiss albino mice. The mice were observed daily for signs of illness. Sick mice were euthanized, and a preparation was made of 10% of the harvested mouse brain suspension made in Eagle's Maintenance Media with 2% serum albumin, 2% glutamine, and 1% antibiotics (penicillin, streptomycin, and amphotericin B) and centrifuged at 3,000 rpm for 10 min. The clarified supernatant fluid was filtered with a 0.45-µm syringe filter and injected intracerebrally into a litter of suckling mice to confirm the isolation.

All the sera were diluted 1:10 in sterile phosphate-buffered saline pH 7.4 (without magnesium and calcium), and viral isolation attempts in Vero cell cultures were made by injecting 100 µL of the diluted sample onto confluent monolayer of Vero cells in 25-cm^2 culture flasks. Flasks

were incubated at 37°C and observed daily for evidence of cytopathogenic effect (CPE). After CPE was evident, the supernatant fluid was clarified by centrifugation at 3,000 rpm for 10 min. Viral RNA was extracted from both the 10% brain suspension from sick mice and from the clarified cell culture media and screened for flavivirus and YFV viral nucleic acid RNA by RT-PCR as described previously.

Differential Diagnosis

IgM antibody tests were performed on all the samples to exclude recent exposure to Ebola virus, Marburg virus, Crimean-Congo hemorrhagic fever virus, West Nile virus, dengue virus, chikungunya virus, Sindbis virus, and Rift Valley fever virus. In addition, Ebola virus was excluded by using an antigen capture ELISA. PCR was performed to exclude the following pathogens: Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, Ebola virus, Marburg virus, rickettsia, leptospira, brucella, and members of the genus *Alphavirus* and *Bunyavirus*. For 17 patients, thick blood smears were checked for *Borrelia* spp. and thin blood smear slides were checked for malarial parasites.

Results

On May 17, 2003, serologic tests and RT-PCR performed at the WHO CCAVHFRR at KEMRI identified YFV as the causative agent of the outbreak in southern Sudan. The diagnosis was supported by subsequent gene sequencing of the amplicons and isolation of the virus. The results obtained are summarized in the Table. Duplicate samples submitted to the Special Pathogens Unit at the National Institute for Communicable Diseases, South Africa, confirmed an outbreak of yellow fever.

Detecting IgM Antibody to Flaviviruses

RT-PCR and Nucleotide Sequencing

Samples from five patients were positive for flavivirus viral nucleic acid and subsequently were positive for yellow fever viral nucleic acid with primers specific for YFV. Analysis of the nucleotide sequences obtained with the flavivirus primers confirmed that the amplicons were from the NS5 region of the yellow fever genome (phylogeny has been performed separately). The serum samples that were positive by PCR were collected from patients on day 1 after onset of illness except for one sample collected on day 8 from a patient with a fatal infection.

Viral Isolation

YFV was isolated, in both suckling mice and cell cultures, from five patients from whom PCR products were obtained. Virus isolation attempts on the remaining 23 serum samples from both suckling mice and Vero cell culture were negative.

Differential Diagnosis

The sera were all negative by using various tests as described for Ebola virus, Marburg virus, Crimean-Congo hemorrhagic fever virus, West Nile virus, dengue virus, chikungunya virus, Sindbis virus, Rift Valley fever virus, rickettsia, leptospira, brucella, and members of the genus *Alphavirus* and *Bunyavirus*. The thick smears were negative for *Borrelia* by Giemsa staining. However, malaria parasites were demonstrated in thin smears from 7 of 17 patients tested; 6 of the patients with malaria also had evidence of YFV infection.

Discussion

Samples from 28 patients were collected from various villages in the Imatong region as follows: 10 were from Locomo village, 3 from Lofi, 4 from Tarafafa, 3 from Imatong, and 1 each from Itohom, Lofulung, Ogolok, Lotodo, Lofong, and Itede; 2 were from unknown locations. A total of 18 patients had possible YFV infections demonstrated by detection of IgM antibody, while 5 patients were confirmed as positive for YFV by RT-PCR and virus isolation. Antibody was not demonstrable in the five patients who had positive RT-PCR results; in 4 patients, the specimens were collected too early (day 1) for antibody to be detectable. In patient 001 (Table), the specimen was collected on day 8 from a patient with a fatal infection. The patient was comatose and in the late stages of infection; antibodies were not found, possibly because of a failure to produce antibodies for a variety of factors, such as nutritional status, age, and immune status. This finding demonstrates the importance of using both modalities in the diagnosis of acute YFV infection.

Plasmodium, the causative agent of malaria, was demonstrated in six patients who were positive for YFV. This finding demonstrates that co-infection with malaria and YFV is possible. Therefore, malaria should not be assumed as the etiologic agent of a disease outbreak solely based on positive screens, without first excluding other causes.

YFV maybe be enzootic in the Imatong Mountains, yet outbreaks have not been previously recorded in this region. Political unrest and population movement of highly susceptible (unvaccinated) people within the Imatong Mountains might have been a predisposing factor for the YFV outbreak in this region. During the civil unrest, large populations of monkeys were reportedly seen. As they became more dependent on human crops for food, the monkeys came closer to human habitations. Weather factors might have also contributed to the timing of the outbreak. The months of March to May 2003 were the rainy

season, and heavy rains were reported around the mountain region. The persistent downpours may have resulted in high vector populations. With no history of human yellow fever in the outbreak zone, the monkey population must be adequate to support continued circulation of the virus in the sylvatic cycle. A combination of these factors likely contributed to the timing of the outbreak.

The early detection of this outbreak was due to EWARN, a WHO-facilitated network that brings together several healthcare providers and laboratories. One of the objectives of EWARN is the early detection and response to outbreaks, such as this one. The rapid response to this outbreak of YFV and subsequent laboratory-based diagnosis clearly demonstrate that an efficient surveillance system can lead to quickly detecting outbreaks and appropriate intervention, potentially saving many lives. At the time of this writing, 80% of the population at risk in Imatong had been vaccinated, with the campaign extending to Torit County and neighboring counties. Vaccination in Torit County had covered 51% of the population at risk (98,705).

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Mr. Onyango is a researcher at the World Health Organization Collaborating Center for Arboviruses and Viral Hemorrhagic Fever Reference and Research Laboratory, based at the Kenya Medical Research Institute. He heads the Molecular Virology section.

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Address for correspondence: Clayton O. Onyango, WHO Collaborating Center for Arbovirus and Viral Hemorrhagic Fever Reference and Research, Kenya Medical Research Institute, P.O. Box 54628, Nairobi, Kenya; fax: +254-20-2714745; email: conyango@nairobi.mimcom.net

Newborn Screening for Congenital Infectious Diseases

Eurico Camargo Neto,*†‡ Rosélia Rubin,† Jaqueline Schulte,† and Roberto Giugliani*‡§

To estimate the prevalence of congenital toxoplasmosis, Chagas disease, cytomegalovirus, and rubella, blood samples on dried blood spot (DBS) from neonates (day 3–20 of life) were screened for immunoglobulin (Ig) M against *Toxoplasma gondii*, cytomegalovirus, rubella virus, and IgG against *Trypanosoma cruzi* by methods used for serum and adapted for use with DBS. Positive samples were further analyzed for IgM and IgG in serum from neonates and mothers. DBS samples from 364,130 neonates were tested for *Toxoplasma gondii*–specific IgM, and 15,873 neonates were also tested for IgM against cytomegalovirus and rubella virus and for *Trypanosoma cruzi*–specific IgG. A total of 195 were diagnosed with congenital toxoplasmosis, 16 with cytomegalovirus, and 11 with congenital rubella. One newborn had a confirmed result for Chagas disease, and 21 mothers had positive serum antibodies. These results suggest that infectious diseases should be considered for future inclusion in programs for newborn screening of metabolic diseases in disease-endemic areas.

Toxoplasmosis infection during pregnancy can cause congenital infection and manifestations, such as mental retardation and blindness (1). Hydrocephalus, intracranial calcification, and retinochoroiditis are the most common manifestations of tissue damage from congenital toxoplasmosis. However, the effect of prenatal treatment on these outcomes is unclear (2), and the best method for preventing and controlling congenital toxoplasmosis is controversial. A neonatal screening program based on detecting immunoglobulin (Ig) M antibodies against *Toxoplasma gondii* alone would identify 70%–80% of congenital toxoplasmosis cases (3). Moreover, prenatal screening has indicated neither the natural history of toxoplasmosis nor the efficacy of antiparasite treatment during pregnancy (4). A study by Guerina et al. (5) showed a prevalence of congenital toxoplasmosis of 1 per 10,000 live births in the United States, where 85% of women of childbearing age are susceptible to acute infection with *T. gondii* (6).

Congenital Chagas disease has been reported, mostly in Latin America (7), where approximately 20 million persons are affected; 90 million others are at risk of being infected by the parasite (8). The high prevalence of the disease has been demonstrated in several Latin American countries (8–10). The evolution of the congenital and reactive forms of the disease has yet to be determined (11). The vertical transmission of *Trypanosoma cruzi* cannot be prevented, but early detection and treatment of congenital infection achieve cure rates close to 100% (12–14). Persons infected by *T. cruzi* can be successfully treated with nifurtimox or benzonidazole (12,14).

Cytomegalovirus is the most common congenital virus infection in the world. Both primary and recurrent infection can result in fetal infection. The birth prevalence of congenital cytomegalovirus infection varies from 0.3% to 2.4%, and at least 90% of congenitally infected infants have no clinical signs (15). The disease causes illnesses ranging from no clinical signs to prematurity, encephalitis, deafness, hematologic disorders, and death (16). Congenital cytomegalovirus infection is described in 30,000 to 40,000 newborns each year in the United States; approximately 9,000 of these children have permanent neurologic sequelae (17). The death rate of symptomatic congenital cytomegalovirus infection is approximately 30% (18). The value of vaccination against congenital cytomegalovirus infection is not known, and screening of newborn infants has been recommended to indicate infants at high risk for deafness and to make early rehabilitation possible (18).

Rubella virus infection during early pregnancy can lead to severe birth defects known as congenital rubella syndrome (19). Sequelae of rubella virus infection include three distinct neurologic syndromes: postinfectious encephalitis after acute infection, a range of neurologic manifestations after congenital infection, and an extremely rare neurodegenerative disorder, progressive rubella panencephalitis, that can follow either congenital or postnatal infection (19). A review of the literature that identified studies about the

*Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; †Nobel RIE Laboratory, Porto Alegre, Brazil; ‡Neonatal Screening Center, Porto Alegre, Brazil; and §Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

prevalence of anti-rubella antibodies from developing countries concluded that congenital rubella syndrome is an under-recognized public health problem and that appropriate data need to be collected to estimate the cost-effectiveness of a potential global rubella control program (20).

Material and Methods

Samples

Blood was obtained by heel puncture and applied on filter paper Schleicher and Schuell 903 (Keene, NH, USA), between day 3 and day 20 of life (mean day 10). The samples were collected in areas throughout Brazil and sent by mail to Porto Alegre, South Brazil, where the tests were performed.

Tests in Filter Paper Dried Blood Spots (DBS)

An indirect enzymatic immunoassay for IgM anti–*Toxoplasma gondii* prepared in-house (21) was used to test the first 78,350 samples. A fluorometric capture enzymatic immunoassay (FEIA) (Neonatal *Toxoplasma gondii*, AniLabsystems, Helsinki, Finland) was used in 285,780 samples. A kit produced by Wiener Laboratory (Rosario, Argentina) to detect IgG against *Trypanosoma cruzi* in human serum was adapted. In brief, a 3.2-mm DBS paper disk was placed in microtiter plates precoated with *T. cruzi*. The serum was eluted with 200 µL of phosphate-buffered saline (PBS)/bovine serum albumin (BSA) buffer on an orbital shaker set at 100 rpm for 60 min and incubated for 14–16 h at room temperature. After washing with PBS/BSA buffer, the protocol was followed according to the manufacturer's instructions with two modifications: the reactions occurred at room temperature, and the incubation times were duplicated, except after the addition of the color reagent. Also, two kits produced by Diesse Diagnostica Senese (Monteriggioni, Italy) were adapted to detect IgM against cytomegalovirus and IgM against rubella in human serum eluted from filter paper. A 3.2-mm DBS paper disk was placed in microtiter plates precoated with an anti-human IgM monoclonal antibody. The serum was eluted with 150 µL of PBS/BSA buffer on an orbital shaker set at 100 rpm for 2 h at room temperature. Afterwards, the protocol was followed according to the manufacturer's instructions with the same modifications made with the IgG Chagas test.

Controls

The cutoff for each test was obtained by testing 97 whole blood samples negative for IgM *Toxoplasma gondii* antibodies, 95 whole blood samples negative for IgG *Trypanosoma cruzi* antibodies, and 86 whole blood samples negative for IgM cytomegalovirus and rubella virus antibodies. The cutoff was established as three times the mean optical density of the negative samples. Negative, cutoff, and positive control samples were prepared in DBS for each test. The sensitivity of the methods was tested with 55 positive IgM *Toxoplasma gondii* samples, 43 IgG positive *Trypanosoma* samples, and 40 positive IgM cytomegalovirus and rubella samples. All samples were over the cutoff point for a preliminary analytical sensitivity of 100%. The presumptive positive samples were confirmed in a new duplicate run.

Confirmatory Serologic Tests

Serum tests were performed on samples from the mothers and neonates. For the first 202 case-patients with possible congenital toxoplasmosis and Chagas disease, an indirect immufluorescence test (Biolab-Meriéux Diagnóstica, Rio de Janeiro, Brazil) was used. Confirmatory serum tests for toxoplasmosis, cytomegalovirus, and rubella (IgM and IgG) were run by microparticle enzyme immunoassay (MEIA) in the Axsym (Abbott Laboratories, Chicago, IL). The FEIA method was used for serum tests and run in parallel with the Axsym, which showed good agreement.

Clinical Examination of Infected Infants

Patients suspected to have congenital toxoplasmosis and cytomegalovirus were given a skull ultrasound, tomography, or x-ray and ophthalmoscopic and audiologic exams. Patients suspected to have congenital Chagas disease and their mothers were evaluated for cardiac and esophageal malformations. Patients suspected to have congenital rubella were evaluated for hearing loss and eye lesions. When the samples were above or maximally 20% below the cutoff value, serum samples from the infant and the mother were requested. All clinical and follow-up information was obtained by contacting the pediatricians or, in rare cases, the families.

A neonate was followed and classified as infected by meeting one of the following criteria: antigen-specific IgM and IgG in the neonate and in the mother, antigen-specific IgM in the neonate only, antigen-specific IgM in the mother only, or increased amount of antigen-specific IgG in the neonate. An increase in the neonate's IgG antibodies excluded maternal origin.

Results

Congenital Toxoplasmosis

We analyzed 364,130 DBS samples for IgM against *Toxoplasma gondii*, and 699 samples were positive; all were recalled for serum confirmation. Serum samples from 594 neonates and 576 mothers were received, and of these, 202 suspected cases were tested by indirect immunofluorescence (IIF) (17 diagnosed with congenital

toxoplasmosis), and 497 were tested by MEIA and FEIA (178 were diagnosed with congenital toxoplasmosis). A total of 195 neonates (1 in 1,867) were confirmed to have congenital toxoplasmosis. The laboratory findings are presented in Table 1, and the clinical findings are summarized in Table 2. The false-positive percentage was 0.16%. All patients with confirmed diagnoses were given sulphadiazine, pyrimethamine, and folinic acid.

Of the 195 patients with congenital toxoplasmosis, 138 (70.7%) were asymptomatic until 7 years of age. One IgM-positive asymptomatic infant also had HIV, and six patients with sequelae received late treatment (6–14 months after diagnosis) and could be asymptomatic if treated early. The follow-up was 1–84 months (mean 30 months).

Congenital Chagas Disease

We analyzed 15,873 DBS samples for Chagas disease and had 36 positive results. Serum samples from 31 neonates and 30 mothers were received for confirmatory tests. Results are shown in Table 1. The prevalence of specific *Trypanosoma cruzi*–specific IgG was estimated in 1 in 756 mothers (false-positive rate of 0.08%). All mothers and neonates had x-rays and echocardiographys. One mother (age 41) had an expanded heart and had a brother with Chagas disease. All others were asymptomatic and are under clinical observation. The antibodies observed in the neonates disappeared in time. The follow-up for positive children was 1–24 months (mean 15 months).

Congenital Cytomegalovirus

A total of 15,873 DBS samples for IgM against cytomegalovirus were analyzed, and 39 were positive. Thirty-two serum samples from neonates and 30 from mothers were received for confirmatory testing, and 16 cases were confirmed (Table 1). In 8 case-patients, the IgG levels decreased in the neonates, and in 15 case-patients, IgG in the neonates' serum only was interpreted by the clinicians as being of maternal origin. Likewise, in one case, antigen-specific IgM was detected only in the mother's serum and antigen-specific IgG only in the neonate's serum. This neonate was not followed. The prevalence of cytomegalovirus was estimated at 1 in 992 live births, and the false-positive was 0.11%. The follow-up of the infected patients showed that 11 were asymptomatic until 2 years of age, including a premature baby (36 weeks' gestation). Laboratory results and clinical findings are presented in Tables 1 and 3. The follow-up was 1–24 months (mean 15 months).

Congenital Rubella

A total of 15,873 DBS samples for IgM against rubella virus were analyzed, and positive results were obtained in 67. Serum samples from 55 neonates and 52 mothers were received, and 16 were positive. Four mothers and one neonate were vaccinated against rubella and were excluded from the sample. The prevalence estimated was at 1 in 1,443, and the false-positive rate was 0.30%. Of 49 neonates and mothers without detectable IgM, 30 were followed until the specific IgG levels decreased. In 19 cases, the infant's IgG levels were interpreted by the pediatrician as being of maternal origin and were not followed. From these cases, three mothers received rubella vaccine before pregnancy. The follow-up of the 11 positive cases is shown in Table 4. The follow-up was 1–24 months (mean 15 months).

Discussion

In screening for congenital toxoplasmosis, 195 neonates had diagnosis confirmed, and 105 (53.8%) had specific IgM. The diagnosis of 41 cases (21%) was only possible by monitoring the specific IgG levels in the infants; 49 (25%) cases were followed because of IgM in the mother's serum samples. Some mistakes may have occurred in the clinical evaluation of neonates in whom IgM were not detected in serum. A prospective study showed that a serologically transient toxoplasmosis occurred in 15% of the cases of unknown pathophysiology, leading to a risk of misdiagnosis and inadequate surveillance (22). The clinical decision not to monitor IgG levels in 123 patients for whom IgM was detected only in the mother's serum suggests that the concept still prevails: in the absence of IgM in the neonate, the IgG is from maternal origin. Several cases might have been misdiagnosed in these cases. In some cases, the beginning of treatment was delayed because of the following: 1) unwillingness of the clinician to treat asymptomatic infants because of the toxicity of the drugs; 2) time elapsed between birth, screening, confirmatory tests, and clinical examinations; and 3) the decision of the family to consult another physician.

Table 1. Serum results in the confirmatory tests

Disease	IgM[a] in mother and neonate	IgM in neonate	IgM in mother	Increase of IgG in neonate
Congenital toxoplasmosis	84	21	49	41
Chagas disease	1[b]	1	1	1[b]
Cytomegalovirus infection	6	9 (2 adopted)	1[b]	1[b]
Congenital rubella	8 (4 mothers vaccinated)	9 (2 adopted)	2[b]	1[b]

[a]Ig, immunoglobulin.
[b]Same neonate.

Table 2. Symptoms and findings in patients with congenital toxoplasmosis

n	Complementary examination	Clinical findings
25	Retinal scar or retinochoroiditis	2 blind, 1 with myopia
14	Intracranial calcifications	1 with cognitive deficiency, 4 with splenomegaly or hepatosplenomegaly (HSM)
7	Retinal scar or retinochoroiditis and intracranial calcifications	2 with splenomegaly or HSM, 2 with neuromotor retardation, 1 microcephaly, 1 hydrocephaly and microophthalmia, 1 died immunosuppressed
7	Other symptoms	Splenomegaly or HSM, neuromotor retardation, microcephaly, hydrocephaly and microophthalmia, 1 died immunosuppressed

Congenital toxoplasmosis is routine in prenatal studies in France, and the efficacy of this program is difficult to estimate, even considering the benefits (23). Moreover, prenatal programs have the risk of invasive methods and, according to Lebech (3), testing for specific IgM shows a better cost-benefit ratio if included in newborn-screening programs.

In 41 confirmatory serum tests for Chagas disease on samples from 21 neonates and 20 mothers, IgM antibodies were found in one neonate. He was treated and remains asymptomatic. IgG levels decreased in all asymptomatic neonates. One mother was identified with cardiac enlargement, and all the others received clinical counseling. In the population studied, most of the samples came from urban areas, and the incidence of mothers with specific antibodies (1 in 756) suggests that the seroprevalence can be higher in rural and disease-endemic areas (9,11,12). Because testing to detect IgM against *Trypanosoma cruzi* is not available, neonatal screening could detect asymptomatic mothers.

IgM against cytomegalovirus was detected in 87.5% of the patients diagnosed with congenital cytomegalovirus; 68.8% were asymptomatic. In 15 neonates, having only IgG antibodies in the serum was interpreted as being of maternal origin by the clinicians. The lack of information about the synthesis of specific antibodies against cytomegalovirus could be justified for the same reasons described previously for congenital toxoplasmosis (22). In a 16-year study, 388 children with congenital cytomegalovirus were evaluated for neurosensorial hearing loss (24). A hearing deficit was observed in 5.2% of the cases at birth and 15.4% in children ≥6 years of age, and neonatal screening for cytomegalovirus infection was suggested (23). Symptomatic cytomegalovirus can occur after maternal recurrent infection, but the incidence of these cases is still not established (16). Seropositive women reinfected by a different strain of cytomegalovirus can transmit the infection to the fetus and deliver a symptomatic child (25,26). In this work, the incidence of congenital infection by the cytomegalovirus was estimated to be 1 in 992. A successful treatment with the combined use of ganciclovir and anti-cytomegalovirus immunoglogulin was reported (27).

Table 3. Pediatric decision and symptoms presented in patients with congenital cytomegalovirus

n	Decision	Symptoms
5	No treatment	Asymptomatic, clinical follow-up
6	Symptomatic; treated with ganciclovir	Microcephaly, intracranial calcifications, deafness, failure to thrive, HSM[a] (initially investigated for galactosemia), progressive muscular atrophy, sepsis, low weight, difficulty swallowing (died), thrombocytopenia, leukopenia

[a]HSM, hepatosplenomegaly.

Because of the mass vaccination to rubella, the high incidence of positive tests was unexpected (1 in 1,443, excluding the positive tests in vaccinated mothers). The results confirmed the findings of Cutts and Vynnycky (20) that the disease is under-recognized in developing countries. As observed with congenital toxoplasmosis and cytomegalovirus, 38.7% of neonates showed only IgG antibodies in the confirmatory tests. No further investigation was made because the clinicians presumed at follow-up that IgG was of maternal origin. Also, rubella vaccination of young women does not seem to be enough to prevent the transmission of the virus in a future pregnancy (28). However, prenatal care and mass vaccination seem to be the better choices to prevent new cases of congenital rubella. The purpose of neonatal screening would be to identify congenitally asymptomatic, infected neonates at birth. In Brazil (170 million persons and approximately 2,400,000 newborns/year), the prevalence of infectious diseases is higher than phenylketonuria (1 in 13,000) and congenital hypothyroidism (1 in 3,500). Congenital toxoplasmosis, with well-defined treatment protocols and a high prevalence, deserves special attention from health authorities, and its inclusion in screening programs should be considered. The follow-up of children until 7 years of age showed that most patients treated were asymptomatic or that the sequelae observed at the time of diagnosis had not progressed. By using the existing programs of newborn screening in the country, the inclusion of congenital toxoplasmosis, cytomegalovirus, and Chagas disease in disease-endemic areas would increase the cost

Table 4. Symptoms and number of patients with congenital rubella

Asymptomatic	Symptomatic
6 (1 adopted)	1 congenital rubella syndrome (died)
	2 with scars in 1 eye
	1 with cataract and received lens implant
	1 with cardiopathy and partial deafness

of the program to approximately U.S.$1.50 per test. Also, treating infectious diseases is cheaper, and the time of treatment is shorter when compared to the expensive and long-term treatment of metabolic diseases. Studies on long-term follow-up of these children are in progress for a better understanding of the efficacy of the treatments and the effectiveness of mass screening.

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Dr. Neto is a researcher at the Biochemistry Department of the Federal University, Porto Alegre, Brazil. His main research interest is the prevention of sequelae caused by congenital metabolism defects and by congenital infections in newborns.

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Address for correspondence: Eurico Camargo Neto, Department of Biochemistry, Federal University of Rio Grande do Sul, Nobel RIE Laboratory and Neonatal Screening Center, Av. Ipiranga, 5000, Porto Alegre, RS, Brazil 90610-000; fax: 55-51-3336-1933; email: eneto.voy@terra.com.br

Candida parapsilosis Characterization in an Outbreak Setting

Duncan M. Kuhn,*[1] Pranab K. Mukherjee,* Thomas A. Clark,† Claude Pujol,‡ Jyotsna Chandra,* Rana A. Hajjeh,† David W. Warnock,† David R. Soll,‡ and Mahmoud A. Ghannoum*

Candida parapsilosis is an important non-*albicans* species which infects hospitalized patients. No studies have correlated outbreak infections of *C. parapsilosis* with multiple virulence factors. We used DNA fingerprinting to determine genetic variability among isolates from a *C. parapsilosis* outbreak and from our clinical database. We compared phenotypic markers of pathogenesis, including adherence, biofilm formation, and protein secretion (secretory aspartic protease [SAP] and phospholipase). Adherence was measured as colony counts on silicone elastomer disks immersed in agar. Biofilms formed on disks were quantified by dry weight. SAP expression was measured by hydrolysis of bovine albumin; a colorimetric assay was used to quantitate phospholipase. DNA fingerprinting indicated that the outbreak isolates were clonal and genetically distinct from our database. Biofilm expression by the outbreak clone was greater than that of sporadic isolates ($p \leq 0.0005$). Adherence and protein secretion did not correlate with strain pathogenicity. These results suggest that biofilm production plays a role in *C. parapsilosis* outbreaks.

The yeast *Candida* is the fourth most common cause of hospital-related bloodstream infections (1). Forty percent of patients who have had *Candida* isolated from their intravenous catheters have underlying fungemia (2), and the case-fatality rate for catheter-related candidemia approaches 40% (3).

Although *C. albicans* is the most commonly isolated yeast, other species are found with increasing frequency, including *C. parapsilosis* (4). *C. parapsilosis* particularly affects critically ill neonates and surgical intensive care unit (ICU) patients (5,6), likely because of its association with parenteral nutrition and central lines (7,8). The affinity of *C. parapsilosis* for foreign material is shown by infections related to peritoneal dialysis catheters (9) and prosthetic heart valves (10), and this characteristic may be important in infections of cancer patients with indwelling access devices (11). *C. parapsilosis* is increasingly responsible for hospital outbreaks, and the hands of healthcare workers may be the predominant environmental source (12).

Our understanding of fungal virulence factors is limited. The surface adherence capacity of *Candida* is likely one such factor, possibly linked to its subsequent ability to form biofilms (13). Clinically obtained *C. albicans* isolates form biofilms (14), which may be important for sustaining infection. Adhesion (15) and biofilm formation (14) may be especially important for *C. parapsilosis*, since indwelling devices appear to be the predominant route of infection (8,11). Total parenteral nutrition (TPN) solutions may promote *C. parapsilosis* adhesion and growth (16). Recently, biofilm-forming potential was cited as a reason that patients with *C. parapsilosis*-infected catheters should have the device removed (17).

Fungi secrete enzymes integral to pathogenesis. Phospholipases (e.g., phospholipase B) and proteases (e.g., secreted aspartyl proteases [SAPs]) are two of the best-characterized. Although phospholipase B expression has been well studied in *C. albicans* (18), the relationship between *C. parapsilosis* virulence and phospholipase phenotype is unclear. The role of SAP and pathogenesis is similarly unclear (19).

We characterized genetic and phenotypic characteristics of isolates from a *C. parapsilosis* outbreak that occurred in a Mississippi community hospital (20) and compared these characteristics with those of isolates obtained from persons with sporadic infections at our tertiary hospital. We performed molecular characterization, comparing *C. parapsilosis* isolates involved in the

*University Hospitals of Cleveland and Case Western Reserve University, Cleveland, Ohio, USA; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and ‡University of Iowa, Iowa City, Iowa, USA

[1]Current affiliation: Division of Pulmonary and Critical Care Medicine, Massachusetts General Hospital, Boston, Massachusetts, USA

outbreak with those from our own clinical collection. We then compared adhesion ability, biofilm production, and secretion of SAP and phospholipase B of the outbreak isolates and our clinical strains.

Methods

Organisms

Outbreak isolates of *C. parapsilosis* were obtained at a Mississippi hospital from April through October 2001. Epidemiologic details regarding the organisms and patients have been published elsewhere (21). We examined five invasive strains (defined as obtained from blood or catheter cultures: 165, 167, 173, 177, 179), and three environmental isolates (from healthcare workers' hands; 313, 317, and 385). Isolates from sporadic infections were from the culture collection at the Center for Medical Mycology, University Hospitals of Cleveland (University Hospitals). *C. parapsilosis* strain P/A71 was obtained from sputum, P92 from blood, and *C. albicans* M61 from an intravascular line (19). The site of isolation of other strains is indicated in the figures. Speciation was performed by using germ tube tests and API20C-AUX methods. Organism propagation has been described previously (21). All specimens were stored and used without patient identifiers, to maintain confidentiality.

DNA Fingerprinting

Isolates were analyzed by Southern blot hybridization using the complex DNA fingerprinting probe Cp3-13 (22), according to published methods (23). Genomic DNA was extracted from cells according to the protocol described by Scherer and Stevens (24). Three micrograms of DNA preparation were digested with a combination of *Eco*RI and *Sal*I (4 U each per microgram of DNA) for 16 h at 37°C, then underwent electrophoresis at 60 V in a 0.7% agarose gel. *C. parapsilosis* strain J940043 was used as a reference, and its DNA was run in the first and last lanes of the fingerprinting gel. The DNA was transferred from the gel to a nylon Hybond N^+ membrane (Amersham, Piscataway, NJ) by capillary blotting, prehybridized with sheared salmon sperm DNA, hybridized overnight with [^{32}P]dCTP-labeled Cp3-13 probe, and viewed by autoradiogram.

Computer-Assisted Cluster Analysis

The autoradiogram image was digitized, unwarped, and straightened, by using the DENDRON software database (25). Processed hybridization patterns were scanned to identify and link common bands. Patterns underwent pairwise comparison: the similarity coefficient (S_{AB}) between the patterns of every pair of isolates A and B was computed according to the formula:

$$S_{AB}=2E/(2E + a + b)$$

where E is the number of bands common to both strains, a is the number of bands unique to strain A, and b is the number of bands unique to strain B (22). The S_{AB} ranges from 0.0 (no common bands) to 1.0 (identical match of all bands). Dendrograms based on S_{AB} values were generated by the unweighted pair-group method with arithmetic average (UPGMA) (26); values of 0.07 were considered the threshold for group association (27).

Adherence Assays

Adherence of *C. parapsilosis* isolates to silicon elastomer (SE) disks was measured by using a modification of earlier methods (28); SE was obtained from Cardiovascular Instrument Corp. (Wakefield, MA) and prepared as described (14). Standardized suspensions of 50 to 200 cells/mL were added onto SE disks. Disks were then washed in phosphate-buffered saline (PBS) to remove nonadherent cells and placed in wells of 12-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). Two milliliters of warm (55°C) liquid SD agar was added per well to completely cover the SE disks and allowed to solidify. Plates were incubated overnight (37°C), and colonies adhering per disk were counted by using a dissecting microscope.

Biofilm Formation and Quantitation

C. parapsilosis biofilms were formed on SE disks as described previously (14). Control disks were handled identically, except that no blastospores were added. Biofilm quantitation was performed as described (21) with dry weight measurements. Dry weight measured total biofilm mass including fungal cells and extracellular matrix.

SAP Assays

Previous authors have described methods of evaluating the ability of *Candida* SAP to degrade bovine serum albumen (BSA) from cells grown in SAP expression media (29). We grew *C. parapsilosis* isolates in yeast nitrogen base (YNB) because we could detect SAP activity using YNB, the use of expression medium resulted in contaminating BSA bands, and our assay utilized the same medium used for examining biofilm formation. To confirm relevance of our findings to those of previous studies, we examined SAP expression of organisms grown in the expression medium and found similar results (data not shown).

After overnight growth, *Candida* cell suspensions were centrifuged (6,000 x *g* for 8 min), and the supernatant was collected, then concentrated by using a Centricon 10,000 NMWL filter centrifuge (Millipore Corp., Billerica, MA). Supernatant protein (500 ng) was incubated at 37°C for 15

min with 0.4 mL of 1% BSA (wt/vol, in 0.1 mol/L citrate buffer, pH 3.2). After incubation, 10 µL sodium dodecyl sulfate (SDS) sample buffer and 7 µL of reducing agent were added to 40 µL of each mixture, and the proteins solubilized by boiling (10 min). Ten microliters of sample was separated by SDS–polyacrylamide gel electrophoresis (PAGE), and the protein bands were visualized by silver staining (SilverXpress Staining Kit, Invitrogen Corp., Carlsbad, CA). The appearance of a 20-kDa band was indicative of SAP activity. Quantitation of this band was determined by using QuantOne software v4.3.0 (BioRad Laboratories, Hercules, CA). Control experiments were performed by adding either no supernatant (100 µL of sodium citrate buffer instead) or supernatant mixed with protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO); 10 µL/mL of supernatant). Protein estimations were performed by using the BioRad D*c* kit (BioRad Laboratories) and BSA as standard.

Phospholipase Assays

A colorimetric assay for free fatty acid (FFA) was used to assess phospholipase activity (30). The incubation mixture for phospholipase (acylhydrolase) activity consisted of 200 µM dipalmitoyl (C16:0) phosphatidylcholine and 200 µmol/L L-palmitoylcarnitine in 0.1% (vol/vol) Triton X-100. Concentrated culture supernatant was added (100 µg of total protein), and the mixture made up to a final volume of 0.25 mL with 0.1 mol/L of sodium citrate, pH 4.0. Reactions were incubated at 37°C for 1 h, then stopped by adding chloroform/methanol (1:2, vol/vol). The reaction products were extracted (31), evaporated to dryness under nitrogen, and taken up in 50 µL of 0.1% (vol/vol) Triton X-100. The relative level of free fatty acids in each sample was determined by using an acyl-CoA-oxidase system assay kit (Roche Molecular Biochemicals, Indianapolis, IN).

Statistical Analysis

Adherence and biofilm experiments were performed in quadruplicate and on separate days. Results for different isolates were normalized to *C. parapsilosis* strain 167 to facilitate meaningful comparisons across multiple experiments (14). Phospholipase and SAP assays were performed at least twice; representative results are shown. Statistical analysis was performed by using StatView v5.0.1 software (SAS Institute, Cary, NC); p values < 0.05 were considered significant.

Results

DNA Fingerprinting Analysis

Isolate relatedness was investigated by using the complex DNA fingerprinting probe Cp3-13 (22). We examined both outbreak strains and our independent University Hospitals' isolates to characterize the relatedness of a range of clinical *C. parapsilosis* strains. As shown in Figure 1, the five invasive strains and one of the three environmental isolates generated identical patterns. The two remaining strains (313 and 385) were hand isolates. The fingerprinting pattern of strain 313 was limited to weak bands, while none were obtained for 385. The patterns of the outbreak isolates were also distinct from those of the University Hospitals' isolates.

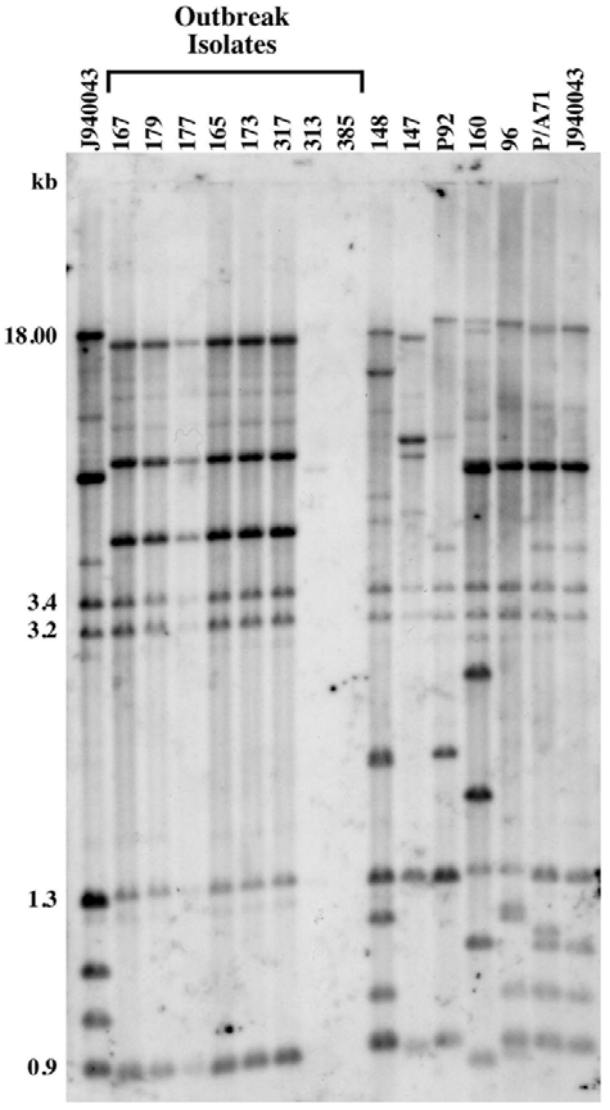

Figure 1. Genetic analysis of *Candida parapsilosis* clinical isolates. Southern blot hybridization patterns of the 14 *C. parapsilosis* test isolates were probed with the Cp3-13 DNA fingerprinting probe. The reference strain J940043 was run in the outer two lanes of the gel. Isolates associated with the hospital outbreak are indicated. Note that while isolates 167, 179, 177, 165, 173, and 317 displayed identical group I patterns, strains 313 and 385 showed patterns typical of non-group I strains with a lack of abundant intense bands. Molecular sizes are presented in kilobases to the left of the panel.

Relatedness among outbreak and University Hospitals' isolates was further assessed through cluster analysis (Figure 2). The six outbreak isolates with an identical fingerprinting pattern had an S_{AB} value close to 1, whereas the dendrogram nodes linking the remaining isolates to each other had an S_{AB} value <0.7, the threshold for relatedness (27). These analyses showed that the six outbreak isolates were identical and belonged to group I strains (22). The remaining isolates appeared moderately related to unrelated at the genetic level, and therefore were non–group I strains. Previous studies have shown that Cp3-13 fingerprinting patterns made up of a few weak bands typically belong to groups II or III, representing a minority of *C. parapsilosis* clinical isolates (22,32). However, internally transcribed spacer region sequencing of strain 313 indicates that it, in fact, belongs to group 1 (D. Warnock, pers. comm.). Alternately, this finding may suggest past genetic exchanges between group I and non–group I strains. The dendrogram also shows that, in addition to being unrelated to the outbreak isolates, the University Hospitals' strains are not related to one another but represent sporadic cases.

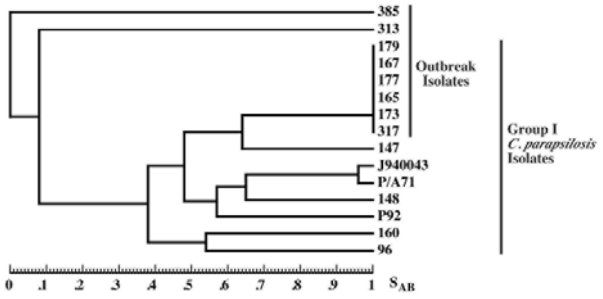

Figure 2. Relatedness of *Candida parapsilosis* clinical isolates. Dendrogram generated from S_{AB}s computed for pairwise comparisons of the 14 *C. parapsilosis* test isolates and the reference strain J940043 fingerprinted with Cp3-13. Note that with the exception of the six identical outbreak isolates, none of the dendrogram nodes exceed an S_{AB} value of 0.7 for the other test isolates.

Adherence

Adherence to substrate, whether natural (endothelium) or artificial (catheter material), is likely the first step in *Candida* pathogenesis (33). As shown in Figure 3, the adherence abilities of *C. parapsilosis* isolates vary widely. Adherence was the same for outbreak isolates of the same clone (167, 165, 173, 317; other clonal isolates were excluded for clarity), regardless of the site of isolation. Adherence was significantly higher than for the two unrelated hand isolates (313 and 385; $p < 0.001$). No relationship was found between the outbreak isolates and University Hospitals' isolates, although the latter exhibited higher values than strain 313 and 385. Among University Hospitals' isolates, no relationship was found between infection site and adherence.

Biofilm Production

We first determined the ability of the *C. parapsilosis* outbreak isolates to form biofilm. All isolates of the same clone (strains 167, 165, 177, 179, 173, and 317) showed a similar pattern of biofilm formation by dry weight (Figure 4A), which suggests that biofilm formation by these isolates is consistent and not site-induced. Except for the unrelated environmental strains 313 and 385, biofilm formation was not significantly different for the various outbreak isolates ($p > 0.05$, when compared with strain 167). However, noninvasive strains 313 and 385 produced significantly less biofilm when measured by dry weight (compared with strain 167; $p < 0.001$ for 385 and $p = 0.001$ for 313).

Figure 4B shows a comparison of biofilm formation by the *C. parapsilosis* outbreak clone with isolates obtained from our University Hospitals' collection, including specimens from different body sites. The outbreak strain 167 produced more biofilm than the University Hospitals' isolates ($p \leq 0.0005$ for comparison of dry weight values of 167 vs. all others), which indicates that outbreak clonal isolates had a higher ability to form biofilms.

For both sets of isolates, biofilm production was examined when TPN solution was substituted for YNB medium because TPN promotes *C. parapsilosis* growth (16). TPN increased the dry weight of biofilms formed by the clonal strain (167) by up to 40% ($p = 0.008$). However, the pattern of results across strains was similar to YNB-based method (not shown).

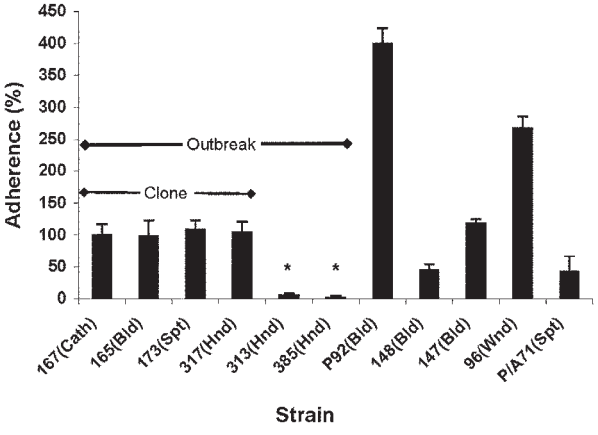

Figure 3. Adherence properties of *Candida parapsilosis* clinical isolates. Graph shows adhesion ability of various *C. parapsilosis* strains, compared to strain 167 from the University Hospitals of Cleveland. Results were normalized to strain 167, which was taken as 100%. Each result is representative of at least two experiments. Error bars represent standard deviation. *$p < 0.001$ for comparison of values of strain 167 vs. strains 313 and 385; all other comparisons had p values > 0.05. (For details of methods used, see text.) Cath, catheter; Bld, bloodstream; Spt, sputum; Hnd, hand; Wnd, wound; Pdf, peritoneal dialysis fluid.

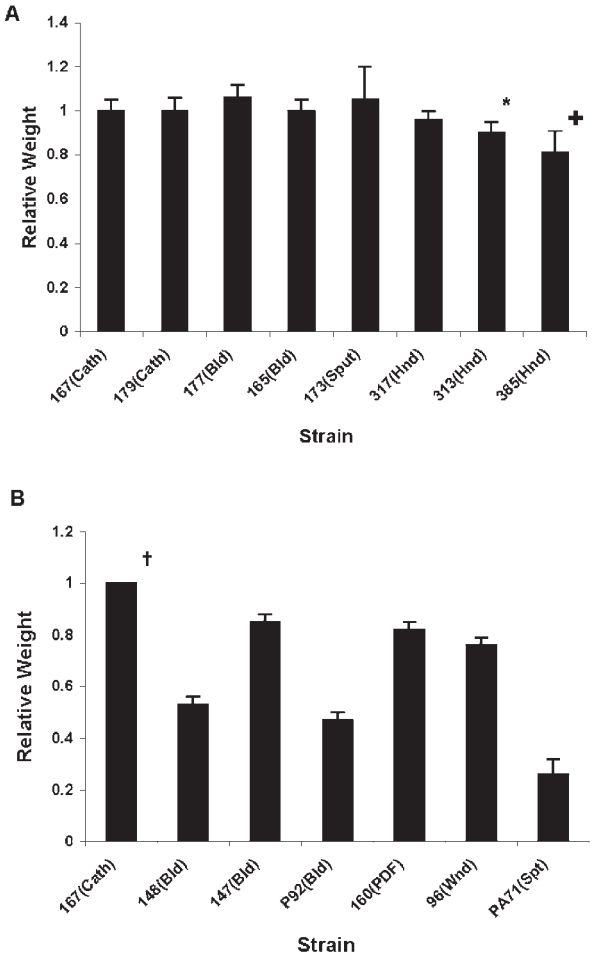

Figure 4. Relative dry weight of biofilms formed by *Candida parapsilosis* clinical isolates. Panel A shows relative dry weight of the *C. parapsilosis* strains from outbreak investigations by the Centers for Disease Control and Prevention from various culture sources. Results were normalized to control *C. parapsilosis* strain 167, which was taken as 100%. Each result is representative of at least two experiments. Error bars represent standard deviation. *p = 0.001 and +p < 0.001 for dry weight comparison of 167 vs. 313 and 385, respectively. Panel B shows relative dry weight of the University Hospitals' *C. parapsilosis* strains from various culture sources, also compared to strain 167. †p ≤ 0.0005 for comparison of dry weight values of 167 vs. all others. (For details of methods used, see text.) Cath, catheter; Bld, bloodstream; Spt, sputum; Hnd, hand; Wnd, wound; Pdf, peritoneal dialysis fluid.

SAP Assays

We measured SAP production by assaying the ability of *C. parapsilosis* supernatant to hydrolyze BSA (29). A representative SDS-PAGE gel is shown in Figure 5A. The appearance of specific digestion products was noted when culture supernatant was added to BSA. Specifically, we observed the presence of a 20-kDa product, which did not appear when the reaction was carried out in presence of a protease inhibitor cocktail (Figure 5A, lanes marked "+"). This indicated that the 20-kDa band was a specific by-product of supernatant protease activity, present in all supernatants (except for strains M61 and 313). Analysis of the protease activity of culture supernatants was performed by densitometric scanning of the 20-kDa band. As seen in Figure 5B, the intensity of the 20-kDa product varied greatly between strains and within the outbreak clonal isolates, and no consistent pattern of protease activity was evident. These results were confirmed in multiple experiments.

Phospholipase Assays

We determined the phospholipase activity of supernatants obtained from cultures of the different *C. parapsilosis* isolates, using a colorimetric assay (30). For comparative purposes, we included the phospholipase activity of *C. albicans* strain M61, since *C. albicans* is a known phospholipase producer. Although phospholipase activity varied among strains (Figure 6), no consistent differences were observed between sources (outbreak vs. University Hospitals), sites (e.g., blood vs. other), or clonality of isolates.

Discussion

We studied a nosocomial outbreak of *C. parapsilosis* (20) and compared outbreak isolates with ones obtained from sporadic infections at our facility. Genetic analysis showed the invasive outbreak isolates (defined as being cultured from blood or catheter [14,34]), as well as at least one hand isolate, were from the same clone. This finding agrees with results of previous epidemiologic studies of *C. parapsilosis* infections, which found predominant clonality (10,35). Since these clones were the same as environmental isolates, the outbreaks appear to have a nosocomial environmental origin. This conclusion also seems to be the case in the Mississippi outbreak. In contrast, the University Hospitals' strains were unrelated, indicating sporadic infection. Our results support assertions that molecular analysis is useful in investigating *C. parapsilosis* outbreaks (36).

Adhesion is likely a critical first step in yeast pathogenesis (33). This property might be expected for *C. parapsilosis*, which is thought to be acquired from exogenous sources, subsequently adheres to indwelling devices, and finally invades the host. However, our results agree with those of DeBernardis (37), who found no difference in adhesion between invasive and skin isolates of *C. albicans*. Other studies on *C. parapsilosis* adherence, in fact, showed an inverse relationship between invasiveness and adherence (38).

The clonal outbreak isolates produced more biofilm than either the unrelated environmental strains, or the University Hospitals' specimens. These results suggest

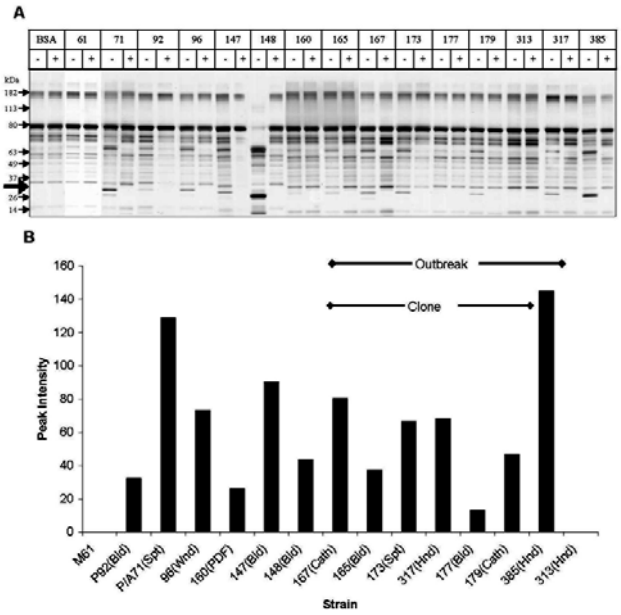

Figure 5. Secretory aspartic protease (SAP) expression by *Candida parapsilosis* clinical isolates. Panel A shows representative sodium dodecyl sulfate–polyacrylamide gel electrophoresis of various *C. parapsilosis* isolates. M, molecular weight marker lane; BSA, bovine serum albumin alone; other lanes show number of isolate; and +, supernatant plus protease inhibitor cocktail. Protease activity is evident from the appearance of lower molecular weight bands representing cleavage products. Thick arrow indicates the 20-kDa protein appearing after protease digestion. (For details of methods used, see text.) Panel B shows densitometric scanning analysis of SAP activity. Strains 177 and 179 were included to demonstrate the heterogeneity in SAP production within the clonal strains. Cath, catheter; Bld, bloodstream; Spt, sputum; Hnd, hand; Wnd, wound; Pdf, peritoneal dialysis fluid.

that biofilm formation is an important component of an outbreak strain's ability to cause infection. Although previous studies of *C. albicans* (14) and *C. parapsilosis* (16,39) have suggested that bloodstream isolates produce more biofilm, further in vivo and clinical studies are needed to confirm these findings. If increased production of biofilm by outbreak isolates can be confirmed, this finding may point to a strategy for determining the significance of *C. parapsilosis* clinical isolates. Finally, our results confirm earlier work, suggesting that TPN promotes the development of *C. parapsilosis* biofilms (39).

Although secreted enzymes are likely virulence factors for all yeast species, patterns of expression vary across species. This variation may be exemplified by SAP phenotypes. Although SAPs may play a role in *C. albicans* adhesion (40), they are not important for the primary mode of invasion (through gut mucosa) because knockout strains do not display attenuated virulence (19). However, SAP may be important for pathogenesis at other sites or stages of infection. Regarding *C. parapsilosis*, DeBernardis et al. (37) found an inverse relationship with invasiveness. Our findings do not support the concept that SAP expression is a critical virulence factor for *C. parapsilosis*. The variability of our results even across a single clone also raises a larger question of the appropriateness of characterizing SAP expression as a stable experimental pathogenic factor in *C. parapsilosis*. Although previous work suggested that SAP expression is stable for a given isolate (41), wide variations exist in relevant mRNA production over fairly short periods (42).

Phospholipases are important virulence factors for *C. albicans* (18) but have not been well studied in *C. parapsilosis*. Although the isolates examined in this article produced phospholipase, no correlation was found between phospholipase activity and site of infection or other virulence factors. Although one article described phospholipase B and protease activity in a few strains of *C. parapsilosis* (43), ours is the first study to conduct a more detailed examination of phospholipase behavior in this species.

Our results also show no apparent correlations across the multiple putative virulence factors studied. This result agrees with Branchini's examination of genotypic variation and slime production in *C. parapsilosis* (44). Other studies that have reported correlation in expression of multiple virulence factors have been for *C. albicans* (45), of uncertain relevance to *C. parapsilosis*.

Although we did not find significant associations between most of the virulence factors and clinical pathologic changes, these results do not mean that these factors are unimportant. Rather, the results suggest that they are not critical to clinical outbreaks. Since all isolates expressed some degree of adhesion (except for hand isolates 313 and 385), SAP, and phospholipase B activity, these phenotypes may be necessary but not sufficient prerequisites for infection. Our results highlight the importance of using outbreak isolates (rather than those from sporadic infections or laboratory stocks) in studies of *Candida* virulence. Definitive analysis of the role of virulence factors will require further genetic analysis and in vivo models examining the behavior of knockout mutants of *C. parapsilosis.*

This study has some limitations. First, the number of isolates is small. Given the nature of *C. parapsilosis* infections and outbreaks, a higher *N* could not be expected, and the outbreak is especially well-characterized (20). Previous work has drawn conclusions from as few as one isolate or from less-characterized groups of isolates (16). Second, genetic analysis of *C. parapsilosis* that uses Cp3-13 fingerprinting may have limitations. Third, our SAP assay is unable to determine the relative contributions of different members of the gene family, of which there are at least 10 (46). Such analysis is beyond the scope of this

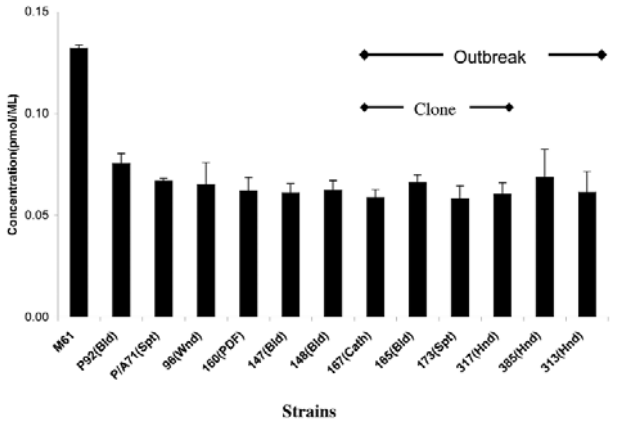

Figure 6. Phospholipase expression by *Candida parapsilosis* clinical isolates. Phospholipase expression as determined by the colorimetric method is shown. *C. albicans* train M61 was included as it is a known phospholipase producer. (For details of the methods used, see text.) Cath, catheter; Bld, bloodstream; Spt, sputum; Hnd, hand; Pdf, peritoneal dialysis fluid.

paper. Fourth, similar limitations exist regarding characterization of phospholipase activity, as *Candida* expresses multiple phospholipases (18). Phospholipase expression varies with environmental conditions (47); however, we performed experiments under standardized conditions, physiologic temperature, and using glucose containing solutions (14).

In conclusion, the genotypic pattern of this *C. parapsilosis* outbreak suggests a clonal outbreak, likely arising from an environmental source and distinct from sporadic infection. The outbreak clone produced more biofilm than all other strains. No clear relationships were apparent for the other putative virulence factors, which suggests that they are not critical to outbreak behavior. Further genetic and in vivo studies are required to confirm these findings. Future analysis of virulence mechanisms likely needs to use outbreak strains, as well as taking into account the interplay of organism, host, and environment.

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Dr. Kuhn completed a fellowship in infectious disease at Case Western Reserve University and University Hospital of Cleveland, where this work was done. He is currently a fellow in pulmonary and critical care medicine at Massachusetts General Hospital, Boston. His research interests include fungal pathogenesis and infections in critically ill patients in general.

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Address for correspondence: Mahmoud A. Ghannoum, Center for Medical Mycology, Dept. of Dermatology, University Hospitals of Cleveland, LKSD 5028, 11100 Euclid Ave., Cleveland, OH 44106, USA; fax: 216-844-1076; email: mag3@po.cwru.edu

Limited Spread of Penicillin-Nonsusceptible Pneumococci, Skåne County, Sweden

Eva Melander,*† Hans-Bertil Hansson,†‡ Sigvard Mölstad,‡ Kristina Persson,† and Håkan Ringberg†

In response to increasing frequencies of penicillin-nonsusceptible pneumococci (PNSP), for which the MIC of penicillin was ≥0.12 mg/L, in Skåne County, southern Sweden, national recommendations were initiated in 1995 to limit the spread of pneumococci with high MICs (≥0.5 mg/L) of penicillin (PRP), especially among children of preschool age. Traditional communicable disease control measures were combined with actions against inappropriate antimicrobial drug use. During the first 6 years that these recommendations were applied in Skåne County, the average frequency of penicillin-resistant pneumococci has been stable at ≈2.6%, as has the average PNSP frequency (7.4%). However, PNSP have been unevenly distributed in the county, with the highest frequencies in the southwest. Simultaneously, the rate of antimicrobial drug use for children <6 years of age was reduced by 20%. Thus the spread of PNSP between and within the municipalities in the county has been limited.

Increasing frequencies of penicillin-nonsusceptible pneumococci (PSNP) (MIC of penicillin ≥0.12 mg/L) became a worldwide problem in the 1980s (1,2). The rapid increase in frequency is likely caused by intercontinental spread of a few PNSP clones for which MICs of penicillin are high (3–6). For a long period, very low PNSP frequencies were noted in Sweden (2%–3%) (7). However, in the early 1990s, the incidence of PNSP increased from 8% to 10% in Skåne, the southernmost county, while the rest of the country reported unchanged low frequencies (8–10). When considered from the perspective of international experience, in which the rate of PNSP rapidly increased when this level of resistance was reached (11), the increasing frequencies of PNSP in Skåne County led to the formation of an expert committee, appointed by the National Board of Health and Welfare. This committee proposed a national strategy in 1995, based on reducing unnecessary use of antimicrobial agents and applying infection control measures to limit the more immediate spread of PNSP for which MICs of penicillin were high (≥0.5 mg/L, PRP), especially in preschool children ages 1 to 6 years. In 1996, infection and carriage with PRP became notifiable by the Swedish Communicable Disease Act. The reasons to choose MIC ≥0.5 mg/L as a limit for intervention were that epidemiologic data on pneumococci suggested that an increased prevalence of strains for which the MIC of penicillin was ≥1.0 mg/L most often was caused by spread of a few already resistant clones, so that those strains could lead to treatment failures, and that those strains were relatively rare in Sweden in 1994 (12). Since the Etest can be hard to interpret, MIC ≥0.5 mg/L was chosen so strains for which the MIC of penicillin was 1.0 mg/L would not be missed. The decision of whether to follow the recommendations of the expert committee is made by each county department for communicable disease control. The recommendations were strictly applied in Skåne County (South Swedish Pneumococcal Intervention Project, SSPIP), while some Swedish counties either did not follow the recommendations at all or only applied parts of the recommendations. For a long period, Skåne County had the highest use of antimicrobial agents in Sweden, especially of macrolides and broad-spectrum antibiotics (13), and in the SSPIP traditional communicable disease control measures are combined with actions aimed at reducing the use of antimicrobial drugs. Experiences from the first 2 years of the SSPIP from parts of Skåne County have previously been reported (14–17). For example, data on individual risk factors for carriage of PNSP have been evaluated. We discuss the overall results of the SSPIP and evaluate the effects of the recommendations in Skåne County during the first 6 years they were implemented.

*Department of Clinical Microbiology, Lund University Hospital, Lund, Sweden; †Regional Center for Communicable Disease Control, Skåne County, Sweden; and ‡Unit of Research and Development in Primary Health Care, Jönköping County, Sweden

Materials and Methods

Study Area

On January 1, 2000, the population of Skåne County was 1,129,424 inhabitants; 93,051 were ≤6 years of age. The county is divided into 33 municipalities. The largest municipality, Malmö, had 259,579 inhabitants and of those, 20,325 were ≤6 years of age. The smallest municipality had 6,745 inhabitants; 581 were ≤6 years. During the study period, ≈76% of the children 1 to 6 years of age were enrolled in day care.

SSPIP

The principles of the SSPIP have been described earlier (16). In brief, all persons in Skåne County with a culture that yields penicillin-nonsusceptible pneumococci with an MIC ≥ 0.5mg/L for penicillin (PRP), regardless of resistance to any other antibiotics, have since 1995 been reported to the Regional Center of Communicable Disease Control (CCDC). Whenever a person with a clinical infection caused by PRP (index case-patient) is identified, nasopharyngeal cultures are obtained from family members and other close contacts to identify asymptomatic PRP carriers (contact case-patients). All carriers are followed weekly with nasopharyngeal cultures at the local primary health care center until two consecutive cultures that yield no growth of PRP (PRP-negative) have been obtained. If the index case is in a child attending any form of group day care, nasopharyngeal cultures are obtained from the other children and staff members at that day care group. Preschool children are restricted from attending day care until they are PRP-negative. In selected cases, eradication therapy with antimicrobial drugs is considered after 2 to 3 months of carriage, or earlier when strong social reasons exist (18).

Microbiologic Methods

The pneumococcal strains considered in this study were recovered from cultures analyzed at the Departments of Clinical Microbiology in Lund, Malmö, Helsingborg, and Kristianstad from July 1, 1995, through June 30, 2001. These four laboratories served the entire population of Skåne County during the study period. The specimen were cultured on blood agar plates, and the isolates were identified as *Streptococcus pneumoniae* on the basis of colony morphology and susceptibility to optochin (19). The strains were screened for penicillin-resistance by using the disk-diffusion method, according to the Swedish Reference Group for Antibiotics (SRGA). The strains were inoculated onto Iso Sensitest agar (Oxoid Ltd, Basingstoke, UK), supplemented according to the recommendations, and the antibiotic disks (Oxoid Ltd) were applied. Inhibition zones were read to the nearest millimeter and interpreted according to SRGA guidelines (20). For pneumococci with an oxacillin 1 μg inhibition zone <20 mm, the MIC of penicillin was determined by the Etest (AB Biodisk, Solna, Sweden) (21). The susceptibility to other antimicrobial agents (erythromycin, tetracycline, trimethoprim-sulfamethoxazole, and clindamycin) was determined by using the disk diffusion method (10). Strains from each patient were registered only once per season, even if multiple cultures were positive for pneumococci. Whether the strains were recovered at a visit to the doctor or by contact tracing or screening was noted. Serotyping to the group level was performed by the quellung reaction, by using antisera from the Statens Seruminstitut, Copenhagen, Denmark (19).

Antimicrobial Drug Use

Data were collected regarding antimicrobial drug prescriptions for outpatient care, served at Swedish pharmacies from July 1, 1995, through June 30, 2001, that were issued for children ages ≤6 years who lived in Skåne County; these data were obtained from the Corporation of Swedish Pharmacies, which owns all Swedish pharmacies and collects and compiles information on all drugs sold in the country (13). The municipality of the patient was registered. The prescribed antimicrobial agents were given as prescriptions per 1,000 inhabitants ages ≤6 years per season, and all prescriptions of phenoxymethylpenicillin (PcV), ampicillin/amoxicillin (including amoxicillin+ clavulanic acid), cephalosporins, trimethoprim-sulfamethoxazole, macrolides, clindamycin, and other antimicrobial drugs (grouped together) were registered.

Results

During the project, the frequency of PNSP carriers from clinical nasopharyngeal cultures has been evaluated. Cultures taken at contact tracing were excluded. From July 1, 1995, until June 30, 2001, the average frequency of PNSP carriers has been stable, approximately 7.4% (2,750 PNSP/34,745 pneumococci) (Figure 1). The highest frequencies (8.1%) were seen in 1997 to 1998. During the 6 years of the recommendations, the levels of PNSP have been unevenly distributed over the county; higher and stable levels were found in the city of Malmö in the southwestern part of the county, (11.3%, 976 PNSP/8,651 pneumococci) and lower, but stable, levels were found in the northeastern part of the county (4.3%, 395 PNSP/9,109 pneumococci). The frequency of PNSP among invasive pneumococcal strains (blood and cerebrospinal fluid cultures) in Skåne County has been low, ≥2.5 % (22 PNSP/858 pneumococci). As with the PNSP in nasopharyngeal cultures, the highest frequencies of invasive PNSP were seen in 1997 to 1998.

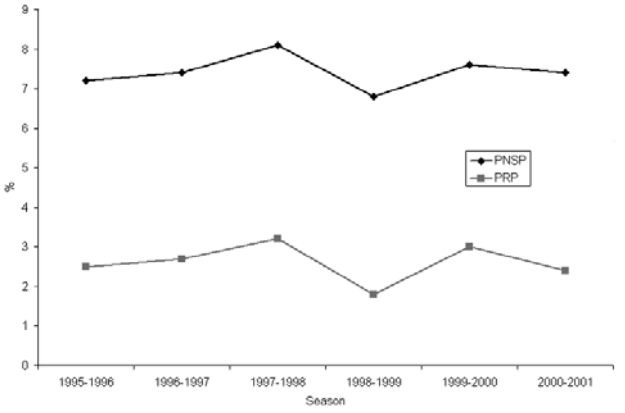

Figure 1. Frequency of penicillin-nonsusceptible pneumococci with MIC for penicillin ≥0.12 mg/L and ≥0.5 mg/L per season in Skåne County, Sweden, expressed in percentage of all pneumococci from clinical nasopharyngeal cultures.

The average frequency of index case-patients with PRP (that is, the number of clinical case-patients with a culture with growth of PRP, divided by the number of pneumococci found in nasopharyngeal cultures) has been rather stable since the start of the project, ≈2.6% (905 PRP/34,745 pneumococci) (Figure 1). In the southwestern part of the county, in the cities of Malmö and Lund and surrounding municipalities, where most cases were found, PRP have been constantly present. However, in several of the smaller municipalities, PRP were prevalent during one or two seasons but then disappeared. In two municipalities, both situated in the northeast, no cases of PRP infection have been found. The proportion of cases with PRP with high MICs (≥2 mg/L) of penicillin has not increased during the 6 years (Table). Carriers of PRP with MIC ≥4.0 mg/L have all been adopted children or immigrants from Eastern Europe or from countries outside Europe.

During the 6-year period, 2,269 PRP carriers (1,865 persons) have been registered at the CCDC in Skåne County. Of the 2,269 PRP carriers, 40% were index case-patients, and 60% were contacts. Few contact cases have been found in persons at the extremes of ages (≤1 year and >65 years). The number of contact cases per index patients in children ≤6 years of age has, on an average, been 1.6 (1.1 to 2.2 per season) and has not increased. The index patients have most often been found through positive nasopharyngeal cultures, and only a minority (n = 13, 1.4%) have been found through positive blood or cerebrospinal fluid cultures. More men carried PRP overall, but in the group ages 18 to 64 years, PRP carriage was more common among women. A clear seasonal variation in the incidence of PRP was found; considerably more cases occurred during the winter months (October–March) than during summer. The median duration of PRP carriage was 21 days (range 2–368 days); 1,704 (75%) of the PRP patients were children 1 to 6 years of age. Seventy-one percent of these children attended day care centers; 6% attended family day care, and 23% were cared for at home. The other contacts have primarily been siblings, parents, or grandparents of young children.

Two hundred and twenty-seven children with a clinical culture showing growth of PRP, led to screening of children and staff at 227 of the county's 1,250 day care centers. The number of contact cases per index case-patient among all children attending these day care centers has, on an average, been 3.46 (2.6–4.2 per season) and has not increased, and the number of contact cases in each day care center has varied from 0 to 25 (median 2). In 24% of the day care center interventions, no contact cases were found. In 45 of the 227 day care centers, more than one PRP serotype was discovered, and 65 day care centers have been investigated twice or more (median 2, range 2–6). Twenty-two of these day care centers had two outbreaks with the same serotype within 6 months. Most of the day care interventions took place in the southwestern part of the county. Only 20 of all screened staff at day care centers were PRP-positive.

Serotyping to the group level was performed for 2,131 (94%) of the 2,269 PRP strains. Twenty-five serotypes of PRP were found. Six serotypes comprised 93% of the strains (serotypes 9, 19, 6, 23, 15, 14). The most prevalent strain, serotype 9, represented an average of 48% of the PRP strains (37%–66% per season). This strain has spread over the county from municipality to municipality in a clear pattern, whereas the other common serotypes have spread more randomly.

The use of antimicrobial agents in outpatient care in Skåne County decreased from the first to the last season in all age groups, but especially in children aged ≤6 years (Figure 2). The main reduction among children ages ≤6 years was caused by a decrease in number of prescriptions for phenoxymethylpenicillin, and the use of macrolides was reduced by 50%. Even though the decreased use of antimicrobial drugs was seen in all municipalities, the municipalities with the highest utilization in 1995–1996 still had the highest use in 2000–2001. Those municipalities were all located in the southwestern part of the county.

Table. Distribution of MICs of penicillin for all registered PRP cases in Skåne County per season[a]

Season	0.5 mg/L, n (%)	1.0 mg/L, n (%)	≥2.0 mg/L, n (%)
1995–1996	217 (33)	290 (44)	151 (23)
1996–1997	132 (36)	140 (37)	102 (27)
1997–1998	189 (42)	214 (47)	52 (11)
1998–1999	124 (46)	140 (52)	4 (2)
1999–2000	183 (59)	111 (35)	18 (6)
2000–2001	124 (62)	75 (38)	0

[a]PRP, pneumococci with MIC ≥0.5 mg/L.

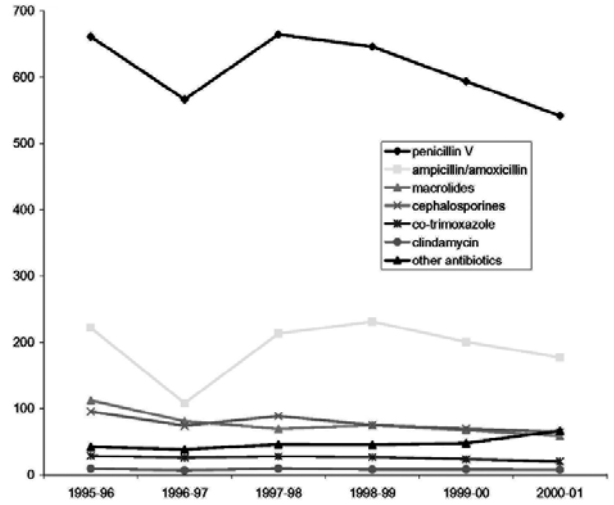

Figure 2. Use of antimicrobial agents in outpatient care among children ages ≤6 years in Skåne County, Sweden, expressed in prescriptions per 1,000 inhabitants per season.

Discussion

In Skåne County, where PNSP (pneumococci with an MIC for penicillin ≥0,12 mg/L) have been prevalent for >10 years, the frequency of PNSP in clinical nasopharyngeal cultures (cultures taken at contact tracing excluded) remained at the same level in 2000 to 2001 as in 1995 to 1996, on average, 7.4%. In the city of Malmö, where the spread of PNSP appears to have started, the average level of PNSP has been higher (11.3%) during the project period. In contrast, the average level of PNSP remained low (4.3%) during the study period in the northern and eastern parts of the county. Furthermore, the number of PRP contact cases per index case among children attending day care centers did not increase during the 6-year period. These data indicate that the rapid spread of PNSP that started in the southwestern part of the county in the beginning of the 1990s (8,9) has been limited. These results are unique in comparison with experience in other countries, where PNSP rates have rapidly increased after reaching an 8% PNSP level (11). The proportion of contacts with PRP with high MICs of the PNSP in Skåne County has not increased during the 6-year period, contrary to results from most other countries, which report increasing frequencies of PNSP with high MICs (22,23).

One of the project's aims was to decrease unnecessary use of antimicrobial agents, especially of macrolides and trimethoprim-sulfamethoxazole, which in studies have been shown to promote spread of PNSP (17,24–27). During the study period, the use of antimicrobial drugs decreased in all municipalities in the county, and the decrease was most prominent in children ages ≤6 years. In this age group, the use of macrolides was cut in half. The use of long-acting macrolides has been extremely low and has comprised <3% of the total use of macrolides among children. Even though the use of antimicrobial agents decreased in all municipalities of Skåne County, those with high usage in 1995 to 1996 also had high usage in 2000 to 2001, and those with low usage in 1995 to 1996 had an even lower usage in 2000 to 2001. Mirroring the use of antimicrobial agents, the occurrence of PNSP has been unevenly distributed in the county, with constant high rates in the southwestern part and low levels in the northern and eastern parts. In cities in the northeastern parts and in the smaller municipalities, with lower antimicrobial agent use, single outbreaks have occurred without any more cases being reported later. A possible explanation for this might be that municipalities in which antibiotics are frequently used have a higher risk of spreading PNSP in the community, as indicated by previously published data from the project as well as by other studies (17,24,28,29).

Many PRP serotypes were found in the county during the six seasons. However, one serotype, serotype 9, has dominated during all six seasons and has spread in a clear pattern from municipality to municipality throughout the county (15), while other strains have spread more randomly in time and geographically. Why this strain has been persistently present for such a long time is unclear.

We chose to present the figures for the frequency of PNSP in clinical cultures over time, excluding cultures taken at contact tracing, since the contacts were not found at random, but rather represented a selected population around an index patient. The exclusion of cultures taken at contact tracing ought to give more fair figures when comparing data from the study period with the "resistance situation" before the project started or with data from other studies. The possibility of excluding all cultures taken at contact tracing retrospectively was not 100% guaranteed, but cultures taken at day care center screenings were easily found and excluded. The rest of the cultures obtained because of contact tracing constituted only a small proportion of cultures, and thus they should not be a source of error.

Although the indication for nasopharyngeal sampling was not changed during the study period, the number of obtained samples has decreased since the start of the project. However, the number of nasopharyngeal cultures increased in 1995 to 1996 compared to 1993 to 1994. One possible explanation for this might be that the health authorities encouraged doctors to take nasopharyngeal samples more frequently during the first years of the project.

Exactly comparable data on PNSP frequencies from other Swedish counties or comparable survey data (nasopharyngeal cultures) from other countries are hard to find. However, since 1996, Swedish law has mandated the

reporting of all PRP strains to the Swedish Institute for Infectious Disease Control. Data from this national register contains information on reported PRP from all over Sweden from 1997 to 2002. Of all reported PRP, the PRP from Skåne County comprised 40% in 1997, but only 10% in 2002 (Figure 3). Furthermore, the recommendations are strictly applied in Skåne County, while some Swedish counties either do not follow the recommendations at all or only apply parts of the recommendations. In some counties, where the recommendations were not applied or not as strictly applied as in Skåne County, the frequencies of PRP have increased from 2000 to 2002 (30,31). Moreover, according to data from the annual Resistance Surveillance and Quality Control Programme, the frequency of PNSP in clinical nasopharyngeal cultures in Sweden has increased from 3.8% in 1994 to 6.2% in 2002 (30,32). In addition, the frequency of PNSP among invasive pneumococcal strains in Sweden has, on average, increased from 1.4% in 1999 to 2.4% in 2002 (30,33), but the frequency of invasive PNSP during the same period in Skåne County has been approximately 2.5% and stable. The numbers of invasive PNSP isolates in Skåne County are very low, and therefore drawing any reliable conclusions from these data is difficult. However, the frequency of PNSP has not increased, as it has in most other countries, and the trends of invasive PNSP seem to follow the trends for PNSP from nasopharyngeal cultures. Compared to the other Nordic countries, who still report low frequencies of invasive PNSP, Sweden has had the lowest frequencies of invasive PNSP between 1999 and 2002 (33).

Although the frequencies of PNSP in Skåne County were not reduced, the spread of PNSP seems to have been limited. Whether this is a result of the actions of the SSPIP among children or of the decreased use of antibiotics in the county, or both, is impossible to tell since these actions were started simultaneously. In other countries, actions against unnecessary use of antimicrobial drugs has been the only measure of combating the spread of PNSP, in most cases with little success, probably in part because the actions have been initiated in a much later phase, when frequencies of PNSP were higher (34–37). Still, further efforts are necessary to reduce the prescribing of antimicrobial agents in the southwestern areas, since the use of antimicrobial agents is still high there and poses the greatest risk factor for PNSP carriage.

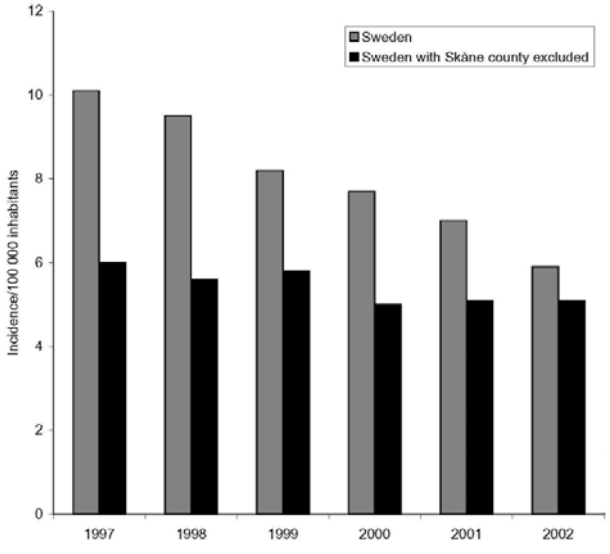

Figure 3. National incidence of penicillin-resistant pneumococci with and without Skåne County included.

Acknowledgments

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Dr. Melander is a physician in the Department of Medical Microbiology, Lund University Hospital, Lund, Sweden. Her main research interests include bacterial antimicrobial resistance and use of antimicrobial agents.

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Address for correspondence: Eva Melander, Department of Clinical Microbiology and Immunology, Lund University Hospital, SE-221 85 Lund, Sweden; fax: +4646135936; email: eva.z.melander@skane.se

Antimicrobial Resistance Incidence and Risk Factors among *Helicobacter pylori*–Infected Persons, United States

William M. Duck,* Jeremy Sobel,* Janet M. Pruckler,* Qunsheng Song,* David Swerdlow,* Cindy Friedman,* Alana Sulka,* Balasubra Swaminathan,* Tom Taylor,* Mike Hoekstra,* Patricia Griffin,* Duane Smoot,† Rick Peek,‡ DavidC. Metz,§ Peter B. Bloom,¶ Steven Goldschmid,¶ Julie Parsonnet,# George Triadafilopoulos,# Guillermo I. Perez-Perez, Nimish Vakil,†† Peter Ernst,‡‡ Steve Czinn,§§ Donald Dunne,¶¶ and Ben D. Gold***

Helicobacter pylori is the primary cause of peptic ulcer disease and an etiologic agent in the development of gastric cancer. *H. pylori* infection is curable with regimens of multiple antimicrobial agents, and antimicrobial resistance is a leading cause of treatment failure. The *Helicobacter pylori* Antimicrobial Resistance Monitoring Program (HARP) is a prospective, multicenter U.S. network that tracks national incidence rates of *H. pylori* antimicrobial resistance. Of 347 clinical *H. pylori* isolates collected from December 1998 through 2002, 101 (29.1%) were resistant to one antimicrobial agent, and 17 (5%) were resistant to two or more antimicrobial agents. Eighty-seven (25.1%) isolates were resistant to metronidazole, 45 (12.9%) to clarithromycin, and 3 (0.9%) to amoxicillin. On multivariate analysis, black race was the only significant risk factor ($p < 0.01$, hazard ratio 2.04) for infection with a resistant *H. pylori* strain. Formulating pretreatment screening strategies or providing alternative therapeutic regimens for high-risk populations may be important for future clinical practice.

The prevalence of *Helicobacter pylori* infection worldwide is approximately 50% (1), as high as 80%–90% in developing countries, and ≈35%–40% in the United States (1). Approximately 20% of persons infected with *H. pylori* develop related gastroduodenal disorders during their lifetime (1). *H. pylori* is an etiologic agent of peptic ulcer disease, primary gastritis, gastric mucosa-associated lymphoid-tissue lymphoma, and gastric adenocarcinoma (2). The annual incidence of *H. pylori* infection is ≈4%–15% in developing countries, compared with approximately 0.5% in industrialized countries (3). Documented risk factors include low socioeconomic status, overcrowding, poor sanitation or hygiene, and living in a developing country (2).

Eradication therapy of symptomatic *H. pylori* infection substantially reduces the recurrence of associated gastroduodenal diseases. Therapy entails complicated regimens of several antimicrobial agents for at least 2 weeks. In general, triple therapy regimens usually entail two of the following antimicrobial agents: metronidazole, amoxicillin, tetracycline, or clarithromycin in combination with a proton pump inhibitor or bismuth (4). The most common causes of treatment failure are patient noncompliance and antimicrobial resistance of the infecting *H. pylori* strain (5). Quadruple regimens are used as a salvage therapy when triple therapy regimens have failed (4). Pretreatment resistance of *H. pylori* has been reported to compromise the efficacy of treatment. For example, therapy regimens containing metronidazole and clarithromycin fail in as many as 38% and 55% of cases, respectively, when used to treat infection with an organism resistant to one of these antimicrobial agents (6). In addition, published data are lacking that describe the effect conferred by polymicrobial resistance on eradication success.

The *Helicobacter pylori* Antimicrobial Resistance Monitoring Project (HARP) is a prospective, longitudinal

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Howard University Medical Center, Washington, DC, USA; ‡Vanderbilt University Medical Center, Nashville, Tennessee, USA; §University of Pennsylvania Medical Center, Philadelphia, Pennsylvania, USA; ¶Atlanta Veterans Affairs Medical Center, Atlanta, Georgia, USA; #Stanford University Medical Center, Palo Alto, California, USA; **New York University School of Medicine, New York, New York, USA; ††Sinai Samaritan Medical Center, Milwaukee, Wisconsin, USA; ‡‡University of Virginia, Charlottesville, Virginia, USA; §§University Hospitals of Cleveland, Cleveland, Ohio, USA; and ¶¶Indiana University Medical Center, Indianapolis, Indiana, USA

network monitoring ongoing national and regional trends of antimicrobial resistance in *H. pylori* isolates in the United States. The network is positioned to document emerging resistance and to assist physicians in formulating therapy recommendations. We present the results of the antimicrobial resistance monitoring and risk factor analysis from data collected prospectively from 1998 through 2002.

Materials and Methods

During the course of the study, HARP consisted of 11 hospital study sites across the United States. The first five patients who sought treatment each month for esophagogastro duodenal endoscopy (EGD) as part of treatment for *H. pylori* infection, confirmed by gastric biopsy, were enrolled at each site. When fewer than five eligible study participants were seen at a study center, those eligible were enrolled. Written, signed consent was obtained from all participants or their guardians. Culture of biopsied material for *H. pylori* was performed at the study site or at the Atlanta Veterans Affairs Hospital. If primary isolation was performed at the HARP study site, isolates were placed in trypticase soy broth (TSB) supplemented with glycerol and stored at –70°C. Isolates were sent to the Centers for Disease Control and Prevention for antimicrobial resistance testing. Samples forwarded to the Atlanta Veterans Affairs Hospital for isolation were packed in 6 pounds of dry ice and maintained at –70°C to –80°C until cultured (up to 1 month). Each specimen was accompanied by a standard case report form containing 145 variables of demographic, clinical, and epidemiologic information.

Laboratory Procedures

Isolates were cultured on heart infusion agar (HIA) with 5% rabbit blood at 37°C in a microaerobic atmosphere (10% CO_2, 85% N_2, 5% O_2) for a minimum of 72 hours or until appearance of *H. pylori* colonies. Gastric biopsy specimens were homogenized by using a micropestle in a microcentrifuge tube, and the homogenates were added to HIA with 5% rabbit's blood and Skirrow media and incubated at 37°C microaerobically for up to 2 weeks. Presumptive *H. pylori* colonies were subcultured for further testing. Isolates were confirmed as *H. pylori* if they demonstrated typical morphologic features by dark-field microscopy and if urease, oxidase, and catalase activities were detected. Also, a polymerase chain reaction amplification assay that used primers targeted at the *ure*A gene provided confirmation (7). Antimicrobial susceptibility testing was performed by the agar dilution method in accordance with National Committee for Clinical Laboratory Standards (NCCLS) protocols, except that defibrinated rabbit blood was used instead of aged sheep blood (8). For antimicrobial agents without an NCCLS-recommended breakpoint, breakpoints were selected after a review of the literature.

Data Collection and Analysis

Case report forms and laboratory data were entered into a MS Access 2000 database and analyzed by using SAS, version 8.2 (SAS Institute, Cary, NC). We defined a resistant case to be an HARP isolate that demonstrated any detectable resistance to metronidazole, clarithromycin, tetracycline, or amoxicillin. HARP study sites were assigned to the following four regions: Northeast (Washington, DC; New York, NY; and Philadelphia, PA), South (Atlanta, GA; Nashville, TN; and Galveston, TX), Midwest (Detroit, MI; Cleveland, OH; Indianapolis, IN; and Milwaukee, WI), and West (Palo Alto, CA). Differences between patients infected with resistant and susceptible strains of *H. pylori* were assessed by using the Fisher exact test, Mantel-Haenszel chi-square, or Student *t* test, as appropriate. A one-way analysis of variance (ANOVA) was used to determine antimicrobial resistance rate trends for clarithromycin and metronidazole. When the difference in the number of study participants that harbored a resistant *H. pylori* infection and those who did not for a given risk factor was ≤ 2, we chose to exclude the risk factor from further analysis because of the possibility of arriving at a spurious association caused by small cell-size variations. This method enhanced the statistical reliability of all possible exposure and resistance associations considered for multivariate modeling. Risk factors that met these criteria were subjected to a chi-square score model selection procedure to obtain the most significant and stable multivariate model. Multivariate analyses were performed by using logistic regression to assess the independent association of identified risk factors with any detectable antimicrobial resistance using two different models.

The first logistic regression model assessed significant univariate risk factors for antimicrobial resistance. A second logistic regression model was created that we conditioned on geographic location of HARP site, previous antimicrobial treatment for *H. pylori* infection, and antacid use to resolve colinearity issues with race.

Results

Of 317 enrolled HARP study participants for whom complete demographic information was available, 205 (65%) were male, 116 (37%) were white, 172 (54%) were black, 14 (4%) were Asian, 3 (1%) were Native American, and 12 (4%) were of other ethnic backgrounds. The median age was 57 years (range 3–94 years). Among males, 97 (31%) were white, 90 (28%) were black, and 9 (3%) were Asian, 1 (0.3%) was Native American, and 8 (3%) were of other ethnic backgrounds. Among females, 19 (6%) were

white, 82 (26%) were black, 5 (2%) were Asian, 2 (0.6%) were Native American, and 4 (1%) were of other ethnic backgrounds. The most common endoscopic diagnoses for conditions of HARP participants were gastric erosions, gastritis, duodenal and gastric ulcers, and esophagitis (Table 1). *H. pylori* resistance was not statistically associated with endoscopic findings.

Among 347 *H. pylori* isolates submitted to HARP from 1998 to 2002, 118 (34%) were resistant to ≥1 antimicrobial agent, 101 (29%) *H. pylori* isolates were resistant to one agent only, and 17 (5%) *H. pylori* isolates were resistant to more than one antimicrobial agent. Three isolates were resistant to amoxicillin (1%), 45 were resistant to clarithromycin (13%), 87 were resistant to metronidazole (25%), and no isolate was found to be resistant to tetracycline. Multiple-agent resistance was observed for clarithromycin and amoxicillin (1 isolate, 0.3%) and clarithromycin and metronidazole (16 isolates, 5%). A test of trend showed no significant trend for resistance to metronidazole, but a significant trend ($R^2 = 0.76$) was noted for a decline in resistance to clarithromycin during the study period (Figure).

Most isolates were submitted by centers in the Northeast region, followed by the South, Midwest, and the West. Submitted *H. pylori* isolates from the Northeast region had the highest frequency of single- and dual-agent resistance (Table 2). The Midwest region submitted *H. pylori* isolates with the second-highest single agent resistance rate, while the southern region had the second-highest dual agent resistance rate. The highest proportion of *H. pylori* isolates resistant to clarithromycin, metronidazole, and amoxicillin was in the Northeast (Table 2).

The four sites reporting the highest proportion of isolates resistant to one agent were Indianapolis, Indiana; Galveston, Texas; Philadelphia, Pennsylvania; and Washington, DC (Table 3). Dual agent resistance was most prevalent in Detroit, Michigan. Indianapolis had the highest overall resistance: 50% of all isolates submitted were resistant to at least one agent. The Atlanta site reported the highest proportion of clarithromycin-resistant isolates (Table 3). The highest proportion of metronidazole resistance was reported from the Indianapolis site. Isolates resistant to amoxicillin were found in Nashville, Tennessee, Philadelphia, and Washington, DC.

Demographic, clinical, and epidemiologic data were analyzed for association with infection by a resistant *H. pylori* strain. Results of univariate analysis of risk factors for antimicrobial resistance are summarized in Table 4. On univariate analysis, significant risk factors for *H. pylori* resistance included past treatment of *H. pylori* infection with clarithromycin, treatment of past *H. pylori* infection for a second time in the past 5 years, treatment of past *H. pylori* infection with clarithromycin more than once, and treatment of past *H. pylori* infection with a proton pump inhibitor more than once. No protective factors for resistant *H. pylori* resistance approached significance.

Table 1. Endoscopy findings on patients enrolled in the *Helicobacter pylori* Antimicrobial Resistance Monitoring Project, 1998–2002[a]

Endoscopic diagnosis	No. of patients (%)
Stomach erosion	88 (27.0)
Gastritis	36 (11.0)
Duodenal ulcers	26 (8.0)
Esophagitis	26 (8.0)
Gastric ulcer	26 (8.0)
Duodenal erosion	24 (7.0)
Barrett esophagus	9 (3.0)
Nodularity	8 (2.0)
Stomach tumor	4 (1.0)
Bleeding[b]	1 (0.3)
Other diagnosis	27 (8.0)

[a]N = 331; *H. pylori* resistance is not associated with disease phenotype.
[b]Cause not specified.

On multivariate analysis, the most significant risk factor for *H. pylori* resistance was previous treatment of *H. pylori* infection within the past 5 years (Table 5). Protective factors for *H. pylori* infection were treatment with an antacid (Mylanta) and submission of specimens from New York, Palo Alto, California, Atlanta, or Nashville. However, a high degree of colinearity was found between geographic location and race in this model. For example, 84% of Washington, DC, participants were black, compared to 20% of participants from Nashville. Accordingly, a second multivariate model was constructed in which geographic location was controlled by conditioning. In this model, black race was the only risk factor significantly associated with infection with a resistant *H. pylori* strain (Table 6).

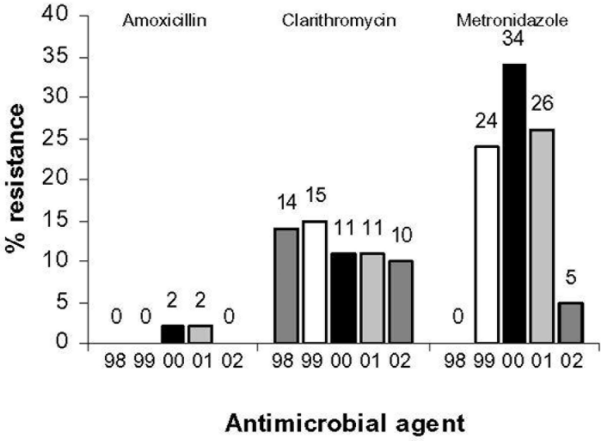

Figure. Resistance of *Helicobacter pylori* isolates submitted to the *Helicobacter pylori* Antimicrobial Resistance Monitoring Project, 1998-2002 (N = 347).1998, n = 7 isolates; 1999, n = 137 isolates. Resistance to tetracycline was found in 0% of isolates during each year of the monitoring project.

Table 2. Resistance of HARP isolates by region[a]

Region	No. isolates	Amoxicillin, no. (%)	Tetracycline, no. (%)	Clarithromycin, no. (%)	Metronidazole, no. (%)	Resistant to 1 agent, no. (%)	Resistant to >1 agent, no. (%)	Resistant to ≥1 agent, no. (%)
Northeast	156	2 (1)	0	23 (15)	47 (30)	54 (35)	9 (6)	63 (40)
South	92	1 (1)	0	13 (14)	13 (14)	19 (21)	4 (4)	23 (25)
West	24	0	0	1 (4)	4 (17)	3 (13)	1 (4)	4 (17)
Midwest	75	0	0	8 (11)	23 (31)	25 (33)	3 (4)	28 (37)
Total	347	3	0	45	87	101	17	118

[a]N = 347; HARP, *Helicobacter pylori* Antimicrobial Resistance Monitoring Project.

Discussion

The emergence of antimicrobial resistance in *H. pylori* represents a serious public health challenge because of the prevalence of infection and incidence of severe sequelae. A 12-fold increase in resistance to antimicrobial agents such as clarithromycin has been documented in cases in which antimicrobial therapy regimens do not eliminate *H. pylori* (9). Once resistance to clarithromycin appears, cross-resistance to all other macrolides also occurs, which eliminates the potential for their use in salvage therapies. In the past 5 years, amoxicillin and tetracycline resistance has also been described (6,10).

H. pylori resistance is amply documented in a variety of study populations. However, most published studies have been based on cross-sectional designs, not prospective population-based surveillance of resistance. A meta-analysis of 20 nationwide clinical trials estimated *H. pylori* resistance to clarithromycin, metronidazole, and amoxicillin to be 10.1%, 36.9%, and 1.4%, respectively (6). These findings were also reflected in smaller single-site studies (11). Resistance rates may be higher in pediatric populations; for example, a study in Michigan demonstrated that clarithromycin resistance in pediatric *H. pylori* strains was 2.5 times higher than that reported in adults in the same region and elsewhere in the United States (12).

Single-site and multicenter surveillance network studies in Britain, Croatia, the Czech Republic, Turkey, Greece, Poland, Russia, Slovenia, Turkey, Estonia, Italy, Bulgaria, Germany, and Belgium have observed primary metronidazole and clarithromycin resistance with rates as high as 40.3% and 10.6%, respectively (13–17). A large prospective surveillance project in northeastern Germany showed primary metronidazole and clarithromycin resistance rates of 26.2% and 2.2%, respectively, in 1,644 clinical *H. pylori* isolates collected from 1995 to 2000 (17). High resistance rates to metronidazole and clarithromycin have been reported in Portugal, Brazil, Hong Kong, Saudia Arabia, and Lebanon (18–22). Overall, *H. pylori* resistance to metronidazole among single and multicenter surveillance networks is greater than all other resistant antimicrobial agents combined.

HARP is the only multicenter network providing ongoing prospective antimicrobial resistance and associated risk factor data for *H. pylori* in North America. The first 4 years of data show that resistance to antimicrobial drugs commonly used to treat *H. pylori* infections is widespread, though rates varied from year to year; this finding was true for both clarithromycin and metronidazole. We speculate that the precipitous decrease in metronidazole resistance in 2002 could be attributed to the reduction in isolates received by HARP, which limited the power and representiveness of our sample.

We performed a two-phased multivariate analysis on risk factors significantly associated with resistance on uni-

Table 3. Resistance of HARP *H. pylori* isolates by site[a]

Location	No. isolates	Amoxicillin, no. (%)	Tetracycline no. (%)	Clarithromycin, no. (%)	Metronidazole, no. (%)	Resistant to 1 agent, no. (%)	Resistant to >1 agent, no. (%)	Resistant to ≥1 agent, no. (%)
Atlanta, GA	37	0	0	7 (19)	5 (14)	4 (11)	4 (11)	8 (22)
Nashville, TN	45	1 (2)	0	5 (11)	5 (11)	11 (24)	0	11 (24)
Galveston, TX	10	0	0	1 (10)	3 (30)	4 (40)	0	4 (40)
Philadelphia, PA	41	1 (2)	0	6 (15)	15 (37)	16 (39)	3 (7)	19 (46)
Washington, DC	93	1 (1)	0	15 (16)	31 (33)	35 (38)	6 (7)	41 (44)
Indianapolis, IN	36	0	0	3 (8)	15 (42)	18 (50)	0	18 (50)
Detroit, MI	18	0	0	3 (17)	4 (22)	3 (17)	2 (11)	5 (28)
Cleveland, OH	6	0	0	0	1 (17)	1 (17)	0	1 (17)
Milwaukee, WI	15	0	0	2 (13)	3 (20)	3 (20)	1 (7)	4 (27)
Stanford, CA	24	0	0	1 (4)	4 (17)	3 (13)	1 (4)	4 (17)
New York, NY	22	0	0	2 (9)	1 (5)	3 (14)	0	3 (14)

[a]N = 347; HARP, *Helicobacter pylori* Antimicrobial Resistance Monitoring Project.

Table 4. Univariate analysis of risk factors for resistance to ≥1 antimicrobial agent among *Helicobacter pylori* isolates submitted to HARP, 1998–2002[a]

Risk factor	Proportion of patients harboring resistant *H. pylori* isolate		Odds ratio (95% CI)
	Exposed, no. (%)	Unexposed, no. (%)	
Zantac use 12 mo before EGD	25 (48)	89 (32)	2.0 (1.1–3.6)[b]
Tums use 12 mo before EGD	19 (51)	95 (32)	2.2 (1.1–4.4)[b]
Tums use 30 d before EGD	12 (55)	102 (33)	2.4 (>1.0–5.8)[b]
Mylanta use 12 mo before EGD	4 (15)	110 (36)	0.3 (0.1–1.0)
Took antibiotic 12 mo before EGD	29 (43)	68 (34)	1.5 (0.9–2.6)
Past treatment of *H. pylori* infection with			
PPI	5 (71)	109 (34)	4.9 (1.0–26)
Clarithromycin	5 (83)	109 (33)	9.9 (1.1–86)
Treatment for *H. pylori* a second time in the past 5 years	11 (65)	107 (32)	3.8 (1.4–11)
Treatment of *H. pylori* infection more than once with			
PPI	8 (80)	106 (33)	8.1 (1.7–39)
Clarithromycin	8 (80)	106 (33)	8.1 (1.7–39)
Age ≥57 years	50 (31)	68 (37)	0.8 (0.5–1.2)
Race/ethnicity			
Black	67 (39)	51 (29)	1.6 (1.0–2.4)
Hispanic	2 (11)	116 (35)	0.2 (0.1–1.0)
Male sex	65 (31)	47 (42)	0.6 (0.4–1.0)
HARP site			
New York, NY	3 (14)	115 (35)	0.3 (0.1–1.0)
Palo Alto, CA	4 (17)	114 (35)	0.4 (0.1–1.1)
Atlanta, GA	8 (22)	110 (36)	0.5 (0.2–1.1)
Nashville, TN	11 (24)	107 (35)	0.6 (0.3–1.2)

[a]N = 347; HARP, *Helicobacter pylori* Antimicrobial Resistance Monitoring Project; EGD, esophagogastro -duodenal endoscopy; PPI, proton pump inhibitor; CI, confidence interval.
[b]These risk factors were not considered for multiv ariate analysis because of small cell -size differences between subjects with a resistant *H. pylori* infection and those without.

variate analysis. In the first-phase, multivariate model, antimicrobial resistance was significantly associated with previous antimicrobial treatment for *H. pylori*, and antacid use was protective. However, in this model, geographic location was highly collinear with race, which necessitated the second-phase multivariate model, in which the contribution of race was assessed by conditioning on geographic location, previous antimicrobial treatment for *H. pylori* infection, and antacid use. This model clearly demonstrated an association between black race and infection with an antimicrobial-resistant *H. pylori*. This association may indicate persistent transmission of resistant strains or in vivo induction of resistance. The association between *H. pylori* infection and blacks in the United States has been amply documented (2,3). However, the association between incidence of *H. pylori* resistance and black race is new. The biologically plausible association between previous antimicrobial use and resistance approached significance in the second-phase model; however, many study participants could not recall whether they were previously treated. This model does not rule out an independent association between geographic location and resistance. Enrollment of additional participants in HARP may sufficiently enhance the statistical power of the study to show such an association, while enrollment in sites serving a more racially diverse population may help clarify the contributions of geographic location as a risk factor for resistant infection.

This study has several limitations. While drawn from various regions in the continental United States, the 11 HARP sites represent a convenience sample of academic medical centers that is not truly representative of the U.S. population. Some participating sites enrolled more patients per month than others, so that cumulative data are biased towards individual study sites. Most of the participating hospital sites serve adult populations, and thus the results largely reflect resistance incidence towards adult patients. Patients undergoing endoscopy for *H. pylori*-related illness constitute a minority of infected persons, whose clinical condition and *H. pylori*-resistance status may not be representative of the total population with underlying *H. pylori* infection. Although this trend has not been documented,

Table 5. Phase I multivariate analysis of risk factors for resistance to ≥1 antimicrobial agent among *H. pylori* isolates submitted to HARP, 1998–2002[a]

Exposure	Odds ratio (95% CI)
Patient treated another time for infection	6.0 (2.0–20)
Took Mylanta in last 12 mo	0.3 (0.1–0.8)
HARP site	
New York, NY	0.2 (0.1–0.7)
Palo Alto, CA	0.2 (0.1–0.7)
Atlanta, GA	0.2 (0.1–0.6)
Nashville, TN	0.4 (0.2–0.9)

[a]N = 347; HARP, *Helicobacter pylori* Antimicrobial Resistance Monitoring Project; CI, confidence interval.

Table 6. Phase II stratified multivariate analysis of risk factors for resistance to ≥1 antimicrobial agent among *H. pylori* isolates submitted to HARP, 1998–2002[a]

Risk factor	Hazard ratio (95% CI)
Black race	2.1 (1.1–3.8)
Age ≥57 y	0.6 (0.3–1.1)
Took antibiotics 12 mo before esphagogastro-duodenal endoscopy	1.9 (0.9–3.7)

[a]N = 235; HARP, *Helicobacter pylori* Antimicrobial Resistance Project; CI, confidence interval.

many public health analysts suspect that the proportion of *H. pylori* infections diagnosed and treated in academic medical centers is decreasing compared with those in community practices. We suspect that this trend may have accounted for the reduced number of isolates submitted to HARP over time. Although these limitations primarily affected our sample size and how the sample represented the population, they did not invalidate the epidemiologic significance of the results generated from this study.

Given the limited population-based surveillance data on *H. pylori* resistance, HARP offers the best available data on *H. pylori* resistance in the United States. The accuracy and usefulness of this prospective, multicenter network can be enhanced in a number of ways. Increasing the number of HARP sites will improve geographic and demographic representation. Given the fact that only 12 study participants <12 years of age were included, enrollment of pediatric-care sites will improve the age representation of the HARP cohort. This feature is particularly important considering the increasing number of reports that describe higher resistance rates in pediatric strains compared to those isolated from adults, particularly resistance to macrolides (12,23). Enrolling study participants in community practices will provide data on resistance rates in *H. pylori* isolates from infected persons not treated at academic medical centers, a population in which resistance rates in *H. pylori* are unknown and may differ significantly from the current HARP cohort. Future HARP findings may serve as the basis for specific therapeutic recommendations and pretreatment antimicrobial susceptibility testing in high-risk populations.

In summary, we have shown that antimicrobial resistance in clinical *H. pylori* isolates is extensive, that it varies from year to year, and that resistant isolates are more common among blacks. Ongoing, prospective surveillance of *H. pylori* resistance is essential to ensure that appropriate data are available to guide the choice of therapy, particularly in high-risk populations.

Mr. Duck is a surveillance epidemiologist in the Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention. His primary research interests include *Helicobacter pylori* antimicrobial resistance, botulism, and bioinformatics.

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Address for correspondence: William M. Duck, Centers for Disease Control and Prevention, Mailstop A38, 1600 Clifton Road, Atlanta, GA 30333, USA; fax: 404-639-2205; email: wdd8@cdc.gov

Respiratory Picornaviruses and Respiratory Syncytial Virus as Causative Agents of Acute Expiratory Wheezing in Children

Tuomas Jartti,* Pasi Lehtinen,* Tytti Vuorinen,† Riikka Österback,† Bernadette van den Hoogen,‡ Albert D.M.E. Osterhaus,‡ and Olli Ruuskanen*

We studied the viral etiology of acute expiratory wheezing (bronchiolitis, acute asthma) in 293 hospitalized children in a 2-year prospective study in Finland. A potential causative viral agent was detected in 88% of the cases. Eleven different viruses were represented. Respiratory syncytial virus (RSV) (27%), enteroviruses (25%), rhinovirus (24%), and nontypable rhino/enterovirus (16%) were found most frequently. In infants, RSV was found in 54% and respiratory picornaviruses (rhinovirus and enteroviruses) in 42% of the cases. In older children, respiratory picornaviruses dominated (65% of children ages 1–2 years and 82% of children ages ≥3 years). Human metapneumovirus was detected in 4% of all children and in 11% of infants. To prevent and treat acute expiratory wheezing illnesses in children, efforts should be focused on RSV, enterovirus, and rhinovirus infections.

Acute expiratory wheezing illnesses (bronchiolitis, acute asthma) are the primary causes of hospitalization in children. An estimated 3% of children without other medical conditions are hospitalized for bronchiolitis (1). The annual hospitalization rate for exacerbation of asthma is 0.15% in children (2). In the United States alone, ≈200,000 children are hospitalized for bronchiolitis and acute asthma each year, which causes a substantial impact on families and the community.

Respiratory viruses are the most important precipitants of acute expiratory wheezing in children (3,4). Bronchiolitis is reportedly induced in infants mainly by respiratory syncytial virus (RSV), and asthma in older children is induced mainly by rhinovirus. The role of rhinovirus in infants is not clear. Furthermore, the roles of other respiratory viruses, e.g., enteroviruses, and the recently discovered human metapneumovirus (HMPV) in the etiology of acute wheezing are not well established (5,6). Investigating the viral origin of acute expiratory wheezing is useful because some antiviral treatments and vaccination are available, and the efficacy of anti-inflammatory treatments may be related to viral origin.

The purpose of the study was to investigate the role of 11 respiratory viruses in children hospitalized for acute expiratory wheezing. The viral etiology was studied for 2 years prospectively to cover outbreaks of all major respiratory viruses. Virus culture, virus antigen detection, polymerase chain reaction (PCR) techniques, and serologic testing were used to optimize the diagnosis of viral infection.

Methods

Study Participants and Definitions

As part of a randomized clinical trial evaluating the efficacy of systemic corticosteroid in the treatment of acute expiratory wheezing in children, we investigated the viral etiology of the infections. From September 1, 2000, through May 31, 2002, a total of 293 children participated in the study in the Department of Pediatrics, Turku University Hospital. Study breaks occurred from June to July 2001 and during Christmas week 2001. Inclusion criteria were the following: age from 3 months to 16 years, hospitalization for acute expiratory wheezing, and written informed consent from the parents. Exclusion criteria were the following: chronic diseases other than asthma or allergy, systemic glucocorticoid treatment within 4 weeks before the study, severe wheezing necessitating intensive-

*Turku University Hospital, Turku, Finland; †Turku University, Turku, Finland; and ‡Erasmus Medical Center, Rotterdam, the Netherlands

care unit treatment, and previous participation in this study. The study protocol was approved by the Ethics Committee of the Turku University Hospital.

Acute expiratory wheezing was called bronchiolitis when it occurred in children <3 years of age. When it recurred ≥2 times in persons of any age or occurred in persons ≥3 years of age, the diagnosis of asthma was used (7). To some extent, bronchiolitis and asthma are expressions of the same pathologic process, and no rigid criteria separate these illnessess. All patients were examined by one of the two study physicians (T.J. and P.L.).

Sample Collection

On patient's admission, a nasopharyngeal aspirate sample was taken through a nostril by inserting a disposable catheter connected to a mucus extractor to a depth of 5 to 7 cm and retracting it slowly while applying gentle suction with an electric suction device. All specimens were obtained without inserting any solution into the nostrils. Disposable plastic gloves were used, and all surfaces were wiped with disinfectant to prevent contamination. Immediately after the secretion was suctioned, two sterile cotton swabs were dipped in the aspirate. The swabs were then placed in vials containing 2 mL of viral transport medium (5% tryptose phosphate broth, 0.5% bovine serum albumin, and antimicrobial agents in phosphate-buffered saline) for virus culture and PCR assays. The rest of the mucus was used for virus antigen detection. The specimens were transported to the laboratory on the same day at room temperature. The tubes for RSV and HMPV PCR assays were frozen at –70°C before processing. Blood samples were collected on patient's admission and 2–3 weeks after discharge from the hospital.

Virologic Methods

Viral antigens for adenovirus; influenza A and B viruses; parainfluenza virus types 1, 2, and 3; and RSV were detected by time-resolved fluoroimmunoassay (8). Immunoglobulin (Ig) G antibodies to the same viruses were measured from paired serum samples by enzyme immunoassays as described earlier (9–11). Purified heat-treated coxsackievirus A9, coxsackievirus B3, echovirus 11, and poliovirus 1 were used as an antigen mixture in enterovirus IgG assays and purified heat-treated coxsackievirus A16, coxsackievirus B3, and echovirus 11 in IgM assays (12). Virus culture was performed according to routine protocols in A549, HeLa, and LLC-Mk2 cell lines and human foreskin fibroblasts (13). The supernatants of cell cultures exhibiting a cytopathogenic effect were further studied by antigen detection for adenovirus; influenza A and B viruses; parainfluenza virus types 1, 2, and 3; and RSV or by reverse transcription (RT)-PCR for enteroviruses and rhinovirus. Nucleic acids for RT-PCR were isolated from the nasopharyngeal samples with a commercial kit (High Pure Viral Nucleic Acid Kit, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. RT-PCR was used to detect enteroviruses and rhinovirus, coronavirus, RSV, and HMPV, as described previously (6,14,15). A case was defined as virus positive if at least one of the tests used was positive for virus. The rates of HMPV, respiratory picornaviruses, and RSV detected during the first study season 2000–2001 have been published (16).

Statistical Methods

The chi-square test was used for intergroup comparisons of different age groups in specific virus groups. The results were analyzed by using SAS software (version 8.2, SAS Institute, Cary, NC).

Results

Patient Characteristics

From September 2000 through May 2002, a total of 661 children were hospitalized for acute expiratory wheezing (Figure 1). Of the 661 patients, 341 did not meet the study criteria: 87 had already participated in the study, 79 were <3 months of age, 55 were not enrolled during study breaks, 48 had had systemic glucocorticoid treatment within 4 weeks, 24 did not need hospitalization, 17 had a chronic disease, 12 had guardians with language difficulties, 11 needed treatment in our intensive care unit, 3 had guardians who were not present, 2 were exposed to varicella, 2 patients' cases were not reported to the study physician, and 1 child was not eligible because of social reasons. The remaining 320 were eligible, but the parents of 27 (8%) children did not give their consent for participation in the study. Eventually, 293 children participated in the study.

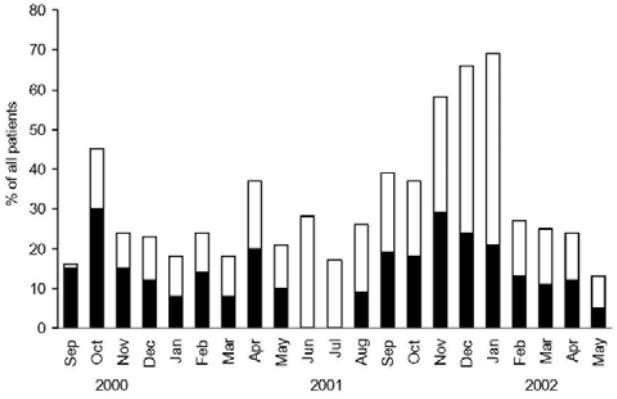

Figure 1. Hospitalized children with expiratory wheezing during the study period. Black indicates included patients.

The median age of the 293 study patients was 1.6 years (range 3 months–15.2 years). Seventy-six (26%) children were <12 months of age, 152 (52%) children were 12–35 months old, and 65 (22%) children were ≥3 years. In 179 children, the clinical diagnosis was bronchiolitis and in 114, acute asthma. Of the children with asthma, 49 were <3 years of age, 53% were boys, 38% experienced atopy, and 41% had parents who smoked.

Virus Infections

A potential causative viral agent was detected in 88% of the cases (Table 1). RSV (27%), enteroviruses (25%), and rhinovirus (24%) were the most common causative agents, resulting in 31%, 28%, and 28% of 258 virus-positive cases, respectively. The viruses in samples identified by the primary picornavirus PCR test but not identifiable in the liquid-hybridization assay were named rhino/enteroviruses (16%). According to our sequence data, these amplicons have shown >90% homology to human rhinoviruses. The remaining eight viruses studied accounted for 18% of the cases, and none of these viruses was detected in >5% of all cases.

Mixed viral infections were found in 57 (19%) cases and were usually associated with respiratory picornaviruses. Coinfection with enteroviruses and RSV was the most common mixed infection (19%), followed by rhinovirus and RSV (14%), rhino/enterovirus and RSV (11%), and enteroviruses and rhinovirus (9%). Of 12 HMPV infections, 5 were associated with other respiratory viruses.

Most of the viruses (84%), respiratory picornaviruses especially, were detected by using PCR (Table 1). Rhinovirus was cultivated in 25 (38%) of 65 specimens with PCR-positive results, enteroviruses in 14 (24%) of 59 specimens with PCR-positive results, and rhino/enterovirus in 1 (2%) of 46 specimens with PCR-positive results. To compare different methods of detecting RSV infection, we selected the patients whose samples were studied with four methods (n = 257). The recovery rate of RSV by IgG serologic testing was 22%; by virus antigen detection, 21%; by virus culture, 20%; and by PCR, 18%.

Seasonality of Virus Infections

Typical of the situation in Finland, a minor RSV epidemic occurred during the spring of 2000, followed by a major epidemic during the winter of 2001 to 2002 (Figure 2). Enterovirus outbreaks were seen during the fall in both 2000 and 2001. Rhinovirus outbreaks occurred during fall and spring of both years. An HMPV epidemic was seen during the winter of 2001. HMPV was detected in 30% of the study children during the 3-month epidemic. During the peak 3 epidemic months of respiratory picornaviruses, from September to November 2000, they accounted for 82% of all cases, and only 5% had other viral causes. During the peak 3 epidemic months of RSV, from November 2001 through January 2002, RSV accounted for 65% of all cases, and other viruses were found in 20%. Influenza A virus epidemics occured in the community from the beginning of October 2000 to the end of March 2001 and from October 2001 to May 2002 (data not shown), but influenza A virus caused only three cases of acute expiratory wheezing for which the patient had to be hospitalized.

Virus Infections by Age

RSV (54%), respiratory picornaviruses (42%), and HMPV (11%) were the most common viruses in infants (Figure 3). Respiratory picornaviruses were detected in 65% and RSV in 22% of the cases in children ages 12–35

Table 1. Positive viral findings in 293 children hospitalized for acute expiratory wheezing[a]

Virus	Virus antigen test; n = 293	Virus culture; n = 292	Virus PCR;[b] n = 291	Virus serology; n = 266	Total; n = 293
Respiratory syncytial virus (RSV)	62 (21)	58 (20)	50 (18)	56 (21)	80 (27)
Enterovirus		14 (5)	59 (20)	27 (10)	72 (25)
Rhinovirus		25 (9)	65 (22)		71 (24)
Rhino/enterovirus		1 (0.3)	46 (16)		46 (16)
Parainfluenza virus type 1	8 (3)	0			8 (3)
Parainfluenza virus type 2	0	0		0	0
Parainfluenza virus type 3	4 (1)	1 (0.3)			5 (2)
Parainfluenza virus type 1 or 3				8 (3)	4 (1)
Adenovirus	0	9 (3)		6 (2)	15 (5)
Human metapneumovirus			12 (4)		12 (4)
Influenza A virus	1 (0.3)	1 (0.3)		2 (0.8)	3 (1)
Influenza B virus	4 (1)	2 (0.7)		2 (0.8)	4 (1)
Coronavirus		0	4 (1)		4 (1)
Mixed viral infection					57 (19)
Total	79 (27)	111 (38)	236 (81)	101 (38)	258 (88)

[a]Data are presented as number of samples positive (% of evaluated samples).
[b]For RSV polymerase chain reaction (PCR), n = 279.

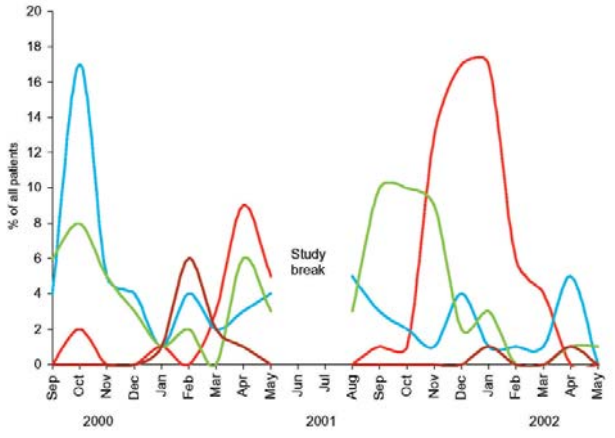

Figure 2. The epidemics of respiratory syncytial virus (red), rhinovirus (blue), enterovirus (green), and human metapneumovirus (brown) during the study period.

months. In children aged ≥3 years, respiratory picornaviruses (82%) were found most frequently.

Comparisons between age groups showed that RSV ($p < 0.001$) and HMPV ($p = 0.0030$) infected infants significantly more often than children in other age groups, adenovirus infected children ages 1–2 years significantly more often ($p = 0.022$), and enteroviruses infected children ages ≥3 years more often ($p = 0.0018$; Figure 3). No other significant differences were found.

Discussion

Our prospective study produced four notable findings. First, respiratory virus infection was detected in up to 90% of hospitalized children with acute expiratory wheezing. Second, respiratory picornaviruses were commonly associated with wheezing in infants. Third, one third of the wheezing children ages ≥3 years were infected with enteroviruses. Fourth, HMPV infections occurred in infants, but mainly during the first study year, and they were associated with only 4% of all cases with expiratory wheezing.

All major studies of the viral origins of expiratory wheezing are presented in Table 2. In studies from the 1960s to the 1990s, viral diagnosis was based on conventional virus culture, antigen detection, and serologic testing, and a viral agent could be established in 20% to 50% of children with expiratory wheezing. Bronchiolitis was mainly considered an RSV infection, with recovery in up to 73% of patients with confirmed cases (30). Lower RSV recovery rates were seen in older children (17,18,21,22,24,27). In the 1990s, viral detection rates increased to 75% to 85%, mainly as result of the increased detection of rhinoviruses by PCR (3,5). Our data confirm that RSV plays a key role in the etiology of bronchiolitis during RSV epidemics. Our findings regarding bronchiolitis give a prominent role also to rhinovirus, which has earlier been considered a common causative agent of wheezing in older children only (3,4,28,31). We found no differences in the distribution of rhinovirus infections by patient age.

This is the first long-term study to report a high association of enterovirus infections with acute expiratory wheezing in children. Enteroviruses, which replicate most prolifically in the gastrointestinal tract, have recently been shown to be associated with upper respiratory infections in 25% to 35% of the cases (32,33). Our findings are in agreement with those of Rawlinson et al. (4), who found enteroviruses by PCR in 29% of the young children with well-documented asthma during the summer. We found enteroviruses mostly in older children.

HMPV was detected in 4% of our patients. A recent study of children hospitalized for acute respiratory tract disease found HMPV in 6% of the cases (34). Bronchiolitis and pneumonitis were the main diagnoses. HMPV predominantly infected infants as seen in our study and in previous studies (4,6). HMPV outbreaks have been reported mainly in mid-winter, which was supported by our study. Notably, the HMPV outbreak with 10 cases was seen during the first study year, and two cases were found during the second year, which suggests that epidemics do not occur every year.

The use of PCR has markedly increased the recovery rates of viruses in acute respiratory infections (3,5). The clinical value of positive respiratory picornavirus PCR tests is, however, questionable as picornavirus RNA has also been detected in 5% to 30% of asymptomatic children

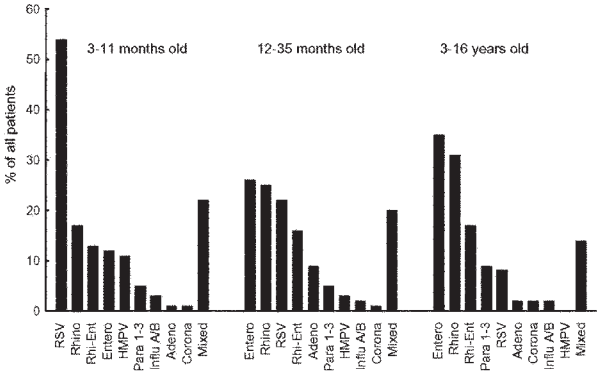

Figure 3. The prevalence of respiratory viruses in hospitalized, wheezing children in different age groups. RSV, respiratory syncytial virus; Rhino, rhinovirus; Rhi-Ent, rhino/enterovirus; Entero, enteroviruses; HMPV, human metapneumovirus; Para 1–3, parainfluenza virus types 1–3; Influ A/B, influenza A and B viruses; Adeno, adenovirus; Corona, coronavirus; Mixed, mixed viral infection. p values are for intergroup comparisons: RSV $p < 0.001$, HMPV $p = 0.003$, enteroviruses $p = 0.0018$, and adenovirus $p = 0.022$.

Table 2. Viral etiology of acute expiratory wheezing in children[a,b]

			Methods for virus detection				Viral identification rates (%)								
Y of study (ref.)	Wheezing episodes	Age (y)	Culture	Antigen	Serology	PCR	RSV	Rhino	Entero	PIV types 1–3	Influ A/B	Adeno	Corona	HMPV	Total pos.
1965 (17)	225	0–16	+				8	3	5	4	0	4			27
1971 (18)	855	0–14	+				9	2	1	8	1	2			25
1975 (19)	1,515	0–12	+				3	12	3	3	2	1			23
1976 (20)	267	1–12	+		+		4	6	1	1	1	3			14
1979 (21)	1,851	0–15	+				7	1		6		2			21
1979 (22)	554	0–12	+				2	13	4	4	2	1			26
1979 (23)	72	5–15	+				1	28		3	10	1			49
1984 (24)	256	2–15	+	+	+		5			4	2	3	2		29
1987 (25)	204	0–12	+	+	+		6	1	5	0	3	2			19
1993 (26)	99	0.2–16	+	+			14	19	3	0	2	0			36
1996 (27)	181	0.3–2	+	+			12	6	1	7		2			26
1999 (3)	70	0.2–16	+	+		+	26	61	1		1		4		83
1999 (28)	132	0.3–14		+		+	21	47	10	4	5	5	5		82
2000 (29)	84	0.7		+		+	54	10	8	0	0	13	0		74
2002 (30)	118	0–1.5				+	53	21		3	3	8	3		74
2003 (4)	179	0.1–17	+	+		+	7	79		1	2			2	88

[a]Sampling period of at least 12 mo.
[b]RSV, respiratory syncytial virus; PCR, polymerase chain reaction; rhino, rhinovirus; entero, enterovirus, PIV, parainfluenza virus; influ, influenza virus; adeno, adenovirus; corona, coronavirus; HMPV, human metapnemovirus; pos., positive.

(3,35). We recently found that the number of positive PCR results for picornavirus markedly decreased over 2 to 3 weeks and disappeared over 5 to 6 weeks after an acute respiratory infection, which suggests that a positive PCR result for picornaviruses is related to acute infection (36). None of the 79 healthy controls were infected with enteroviruses, but 16% were positive for rhinovirus or nontypeable rhino/enterovirus (36). In detecting RSV infections, PCR was no more sensitive than virus culture, antigen detection, or serologic testing. This finding is in contrast to the results of previous studies, especially in adults (37). In children too, PCR has been almost 1.5 times more sensitive than culture and antigen detection (38). These differences may be explained by the greater sensitivity of the nested RT-PCR used in those studies. Compared to children, adults may also have lower titers of viruses in the nasopharynx, which favors PCR diagnosis over virus culture or antigen detection. We likely did not miss many cases of adenovirus, parainfluenza virus, or influenza virus infections because PCR has only modestly increased sensitivity to those viruses compared to virus culture and antigen detection (39,40).

Our study has some limitations. First, and most important, we studied fewer than half of the children admitted to our hospital for acute expiratory wheezing. However, throughout the study, we enrolled approximately half of the patients hospitalized for expiratory wheezing each month. Since the respiratory virus season is the main factor determining the viral cause of acute illness, any seasonality bias is largely excluded. Several infants with RSV infection were missed, because infants <3 months of age were not included. However, during the summer study break, when rhinovirus and enteroviruses are normally circulating in the community, children were not enrolled. This balances the ratio of missed RSV cases to picornavirus cases. We therefore believe that our sample reliably represents the whole patient population hospitalized during the study years. Furthermore, we only analyzed viral infections. *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* have been detected in 5% to 25% of children with acute wheezing, but the clinical importance of these findings remains to be determined (7,28).

In conclusion, this study showed that acute expiratory wheezing necessitating hospitalization was most often associated with RSV, enterovirus, and rhinovirus infections. Acute expiratory wheezing in infants may be a risk factor for childhood asthma (31). Therefore, efforts should focus on developing antiviral agents and vaccines against RSV and respiratory picornaviruses.

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Dr. Jartti is a fellow in pediatric allergology at Turku University Hospital, Turku, Finland. He is interested in the pathogenesis and treatment of acute expiratory wheezing illnesses with a special interest in respiratory viral infections.

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Address for correspondence: Tuomas Jartti, Sirkkalankatu 4 C 59, FIN-20520 Turku, Finland; fax: 358-9-471 86416; email: tuomas.jartti@hus.fi

Antimicrobial Resistance among *Campylobacter* Strains, United States, 1997–2001

Amita Gupta,* Jennifer M. Nelson,* Timothy J. Barrett,* Robert V. Tauxe,* Shannon P. Rossiter,* Cindy R. Friedman,* Kevin W. Joyce,* Kirk E. Smith,† Timothy F. Jones,‡ Marguerite A. Hawkins,§ Beletshachew Shiferaw,¶ James L. Beebe,# Duc J. Vugia, Terry Rabatsky-Ehr,†† James A. Benson,‡‡ Timothy P. Root,§§ and Frederick J. Angulo* for the NARMS Working Group**

We summarize antimicrobial resistance surveillance data in human and chicken isolates of *Campylobacter*. Isolates were from a sentinel county study from 1989 through 1990 and from nine state health departments participating in National Antimicrobial Resistance Monitoring System for enteric bacteria (NARMS) from 1997 through 2001. None of the 297 *C. jejuni* or *C. coli* isolates tested from 1989 through 1990 was ciprofloxacin-resistant. From 1997 through 2001, a total of 1,553 human *Campylobacter* isolates were characterized: 1,471 (95%) were *C. jejuni*, 63 (4%) were *C. coli*, and 19 (1%) were other *Campylobacter* species. The prevalence of ciprofloxacin-resistant *Campylobacter* was 13% (28 of 217) in 1997 and 19% (75 of 384) in 2001; erythromycin resistance was 2% (4 of 217) in 1997 and 2% (8 of 384) in 2001. Ciprofloxacin-resistant *Campylobacter* was isolated from 10% of 180 chicken products purchased from grocery stores in three states in 1999. Ciprofloxacin resistance has emerged among *Campylobacter* since 1990 and has increased in prevalence since 1997.

Campylobacter is the most common cause of bacterial gastroenteritis in the United States, causing an estimated 2.4 million human infections annually (1). Diagnosed infections have declined in recent years. In 2001, FoodNet surveillance identified 13.4 diagnosed *Campylobacter* infections per 100,000 persons (2). Approximately 95% of diagnosed *Campylobacter* infections are due to *C. jejuni* (3). Although most *Campylobacter* infections cause an acute, self-limited illness characterized by diarrhea, fever, and abdominal cramps, severe infections do occur (4). Antimicrobial treatment can shorten the duration of illness and may be life-saving in invasive infections (5–7). Fluoroquinolones (e.g., ciprofloxacin) are often prescribed empirically for the treatment of gastroenteritis and for *Campylobacter* infections in adults (6,8). Quinolones (e.g., nalidixic acid), although now seldom used for treatment in the United States, are frequently used to screen for fluoroquinolone resistance because of the close correlation between quinolone and fluoroquinolone resistance among *Campylobacter*. Macrolides, such as erythromycin, are also prescribed to treat *Campylobacter* infections (4,9).

Fluoroquinolone-resistant *Campylobacter* infections in humans were first detected in Europe in the late 1980s (10–12). Subsequently, an increasing proportion of *Campylobacter* isolates around the world have been found to be fluoroquinolone-resistant (13). Studies in the United States, Europe, and New Zealand have identified poultry as a principal source of *Campylobacter* infection (14–16). Quinolones have been available in human medicine since the mid-1960s, and the first fluoroquinolone (ciprofloxacin) was approved for use in humans in 1986. Two fluoroquinolones, sarafloxacin and enrofloxacin, were approved for use in poultry by the U.S. Food and Drug Administration (FDA) in 1995 and 1996, respectively (17). These fluoroquinolones were the first ones approved in food animals; subsequently, other fluoroquinolones have been approved for veterinary use but not for use in poultry (18).

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA, †Minnesota Department of Health, Minneapolis, Minnesota, USA; ‡Tennessee Department of Health, Nashville, Tennessee, USA; §Maryland Department of Health and Mental Hygiene, Baltimore, Maryland, USA; ¶Oregon Department of Human Services, Portland, Oregon, USA; #Colorado Department of Public Health & Environment, Denver, Colorado, USA; **California Department of Health Services, Berkeley, California, USA; ††Connecticut Department of Public Health, Hartford, Connecticut, USA; ‡‡Georgia Department of Human Services, Atlanta, Georgia, USA; and §§New York State Department of Health, Albany, New York, USA

To investigate the epidemiology of fluoroquinolone-resistant *Campylobacter* in the United States, we reviewed national surveillance data to determine the prevalence of antimicrobial resistance, particularly ciprofloxacin resistance, among *Campylobacter* isolates; conducted a case-control study to determine the proportion of ciprofloxacin-resistant infections that were domestically acquired; and performed a retail survey to determine the prevalence of ciprofloxacin-resistant *Campylobacter* contaminating chicken products sold in selected supermarkets.

Methods

National Surveillance for Resistance in *Campylobacter*

1989–1990 Sentinel County Study

From 1989 to 1990, a national county-based survey of antimicrobial susceptibility among *Campylobacter* isolates was conducted. Sentinel clinical laboratories in 19 counties participated. The methods of this survey are described elsewhere (19,20). Briefly, the first five sporadic *Campylobacter* isolates identified each month were forwarded to the Centers for Disease Control and Prevention (CDC). Patients with *Campylobacter* infection were interviewed with a standard questionnaire, which included information about clinical illness and exposures (i.e., food, animal, and foreign travel) during the 2 weeks before illness onset. Isolates were determined to be *Campylobacter* by dark-field microscopic examination and hippurate hydrolysis (20–22). Hippurate-positive isolates were considered *C. jejuni*. All isolates with questionnaires received during the first 4 months of the study underwent susceptibility testing. Because of a shortage of reagents, a random sample of 50% of isolates with completed questionnaires received during the last 8 months of the study was further characterized. Isolates were tested for susceptibility to azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid, and tetracycline by using broth microdilution methods (19). In 2003, a retrospective analysis of the hippurate-negative isolates was completed; these isolates were speciated by using methods described below.

National Antimicrobial Resistance Monitoring System (NARMS), 1997–2001

NARMS for enteric bacteria is a collaboration between CDC, the Food and Drug Administration, and state and local health departments. The system monitors patterns of antimicrobial drug resistance. NARMS methods are described in detail elsewhere (23).

In brief, isolates were tested for viability, confirmed as *Campylobacter*, and identified to the species level by using the hippurate hydrolysis test according to published methods (21,22). Hippurate-negative *Campylobacter* in which the hippuricase gene could be detected by polymerase chain reaction (PCR) were identified as *C. jejuni* (24). Isolates that tested negative for the hippuricase gene but positive for a *C. coli*-specific ceuE sequence were identified as *C. coli* (25). Isolates that could not be identified as either *C. jejuni* or *C. coli* by these PCR assays were referred to the National *Campylobacter* Reference Laboratory at CDC for identification with genotypic (e.g., 16S rRNA sequencing) and phenotypic methods (21).

Isolates were tested with the E-test system (AB BIODISK, Solna, Sweden) to determine MICs for six antimicrobial agents: chloramphenicol, ciprofloxacin, clindamycin, erythromycin, nalidixic acid, and tetracycline. Beginning in 1998, azithromycin and gentamicin were also included. When available, National Committee for Clinical Laboratory Standards interpretive criteria for *Enterobacteriaceae* MICs were used; ciprofloxacin resistance was defined as MIC $\geq$4 μg/mL, and erythromycin resistance was defined as MIC $\geq$8 μg/mL (26). Multidrug resistance was defined as resistance to two or more of the original six antimicrobial agents.

We used a multivariable logistic regression model to assess changes in the proportion of isolates with antimicrobial drug resistance from 1997 through 2001 because the population under surveillance more than doubled from 1997 to 2001, and substantial site-to-site variation in prevalence of antimicrobial drug resistance was identified (i.e., uncertainty was found in the denominators for calculating rates). The model was for antimicrobial drug resistance as a function of year and included main effects adjustments for age categories and site-to-site variation in prevalence. Within the available data, site by year interaction was not a significant factor but because the catchment areas expanded, the hypothesis of site by year interaction could not be fully tested.

1997 Retrospective Case-Comparison Study

Using NARMS isolates, we conducted a retrospective case-comparison study in four NARMS sites (California, Connecticut, Georgia, and Oregon). Persons with ciprofloxacin-resistant (CipR) *Campylobacter* infection identified in 1997 were compared with persons in whom the diagnosis of ciprofloxacin-sensitive (CipS) *Campylobacter* infection was made that same year. We compared up to two CipS cases for each CipR case and matched cases by geographic site and date of stool specimen collection. All case-patients were interviewed by telephone, usually within 8 weeks of their illness onset, about demographics, clinical information, and exposures (e.g., antimicrobial drug use in the 4 weeks before illness onset, foreign travel, and consumption of poultry and raw milk in

the 7 days before illness onset) with a standardized questionnaire.

1999 Retail Survey

Three NARMS-participating state health departments (Georgia, Maryland, and Minnesota) participated in a survey of retail chicken products. From January to June 1999, each site purchased a convenience sample of 10 whole broiler chickens per month from supermarkets located within the state. State public health laboratories at each site tested the samples for *Campylobacter*. Carcass rinse samples were centrifuged, and pellets were incubated in enrichment broth and plated onto *Campylobacter* blood agar plates according to methods published elsewhere (27); neither media contained quinolone or macrolide antimicrobial agents. *Campylobacter* isolates were forwarded to CDC for species identification and antimicrobial susceptibility testing according to NARMS methods.

Results

National Surveillance

1989–1990 Sentinel County Study

Two hundred ninety-eight patients were interviewed, and their *Campylobacter* isolates were tested. Of these isolates, 289 (97%) were *C. jejuni*, 8 (3%) were *C. coli*, and 1 (0.3%) was a *C. lari*. None were resistant to ciprofloxacin, and 3 (1%) of 294 were resistant to nalidixic acid (MIC ≥ 32 μg/mL); 1 isolate was *C. lari*, which is inherently resistant to nalidixic acid (28), and 2 were *C. jejuni*. The *C. lari* isolate was resistant to ofloxacin (MIC = 8 μg/mL), intermediately resistant to norfloxacin (MIC = 8 μg/mL) but susceptible to ciprofloxacin (MIC = 2 μg/mL).[1] The two nalidixic acid-resistant *C. jejuni* isolates were susceptible to ciprofloxacin (MIC = 0.5 μg/mL) and norfloxacin and ofloxacin (MIC ≤ 2 μg/mL). The proportion of the isolates resistant to tetracycline was 42% (124/295). The resistant proportion for the other antimicrobial agents tested were as follows: erythromycin 3% (8/295), clindamycin 2% (6/295), azithromycin 2% (5/294), chloramphenicol 0% (0/295), and gentamicin 0% (0/295). Travel history was available for 296 patients with *Campylobacter* infection; 23 (8%) patients traveled outside of the United States in the week before illness onset. Of the persons with available information, 32 (11%) of 295 had taken an antimicrobial agent in the 30 days before illness onset, 46 (15%) of 298 were hospitalized, and 241 (81%) of 297 were treated with an antimicrobial agent for their illness. Among the 234 persons for whom treatment data were available, the most common agents used for treatment were erythromycin (62%), ciprofloxacin (19%), and trimethoprim-sulfamethoxazole (5%). Of the three patients with nalidixic acid-resistant infections, none traveled outside the United States, and none were treated with a quinolone or fluoroquinolone in the month before illness.

NARMS, 1997–2001

From 1997 to 2001, a total of 1,932 presumptive *Campylobacter* isolates were received at CDC through NARMS; 193 (10%) were excluded because they were not viable, 104 (5%) were not in accordance with the one-a-week sampling method, 39 (2%) were determined not to be *Campylobacter*, 22 (1%) were duplicates, and 21 (1%) were contaminated cultures. Of the 1,553 (80%) isolates further characterized and included in this analysis, 1,471 (95%) were *C. jejuni*, 63 (4%) were *C. coli*, 7 (0.4%) were *C. upsaliensis*, 5 (0.3%) were *C. fetus*, 2 (0.1%) were *C. lari*, and 5 (0.3%) were undetermined (i.e., determination by 16S study did not identify a species). Forty-five percent of case-patients were female; the median age was 33 years (range <1–96). Among 1,439 isolates with known source of specimen collection, 1,426 (99%) were from stool samples, and 13 (1%) were from blood samples. Among blood isolates, eight were *C. jejuni*, two were *C. fetus*, two were *C. upsaliensis*, and one was *C. lari*.

The results of susceptibility testing among *Campylobacter* isolates by species are shown in Table 1. Resistance to ciprofloxacin among all *Campylobacter* isolates was 13% in 1997 and 19% in 2001. Resistance to erythromycin among all *Campylobacter* isolates was 2% in 1997 and 2% in 2001. The results of antimicrobial susceptibility testing by year for isolates of the most common species, *C. jejuni*, are shown in Table 2

The prevalence of ciprofloxacin-resistant *Campylobacter* ranged from 0% (0/14) in Tennessee in 1999 to 26% (14/53) in Georgia in 2001. By using a multivariate logistic regression model and controlling for age and site-to-site variation in prevalence, the proportion of all *Campylobacter* isolates resistant to ciprofloxacin and nalidixic acid in 2001 was significantly higher than the proportion of isolates resistant to ciprofloxacin in 1997 (data only shown for ciprofloxacin in Table 3). The remaining antimicrobial drugs had no statistically significant change in resistance over time (data not shown).

Fifty-one percent of *Campylobacter* isolates were resistant to ≥1 drug, 18% were resistant to ≥2 drugs, and 10% were resistant to ≥3 drugs. The most common multidrug resistance (i.e., ≥2 drugs) pattern included ciprofloxacin, nalidixic acid, and tetracycline.

[1]This isolate was reported to be ciprofloxacin resistant in reference 20; more recently established interpretive criteria define it as susceptible.

Table 1. Antimicrobial resistance among *Campylobacter* isolates by species, National Antimicrobial Resistance Monitoring System 1997–2001

	% resistant						
Antimicrobial agent	*C. jejuni* (n = 1,471)	*C. coli* (n = 63)	*C. upsaliensis* (n = 7)	*C. lari* (n = 2)	*C. fetus* (n = 5)	Undetermined[a] (n = 5)	Total (n = 1,553)
Azithromycin[b]	2	9	0	0	0	0	2
Chloramphenicol	0.3	5	0	0	0	40	0.6
Ciprofloxacin	16	30[c]	14	0	0	0	16
Clindamycin	1	9	0	0	0	20	2
Erythromycin	2	8	0	0	0	20	2
Gentamicin[b]	0	2	0	0	0	0	0.1
Nalidixic acid	17	36[c]	14	100	80	20	18
Tetracycline	43	43	0	0	20	0	43

[a]Undetermined isolates were hippurate-negative *Campylobacter* that could not be further speciated with available polymerase chain reaction primers.
[b]For azithromycin and gentamicin, only 1,336 isolates were tested.
[c]Comparison of proportion of resistant *C. coli* to resistant *C. jejuni* was statistically significant for ciprofloxacin and nalidixic acid but not tetracycline ($p < 0.01$)

1997 Retrospective Case-Comparison Study

Sixteen (57%) of 28 ciprofloxacin-resistant *Campylobacter* (CipR) case-patients and 31 ciprofloxacin-sensitive (CipS) case-patients were interviewed. The median age was 46 years (range 9–76 years) for CipR patients and 24 years (range 1–87 years) for CipS patients (Wilcoxon rank-sum, $p = 0.08$). CipR patients did not differ significantly from CipS patients in terms of sex (40% vs. 42% female, $p = 0.1$), race (87% vs. 77% white, $p = 0.08$), and place of residence (87% vs. 61% urban/suburban areas, $p = 0.06$). Five (31%) CipR patients were hospitalized for gastroenteritis compared with 1 (3%) CipS patient (matched odds ratio [mOR] = 13.6, 95% confidence interval [CI] 1.4 to 130.1). Eight (57%) CipR case-patients reported having bloody diarrhea compared with eight (30%) CipS patients (mOR = 3.2, 95% CI 0.8 to 12.1). Seven (44%) of 16 CipR patients compared with 1 (3%) of 31 CipS patients traveled to a foreign country in the 7 days before illness onset (mOR = 23.3, 95% CI 2.5 to 215.6); 5 (71%) of 7 CipR patients traveled to Europe while 1 CipS patient traveled to the Caribbean. Among all case-patients, 35 of 47 reported treatment with an antimicrobial agent for their illness. Of those who recalled the name of the antimicrobial drug, 75% reported taking a fluoroquinolone, 16% reported taking a macrolide, and 8% took trimethoprim-sulfamethoxazole (TMP/SMX). One CipR patient and one CipS patient took fluoroquinolones between onset of illness and collection of stool specimens. Among the eight CipR patients who did not travel and did not take fluoroquinolones between illness onset and stool specimen collection, seven (87%) consumed poultry in the 7 days before infection; this finding was not statistically different from that in CipS patients. No other exposures were significantly associated with ciprofloxacin-resistant infection, including having pets, drinking raw milk, or being exposed to a farm (data not shown).

1999 Retail Survey

Among the 180 retail chicken products purchased, representing 18 domestic brand names from 22 grocery stores, *Campylobacter* was isolated from 80 (44%) samples. Sixty-two (77%) were *C. jejuni*, 16 (20%) were *C. coli*, and 2 (2%) were undetermined (i.e., determination by 16S study did not identify a species). The prevalence of *Campylobacter* isolated was 33% (20 of 60) in Georgia, 37% (22 of 60) in Maryland, and 63% (38 of 60) in Minnesota. This difference among sites was in part due to the difference in isolation rates of *C. coli*; 14 (87%) of the

Table 2. Antimicrobial resistance among human *Campylobacter jejuni* strains, 1990–2001

	% resistant						
Antimicrobial agent	1989-1990 (n = 286)[a,b]	1997 (n = 209)	1998 (n = 297)	1999 (n = 294)	2000 (n = 306)	2001 (n = 365)	Total (n = 1,757)
Azithromycin[c]	1	–	1	3	2	2	1
Chloramphenicol	0	1	1	0.3	0	0	0.3
Ciprofloxacin	0	12	14	18	14	18	13
Clindamycin	1	1	1	1	1	2	1
Erythromycin	1	1	2	2	1	2	2
Gentamicin[c]	0	–	0	0	0	0	0
Nalidixic acid	1	13	16	20	16	19	14
Tetracycline	42	47	46	46	39	40	43

[a]1989–1990 U.S. sentinel county study used different sampling and laboratory methods (microbroth dilution testing) than NARMS (Etest). However, studies have concluded that broth microdilution and Etest give equivalent results for ciprofloxacin susceptibility testing of *Campylobacter* (44).
[b]In 1989–1990 U.S. county study, only 285 isolates were tested for azithromycin and nalidixic acid susceptibility.
[c]For azithromycin and gentamicin, only isolates received between 1998 and 2001 were tested (N = 1,262).

Table 3. Trend analysis of the proportion of fluoroquinolone-resistance among *Campylobacter*, National Antimicrobial Resistance Monitoring System, 1997–2001

Y	Unadjusted OR[a] (95% CI)	Adjusted OR[b] (95% CI)
1997[c]	1.0	1.0
1998	1.0 (0.6 to 1.7)	1.3 (0.7 to 2.4)
1999	1.4 (0.9 to 2.3)	2.1 (1.2 to 3.9)
2000	1.1 (0.7 to 1.8)	1.5 (0.8 to 2.8)
2001	1.6 (1.0 to 2.5)	2.5 (1.4 to 4.4)

[a]OR, odds ratio; CI, confidence interval.
[b]Adjusted odds ratios were calculated by using logistic regression model, which accounted for site-to-site variation in prevalence.
[c]1997 was the reference value.

16 *C. coli* isolates came from retail chickens purchased in Minnesota. A ciprofloxacin-resistant strain of *Campylobacter* was identified in 10% of the 180 retail chicken products tested, and an erythromycin-resistant strain was identified in 2% of chicken products (Table 4). The distribution of ciprofloxacin MICs in *Campylobacter* species of retail chicken and human isolates was similar. For both human and poultry *Campylobacter* isolates, MICs were predominantly ≤0.5 or ≥32 μg/mL with few intermediate phenotypes (Figure 1).

Discussion

Fluoroquinolone-resistant *Campylobacter* have emerged over the last decade in the United States. In 1990, no ciprofloxacin-resistant human isolates were identified in a national sentinel county-based survey. From 1997 to 2001, the prevalence of ciprofloxacin-resistant *Campylobacter* increased significantly from 13% to 19%. These data are consistent with four prior surveillance studies from humans conducted in the United States: 1) a hospital-based study in Pennsylvania conducted from 1982 to 1991 found no fluoroquinolone resistance among *C. jejuni* isolates (29), 2) a second study at the same Pennsylvania hospital found a sharp increase in ciprofloxacin resistance among *C. jejuni* from 8% in 1996 to 40% in 2001 (30), 3) a study conducted in Wisconsin between 1992 and 1995 found 12% of the *C. jejuni* to be ciprofloxacin-resistant (31), and 4) a study in Minnesota showed an increase in quinolone-resistant *C. jejuni* isolates from 1.3% in 1992 to 10.2% in 1998 (Figure 2) (18). The emergence of fluoroquinolone resistance among *Campylobacter* isolates in the 1990s has occurred while resistance to other antimicrobial agents has remained stable. Specifically, resistance to the macrolides, azithromycin and erythromycin, which are commonly used antimicrobial agents in humans (32), has remained low (1%–3%).

Our retrospective case-comparison study showed that patients with ciprofloxacin-resistant *C. jejuni* infections were more likely to be hospitalized compared to patients with ciprofloxacin-susceptible infections. These results, however, are based on a small number of patients, and age could have been a confounder. Other studies have found that patients infected with fluoroquinolone-resistant *Campylobacter* have a longer duration of diarrhea than patients with fluoroquinolone-susceptible isolates, although no difference in hospitalization rates have been reported (18,33). These findings may have clinical implications. Ciprofloxacin is commonly used to treatment severe *Campylobacter* infections and other intestinal infections in adults, so the rise of fluoroquinolone resistance may result in ineffective treatment when fluoroquinolones are used. Macrolides, which are efficacious in treating *Campylobacter* (5,34), should still be considered the first-line drugs for severe *Campylobacter* infections, as resistance to this class remains low.

Our study also identified foreign travel, particularly to Europe, to be associated with ciprofloxacin-resistant *C. jejuni* infection. High rates of fluoroquinolone-resistant *Campylobacter* have been reported from southern Europe and other regions of the world (13). Studies in northern Europe have associated fluoroquinolone use in food animals, particularly poultry, as a source for human infection with fluoroquinolone-resistant *Campylobacter* (13). Nevertheless, while foreign travel was a risk factor in our study, over half of the ciprofloxacin-resistant infections were domestically acquired. Ciprofloxacin resistance was not associated with use of fluoroquinolones before specimen collection, which suggests that fluoroquinolone-resistant organisms did not result from individual use of fluoroquinolones. A more recent, larger case-control study of patients infected with ciprofloxacin-resistant *Campylobacter* infections found similar results to our study; 58% of illnesses were domestically acquired, and none of the patients took fluoroquinolones after illness onset and before specimen collection (35).

Our 1999 survey of retail chicken sold in selected supermarkets provided ecologic evidence that chicken

Table 4. Antimicrobial resistance among *Campylobacter* isolates from retail chicken, by species, National Antimicrobial Resistance Monitoring System, 1999

	% resistant		
Antimicrobial agent	*C. jejuni* (n = 62)	*C. coli* (n = 16)	Other[a] (n = 2)
Azithromycin	6	0	0
Chloramphenicol	0	0	50
Ciprofloxacin	24	19	50
Clindamycin	5	0	0
Erythromycin	6	0	0
Gentamicin	0	6	0
Nalidixic acid	29	37	50
Tetracycline	69	50	50

[a]One isolate was undetermined (i.e., hippurate-negative *Campylobacter* that could not be further speciated by 16S polymerase chain reaction study), and one isolate was an unknown *Campylobacter* that could not be further characterized.

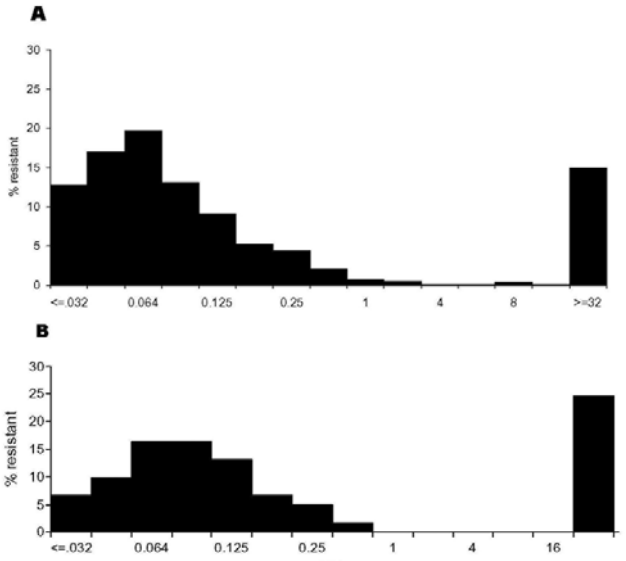

Figure 1. Distribution of ciprofloxacin MICs among *Campylobacter jejuni* isolated from humans and retail chicken. A, human isolates, 1997–2001; N = 1,471. B, grocery store purchased chicken isolates, 1999; N = 62.

may be a source of domestically acquired ciprofloxacin-resistant *Campylobacter* infections; 10% of retail chickens were contaminated with ciprofloxacin-resistant *Campylobacter*. Other studies have shown that *Campylobacter*, including ciprofloxacin-resistant *Campylobacter*, are commonly isolated from retail poultry meats. A survey of retail meats purchased in the Washington, D.C., area isolated *Campylobacter* species from 71% of chicken and 14% of turkeys tested; (36) 25% of the *C. jejuni* isolates and 40% of the *C. coli* isolates were resistant to ciprofloxacin (37). A second survey in Minnesota isolated *Campylobacter* from 88% of retail chicken meats purchased in 1997, including *C. jejuni* in 74% and *C. coli* in 21%. Ciprofloxacin-resistant *Campylobacter* was identified in 20% of retail chicken products (18). In this study, comparison of molecular subtypes from human and retail chicken quinolone-resistant *C. jejuni* isolates found that six of seven subtypes were indistinguishable from each other.

In the United States, the FDA has approved the use of fluoroquinolones at different times for humans and food animals. Fluoroquinolones have been commonly used in humans for treating intestinal and other infections since 1986 (32). The first fluoroquinolones to be FDA-approved for use in food animals in the United States were sarafloxacin in 1995 and enrofloxacin in 1996. These fluoroquinolones were approved for use in chickens and turkeys to treat bacterial respiratory infections principally caused by *E. coli*. These agents are typically administered to the entire poultry house (often >20,000 birds) through drinking water, which results in the treatment of sick and healthy birds with various concentrations of fluoroquinolones. The extent of fluoroquinolone use in chickens and turkeys in the United States is not known; manufacturers and farmers are not required to report these data. The Animal Health Institute has estimated that 1%–2% of the approximately 8 billion broiler chickens slaughtered each year in the United States are treated with fluoroquinolones (38). An experiment with *Campylobacter*-infected chickens treated with enrofloxacin and sarafloxacin showed that ciprofloxacin resistance rapidly developed among *Campylobacter* (39).

An association between the approval of fluoroquinolones for use in food-producing animals and the development of fluoroquinolone-resistant *Campylobacter* in animals and humans has been noted in several countries. The approval of fluoroquinolones for use in food animals has been followed temporally by a rise in ciprofloxacin-resistant *Campylobacter* and other enteric pathogens isolated from animals and humans in Denmark, the Netherlands, and Spain (13,40). After the use of oral fluoroquinolones in pigs was discontinued in Denmark in 1999, nalidixic acid resistance among *C. coli* isolates from pigs decreased from 17% in 1998 to 5% in 2001 (41). In the United States, FDA has recently conducted a quantitative risk assessment and concluded that fluoroquinolone use in chickens and turkeys results each year in >10,000 human infections with fluoroquinolone-resistant *Campylobacter* in persons who seek medical care and are treated with fluoroquinolones (42). FDA proposed the withdrawal of approval of fluoroquinolones for use in poultry in October 2000 (43). This is the first time a proposal has been made to withdraw an approval for an antimicrobial used in agriculture because of associated emergence of resistance in humans. The manufacturer of sarafloxacin has since withdrawn this product from the market, but the manufacturer of enrofloxacin continues to

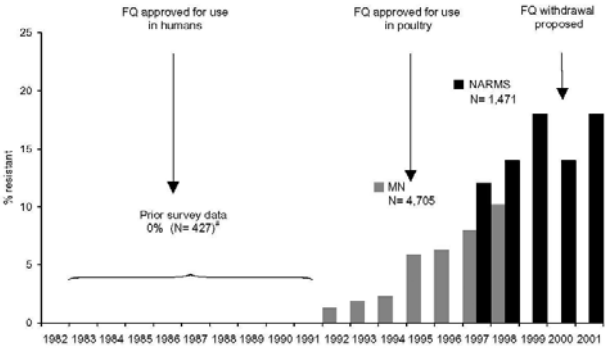

Figure 2. Quinolone- and fluoroquinolone-resistant *Campylobacter jejuni* in the United States, 1982–2001. FQ, fluoroquinolone; MN, Minnesota quinolone resistance among *C. jejuni* strains data (adapted from 18), NARMS, National Antimicrobial Resistance Monitoring System. Prior survey data adapted from reference 19 and 30.

market enrofloxacin for use in poultry in the United States.

Our studies had several limitations. The retrospective case-comparison study did not assess exposures among travelers and therefore cannot assess the possibility that the travelers may have acquired ciprofloxacin-resistant *Campylobacter* from eating poultry or other foods while traveling. Routine surveillance for antimicrobial susceptibility among *Campylobacter* did not start until 1997, and therefore we cannot identify national trends in antimicrobial resistance from 1991 to 1996. Other limitations are evident in NARMS *Campylobacter* surveillance, including the use of sentinel clinical laboratories in some states and some variation in the isolation procedures. However, these limitations are not likely to be associated with an increased (or decreased) likelihood of selecting antimicrobial-resistant isolates for submission to NARMS since the antimicrobial resistance pattern of the isolates were not known when the isolates were selected. Lastly, because NARMS *Campylobacter* surveillance was not nationwide and resistance may differ regionally, generalization to the U.S. population should be done with caution.

In summary, we describe the emergence over the last decade of fluoroquinolone-resistant *Campylobacter* infections in the United States. As of 1997, more than half of such infections were domestically acquired. In 1999, fluoroquinolone-resistant *Campylobacter* organisms were present on a substantial fraction of chickens sold at supermarkets in three widely separated locations in the United States. Continuing national surveillance of human infections and prospective national monitoring of the frequency of contamination of poultry at retail would provide useful ongoing information. Clinicians should include macrolides, such as azithromycin, as a first-line treatment of severe *Campylobacter* infections.

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Dr. Gupta is a former EIS fellow of the Foodborne and Diarrheal Diseases Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention, and is now on the faculty at Johns Hopkins University, Division of Infectious Diseases. Her research interests include diarrheal diseases and more recently HIV and coexisting conditions in India and other resource-poor settings.

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Address for correspondence: Amita Gupta, Johns Hopkins University, Division of Infectious Diseases, 1830 East Monument Street, Room 450E, Baltimore, MD 21287, USA; fax: 410-614-8488; email: agupta25@jhmi.edu

Emerging Issues in Infective Endocarditis

Beverley C. Millar* and John E. Moore*

Infective endocarditis, a serious infection of the endocardium of the heart, particularly the heart valves, is associated with a high degree of illness and death. It generally occurs in patients with altered and abnormal heart architecture, in combination with exposure to bacteria through trauma and other potentially high-risk activities involving transient bacteremia. Knowledge about the origins of endocarditis stems from the work of Fernel in the early 1500s, and yet this infection still presents physicians with major diagnostic and management dilemmas. Endocarditis is caused by a variety of bacteria and fungi, as well as emerging infectious agents, including *Tropheryma whipplei*, *Bartonella* spp., and *Rickettsia* spp. We review the evolution of endocarditis and compare its progression with discoveries in microbiology, science, and medicine.

Endocarditis is a noncontagious chronic infection of the valves or lining of the heart, mainly caused by bacteria, although fungi can also be associated with this infection (1). The risk of infection of heart valves in persons predisposed to acquiring infective endocarditis increases with the following conditions: congenital heart disease, rheumatic fever, major dental treatment, open heart surgery, and genitourinary procedures. New evidence is growing that changes in social behavior, such as an increase in the incidence of body piercing, excessive alcohol consumption, and the use of intravenous self-administered illicit drugs may also predispose a susceptible person to an increased risk of acquiring endocarditis. The patient may exhibit any of the following signs and symptoms: fatigue and weakness; weight loss; fever and chills; night sweats; heart murmur; aches and pains; painful nodes in the pads of fingers and toes; red spots on skin of palms and soles; nail abnormalities; swelling of feet, legs, and abdomen; shortness of breath with activity; and blood in the urine. A medical history, physical examination, and echocardiogram are usually performed. Blood samples are usually taken, and the physical and biochemical properties of the blood are investigated. Endocarditis is usually curable provided an early diagnosis is made, and the patient receives the appropriate antimicrobial treatment; the time needed for recovery is approximately 6–8 weeks. The patient generally requires long-term antimicrobial drugs (4–6 weeks), hospitalization, and in some cases, valve replacement. A number of complications may be associated with the disease such as blood clots, stroke, heart rhythm problems, abscesses, and other infections. Infective endocarditis is associated with severe illness and death and generally occurs in patients with altered and abnormal heart architecture who have been exposed to bacteria through trauma and other potentially high-risk activities.

In 1885, Sir William Osler presented three Gulstonian Lectures on the topic of malignant endocarditis, which gave a comprehensive account of the disease and outlined the difficulties in its diagnosis (2). The disease had, in fact, been described by a French Renaissance physician, Jean François Fernel, approximately 350 years previously (3). More than 100 years after Osler's lectures, this serious infection can still remain a diagnostic and therapeutic dilemma. Its name has been changed several times, first to "bacterial endocarditis" and subsequently to "infective endocarditis" after the observation that microbiologic agents other than bacteria may cause the disease. In the early years of the new millennium, infective endocarditis still proves to be difficult to diagnose and is associated with a high death rate (21%–35%). Although many developments have taken place with respect to antimicrobial drug therapy in the treatment of the disease, its incidence is continuing to rise, with 3.3 cases per 100,000 population per year in the United Kingdom, with similar figures for the United States and 1.4–4.0 cases per 100,000 population per year in Europe as a whole (4). The reasons for this rise are the following: 1) longer survival of patients with degenerative heart diseases, 2) increased use of antibiotics, 3) increased incidence of prosthetic heart valves, 4) congenital heart disease in younger children, 5) increase in bicuspid valve disease, 6) advances in medical and surgical treatments, 7) increase in the number of injection drug

*Belfast City Hospital, Belfast, Northern Ireland, United Kingdom

users, and 8) more sensitive and specific diagnosis. Generally, the incidence is higher in men than in women (2:1), and the average age group affected is in the fifth decade (2).

Historical Perspective

A historical description of developments in endocarditis closely reflects concurrent developments in laboratory medicine, particularly microbiology. Much of the innovations and developments relating to infective endocarditis were made by physicians in Europe, particularly in France (Appendix). Important contributions were, however, made by several German physicians, particularly in association with the birth of bacteriology (Appendix). More recently, the United States has played a strong role in helping define guidelines and diagnostic criteria that facilitate diagnosing infective endocarditis, including the Beth Israel (5), Duke (6) (Table 1), and modified Duke criteria (7,8) (Table 2). In addition, the American Heart Association has published several seminal articles on the antibiotic treatment and prevention of infective endocarditis (9).

For approximately the first 200 years after the disease was initially described, the anatomy of the heart and heart valves in the diseased state of infective endocarditis was comprehensively elucidated in medical anatomical sketches made after postmortem examination. (For a comprehensive account of the early description of endocarditis, see Contrepois [10].) Not until the early to mid-1800s were descriptions recorded of the medical signs and symptoms of the disease in live patients. Such descriptions included the detection of cardiac murmurs, after percussion and auscultation. Detection of such murmurs was aided by the development of the stethoscope in 1816. From 1830 to 1840, elevated body temperature was recorded as an important symptom of the disease. However, not until the late 1800s and early 1900s was a comprehensive synthesis of information formed by various scholars in Europe and North America, including Sir William Osler in Canada (2) and Thomas Horder in England (11) (Appendix). Osler and Horder were instrumental in establishing fundamental mechanisms regarding the pathophysiology of infective endocarditis and are, to a large degree, responsible for how

Table 1. Original Duke criteria for the diagnosis and classification of infective endocarditis[a]

Major criteria	Minor criteria	Diagnosis
1. **Positive blood culture** i) Typical organism in ≥2 blood cultures in the absence of a primary focus (*Staphylococcus aureus*, enterococci, viridans streptococci, *Streptococcus bovis*, HACEK) ii) Persistently positive blood culture drawn more than 12 h apart or all ¾ drawn at least 1 h apart between first and last	1. **Predisposition** Heart condition Drug abuse	1. **Definite** 2 Major 1 Major and 3 minor 5 Minor pathologic/histologic findings
2. **Evidence of endocardial involvement** i) Positive echocardiogram (TOE) Oscillating intracardiac mass on valve, implanted material or supporting structures in path of regurgitant jets Abscess New partial dehiscence of prosthetic valve ii) New valvular regurgitation	2. **Fever** >38°C	2. **Possible** Findings fell short of the definite but not rejected categories
	3. **Vascular phenomena** Major arterial emboli Janeway lesions Septic pulmonary infarcts	3. **Rejected** Alternate diagnosis Resolution of the infection with antibiotic therapy for ≤4 days No pathologic evidence after antibiotic therapy
	4. **Immunologic phenomena** Osler's nodes Roth spots Rheumatoid factor Glomerulonephritis	
	5. **Microbiologic evidence** Positive blood culture not meeting major criteria Positive serologic finding	
	6. **Endocardiographic evidence** Consistent with infective endocarditis but not meeting the major criteria	

[a]Source: (6); HACEK, *Haemophilus aphrophilus*, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae* group; TOE, transesophageal echocardiogram.

Table 2. Recent suggested modifications to the Duke criteria for the diagnosis of infective endocarditis (IE)[a]

Microbiologic	Biochemical	Clinical
Blood culture Bacteremia due to *Staphylococcus aureus* should be considered a major criterion regardless of whether the infection is nosocomially acquired or whether a removable source of infection is present Serology Positive for *Coxiella burnetii* (major criterion) Positive for *Bartonella* spp. Positive for *Chlamydia* spp. Molecular Evidence for the presence of bacterial or fungal DNA in blood or valve material (major criterion)	Elevated level of CRP >100 mg/L Elevated ESR defined as more than one and a half times higher than normal, i.e. , >30 mm/h for patients <60 years of age >50 mm/h for patients >60 years of age	Possible endocarditis now defined as one major and one minor criterion or three minor criteria Omission of criterion "echocardiogram consistent with IE but not meeting major criterion" Newly diagnosed clubbing Evidence of splinter hemorrhages Petechiae Microscopic hematuria (disregarded for patients with positive urine cultures, menstruating women, patients with end-stage renal disease and patients with urinary catheters) Presence of central nonfeeding venous lines or peripheral venous lines (minor) Purpura

[a]Sources: (7,8); CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

we view endocarditis today. The Figure (online only; available at: http://www.cdc.gov/ncidod/EID/vol10no6/03-0848-G..htm) and Appendix chronologically map the history of infective endocarditis, including diagnostic developments, treatment, and prevention, and emerging causal agents.

The birth of bacteriology as a separate discipline of pathology gave rise to the introduction of the important description of microbiology in the etiology of infective endocarditis. With the early technical innovations of Pasteur in France in the 1880s, routine blood cultures were introduced in the late 19th century as an important part of laboratory investigation into the microbiologic causes of infective endocarditis. Although causal agents of infective endocarditis could now be detected and clearly described, little could be achieved in terms of their eradication because the existence of antibiotics was as yet unknown. However, in Germany, Gerard Domagk, bacteriologist and pathologist, was appointed as director of the I.G. Farbenindustrie (Bayer) Laboratory for Experimental Pathology and Bacteriology in Wuppertal in 1925. Domagk was innovative in that he began to experiment with dyes, looking for their possible effects against various infections. He described the effect of prontosil red against streptococcal infections in mice; the active component of prontosil was later described as sulfanilamide. At approximately the same time, Sir Alexander Fleming discovered the antibacterial effects of a secondary metabolite (penicillin), produced from a filamentous fungus. Such discoveries were revolutionary because medicine now had an effective means of treating bacterial infections, including infective endocarditis, caused by a wide variety of bacterial pathogens, most notably *Streptococcus* species. Since wild-type pathogens had not had sufficient time to develop resistance to these newly described antimicrobial agents, treatment failures due to resistance were infrequent. Fleming did observe, however, that some organisms were resistant to penicillin and suggested that the phenomenon be followed up. Approximately 60 years later, the marked increase in resistance to antimicrobial agents is cause for concern on all continents. The tangible consequence is that clinicians may have fewer antimicrobial agents to treat both benign and serious infections, including infective endocarditis. To combat the threat of such a "postantibiotic era," the global pharmaceutical industry has responded by producing novel antimicrobial agents, including the carbapenems (imipenem and ertapenem), the oxozolidones (linezolid), and improved antifungal agents (caspofungin and voriconazole), which prolong antimicrobial effectiveness before the problem of resistance evolves with such new agents.

Over the past century, streptococci and staphylococci have remained the main causative organisms associated with infective endocarditis, with an increase in cases due to staphylococci associated with injection drug users and HIV patients. With substantial advances made in the isolation and identification of microorganisms, scientists now recognize a wide spectrum of causal organisms. Although rare, infective endocarditis is caused by gram-negative organisms such as the HACEK (*Haemophilus aphrophilus*, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, *Kingella kingae*) group, *Bartonella* spp., and *Coxiella burnetii*. More recently, cases of fungal endocarditis have increased, particularly in postoperative patients, injection drug users, and immunocompromised patients (4).

A history of rheumatic fever can serve as a risk factor for acquiring infective endocarditis. The incidence of rheumatic fever, which was common as recently as a century ago, is relatively rare today (12). This decline in the incidence of rheumatic fever has not been mirrored by a pro rata decrease in the incidence of infective endocarditis,

which suggests that additional etiologic factors are becoming more important in acquiring endocarditis.

Current Trends and Future Concerns

Although endocarditis has been documented for approximately 450 years, the diagnostic challenges and treatment dilemmas are as real today as they were in the time of Fernel (3). Major advances have been made in the diagnosis of endocarditis, in both laboratory and clinical (imaging) parameters, but we are witnessing the emergence of several newly described causal bacterial species, such as *Tropheryma whipplei* and *Bartonella* spp., as well as sporadic case reports of unusual and uncommon causal organisms, including *Finegoldia* sp., *Gemella* spp., and *Abiotrophia defectiva*. In addition, since diagnostic methods, mainly 16S rDNA polymerase chain reaction (PCR) and sequencing, are now beginning to identify such infections, no evidence base exists to help determine effective antimicrobial drug regimens to successfully treat endocarditis caused by such organisms. Furthermore, as specimens from many of these infections are culture-negative, conventional antibiotic susceptibility testing does not help the cardiologist decide on the most suitable antimicrobial drug regimens. Another current concern is that we may be returning to a time in which we are largely unable to successfully treat simple infections from panresistant organisms, a scenario that some have described as the postantibiotic era. Indeed, in Northern Ireland, we have now witnessed our first cases of penicillin-resistant pneumococcal meningitis and endocarditis. The increasing incidence of congenital heart disease in children and changing social trends accentuate risk factors for endocarditis.

Endothelial cell dysfunction, resulting from a combination of atypical mechanical forces due to altered cardiac architecture and microbial infection, may lead to an episode of infective endocarditis. Because the endothelium helps regulate vascular tone, inflammation, thrombosis, and vascular remodeling, any insult to the host endothelium may result in infective endocarditis, in which the valves may show changes in the synthetic, morphologic, and metabolic functions of the valvular endothelial cells (13).

Cases in Well-known Persons

Although a relatively uncommon infection, infective endocarditis has been the primary cause of death of several well-known persons, particularly those involved with the arts. One of the late 19th and early 20th century's most influential composers, Gustav Mahler (1860–1911), died from streptococcal endocarditis (10,14). The first sign of valvular problems was observed in 1907, where a compensated mitral contraction was noted. For the next 3 years, he showed little evidence of symptoms of valvular disease until late 1910, when he spent Christmas and the New Year's holiday nursing a sore throat. He was in New York City where he conducted a Philharmonic Orchestra concert on January 17, including the first performance of a revised version of his fourth symphony. On February 24, he became ill with endocarditis, initially diagnosed as influenza. He was attended by one of the most prominent physicians in the city, Emanuel Libman, an important exponent of the value of bacterial blood cultures. Libman demonstrated the presence of viridans streptococci in a large volume (200 mL) of blood drawn from Mahler. Mahler's initial treatment consisted of a "serum treatment" of the times, as well as Metchnikoff's Bulgarian Milk. The latter treatment appeared to work, until early May when blood cultures returned positive with viridans streptococci. The endocarditis was now very marked, with septic abscesses beginning to appear in other parts of his body. On May 18, Mahler died. His untimely death prevented society from hearing him conduct a completed version of his tenth symphony as well as his own opportunity to hear the first public performance of his ninth symphony, which took place on June 26, 1912, by the Vienna Philharmonic Orchestra.

Ottorino Respighi (1879–1936) was an Italian composer who died at the age of 57 from endocarditis. The first signs of Respighi's endocarditis were noted in late 1935, when he was working on his opera Lucrezia; at that time, he was observed to be extremely fatigued, but the cause was unknown (14). In January 1936, *S. viridans* endocarditis was noted when this organism was isolated from his blood. Although sulfonamide drugs were dispatched from Berlin for his treatment, the treatment was unsuccessful, possibly due to the advanced stages of sepsis.

One of Scotland's most famous poets, Roberts Burns (1759–1796), perhaps best known for writing Auld Lang Syne, also had infective endocarditis. He died in July 1796 at the age of 37 years (15). Some historians claim that Burns's work in his teenage years on his father's tenant farm in southwest Scotland did the primary damage to his health. However, Burns's history of rheumatic fever likely predisposed him to infective endocarditis. Burns was attended medically by William Maxwell (1760–1834), who described Burns's symptoms as "flying gout" and prescribed sea-bathing in country quarters and horse riding, so-called cures that probably hastened Burns's death. However, Burns's affinity for alcohol may have contributed to the suppression of his immune system, thus hastening the illness and ultimately his death.

One of the most famous physicians to die of endocarditis was Alois Alzheimer (1864–1915). Alzheimer is most widely known for his description of an "unusual disease of the cerebral cortex," which affected a woman in her fifties, causing memory loss, disorientation, hallucinations, and

ultimately her death at age 55. The disease was named after him by his senior mentor at the Munich Medical School, Emil Kraepelin. Alzheimer was also cofounder and copublisher of the journal Zeitschrift für die gesamte Neurologie und Psychiatrie. Alzheimer's last position was professor of psychiatry at the University of Breslau (now Wroclaw, Poland), which he held for the last 3 years of his life. Historians report that a severe cold was the beginning of Alzheimer's final illness, but endocarditis was responsible for his death at the age of 51 years (16).

Orville Gibson, guitar manufacturer (1856–1918), was another musician who died from endocarditis (17). Gibson's patent contained his ideas for the construction of a mandolin with a carved top and back and with sides, which were constructed from a solid section of wood rather than from thin strips. In 1902, Gibson's physical and mental health began to fail, and he had a history of poor health until 1911. He returned to the St. Lawrence State Hospital, Ogdensburg, New York, in August 1916, a psychiatric center. On August 21, 1918, Gibson died of endocarditis while a patient in the institution.

Rudolph Valentino (1895–1926), a famous actor of the silent screen, also had endocarditis, which also led to his death (18). Valentino had a perforated gastric ulcer closed on August 15, 1926; however, he died from endocarditis on August 23, 1926, at the age of 31 years.

More recently, endocarditis has been described as the cause of death for John Glascock (1951–1997), the recording bass player with the rock band Jethro Tull. Glascock had a tooth abscess, which was believed to be the site of entry for an infectious agent that caused endocarditis. Endocarditis developed in Brian Littrell (1975–), singer with the Backstreet Boys, at the age of 5 years (he was born with a ventricular septal defect, although surgery was not recommended at the time) (19). Brian was admitted to St. Joseph's Hospital, Lexington, Kentucky, where he received extensive intravenous therapy. Endocarditis also developed in a young American actor, Sebastian Hitzig, after he accidentally stepped on a toothpick contaminated with *Staphylococcus aureus*.

In conclusion, considering infective endocarditis to be an "emerging" problem in the 21st century may seem unusual, given that the illness has been well documented over the last 450 years. However, such emergence can be attributed to several factors: 1) the emergence of antimicrobial resistance in classic infective endocarditis microflora, namely, the gram-positive cocci; 2) the existence of antimicrobial resistance in complex ecologic biofilms; 3) the changing pattern of causal agents now regarded as important pathogens of infective endocarditis, e.g., *Bartonella* spp., *T. whipplei*, and fungi; and 4) changing epidemiologic trends of persons who acquire infective endocarditis, including injection drug users, persons with HIV/AIDS, children with congenital heart defects, and persons undergoing body piercing. Furthermore, the way we provide inpatient medical care has also been associated with the emergence of nosocomial infective endocarditis, which can result from invasive procedures such as catheterization, although no cardiac surgery has been performed. The next 100 years will likely witness the emergence of even more changing trends of infective endocarditis, which as yet have not been well recognized.

Although this "old" disease has evolved over the last 450 years, diagnostic and treatment options have developed in tandem, and the prognosis of this disease has markedly improved. However, the emergence of novel etiologic agents, changing social trends, and increased antimicrobial resistance have allowed this disease to remain evasive, which will require new approaches, particularly relating to treatment options in the future.

Appendix. Chronology of important scientific and medical events in the history of infective endocarditis[a]

Year	Scientist/physician, Country	Major findings
1554	Jean François Fernel, France	Earliest report of endocarditis in book Medicini
1669	Richard Lower, England	Accurately described tricuspid valve endocarditis
1646	Lazarus Riverius, France	Described unusual "outgrowths" from autopsy of patient with endocarditis; detected murmurs by placing hand on patient's chest
1708	Giovanni Maria Lancisi, Italy	Described unusual structures in entrance of aorta
1715	Raymond Vieussens, France	Described abnormality in aortic mitral valve
1749	Jean-Baptiste Sénac, France	Described valvular lesions
1769	Giovanni Battistu Morgagni, Italy	Linked infectious disease and endocarditis; observed association with the spleen
1784	Eduard Sandifort, France	Accurately drew intracardiac abnormalities
1797	Matthew Baillie, England	Showed relationship between rheumatism and heart disease
1799	Xavier Bichat, France	Described inflammatory process associated with endocarditis
1806	Jean Nicholas Corvisart, France	Described unusual structures in heart as "vegetations," syphilitic virus as causative agent of endocarditis, and theory of antiviral treatment of endocarditis
1809	Allan Burns, England	Indicated vegetations were not "outgrowths" or "buds" but particles adhering to heart wall
1815	Friedrich Kreysig, Germany	Elucidated inflammatory processes associated with endocarditis
1816	Théophile Laënnec, France	Invented cylindrical stethoscope to listen to heart murmurs; dismissed link between venereal disease and endocarditis

Appendix continued. Chronology of important scientific and medical events in the history of infective endocarditis

Year	Scientist/physician, Country	Major findings
1832	James Hope, England	Confirmed Laënnec's observations
1835–40	Jean-Baptiste Bouillaud, France	Named endocardium and endocarditis; described symptoms; prescribed herbal tea and bloodletting as treatment regimen; described link between acute rheumatoid arthritis and endocarditis
1852	William Senhouse Kirkes, England	Described consequences of embolization of vegetations throughout body. Described cutaneous nodules (named "Osler's nodes" by Libman)
1858–71	Rudolph Virchow, Germany	Examined fibrin vegetation associated with endocarditis by microscope; coined term "embolism;" discussed role of bacteria, vibrios, and micrococci in endocarditis
1861	Jean-Martin Charot, France	Confirmed Virchow's theory on emboli
1861	Alfred Vulpian, Germany	Confirmed Virchow's theory on emboli
1862	Etienne Lancereaux, France	Described granulations or foreign elements in blood and valves, which were motile and resistant to alkalis
1868–70	Samuel Wilks, England	Described infected arterial blood as originating from heart; proposed scarlet fever as cause of endocarditis
1869	Emmanuel Winge, Norway	Established "parasites" on skin transported to heart and attached to endocardium; named "mycosis endocardii"
1872	Hjalmar Heiberg, Norway	Detected microorganisms in vegetations of endocarditis
1878	Edwin Klebs, Germany	All cases of endocarditis were infectious in origin
1878	Ottomar Rosenbach, Germany/ Poland	Combined experimental physiology and infection to produce animal model of endocarditis in rabbit; noted valve had to be damaged before bacteria grafted onto valve
1878	Karl Koester, Germany	Micrococci enter vessels that valves were fitted into; valves exposed to abnormal mechanical attacks over long period created favorable niche for bacterial colonization
1879	Joseph Hamburg, Germany	Virchow's student; employed early animal model of endocarditis
1879	Germain Sée, France	Proposed etiology of endocarditis was based on infectious model and treatment should focus on eliminating "parasitic infection"
1880	Jacques Doleris, France	Working with Pasteur, proposed use of routine blood cultures
1881–86	Arnold Netter, France	Believed endocarditis could appear during various infections; noted translocation of respiratory pathogen from pulmonary lesion to valve through blood
1883	Michel Peter, France	Believed microorganisms were result, not cause, of endocarditis
1884	Joseph Grancher, France	Named disease "infective endocarditis"
1886	Valimir Wyssokowitsch and Johannes Orth, Germany	Demonstrated various bacteria introduced to bloodstream could cause endocarditis on valve that had previous lesion
1885	Sir William Osler, Canada	Synthesized work of others relating to endocarditis
1899	Hermann Lenhartz, Austria	Described streptococcal, staphylococcal, pneumococcal, and gonococcal endocarditis
1903	Hugo Schottmüller, Germany	First described "endocarditis lenta"
1909	John Alexander Mullen, Canada	Credited by Osler as first to observe cutaneous nodes (named "Osler's nodes" by Libman) in patients with endocarditis
1909	Sir Thomas Horder, England	Analyzed 150 cases of endocarditis and published diagnostic criteria relating to signs and symptoms
1910	Emmanual Libman, USA	Described initial classification scheme to include "subacute endocarditis," with clinical signs/symptoms; absolute diagnosis required blood cultures
1981	Von Reyn, USA	Described Beth Israel criteria based on strict case definitions
1994	David Durack, USA	New criteria utilizing specific echocardiographic findings
1995	American Heart Association, USA	Antibiotic treatment of adults with infective endocarditis caused by streptococci, enterococci, staphylococci, and HACEK[a] microorganisms
1996	Pierre Fournier, France	Modified Duke criteria to allow serologic diagnosis of *Coxiella burnetii*
1997	American Heart Association, USA	Guidelines for preventing bacterial endocarditis
1997	Lamas and Eykyn, UK	Suggested modifications to Duke criteria for clinical diagnosis of native valve and prosthetic valve endocarditis: analysis of 118 pathologically proven cases
1998	Working Party of the British Society for Antimicrobial Chemotherapy, UK	Guidelines for antibiotic treatment of streptococcal, enterococcal, and staphylococcal endocarditis
1998	Endocarditis Working Group of the International Society for Chemotherapy, Europe	Antibiotic treatment of infective endocarditis due to viridans streptococci, enterococci, and other streptococci; recommendations for surgical treatment of endocarditis
2000	Jennifer Li, USA	Updated and modified Duke criteria
2002	Beverley C. Millar, UK	Modified Duke criteria to include a molecular diagnosis of causal agents (20)
2001–2003	Didier Raoult, France	Described etiology of *Bartonella* spp., *Tropheryma whipplei*, and *Coxiella burnetii* in endocarditis

[a]HACEK, *Haemophilus aphrophilus, Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens, Kingella kingae* group, *Bartonella* spp., and *Coxiella burnetii*.

Dr. Millar is a clinical scientist in molecular medical microbiology at the Northern Ireland Public Health Laboratory, Belfast City Hospital. She has an active research interest in the molecular diagnosis of infectious diseases, in particular, endocarditis.

Dr. Moore is a clinical scientist in medical microbiology at the Northern Ireland Public Health Laboratory, Belfast City Hospital. His research interests include the application of molecular techniques to aid in patient management, particularly of those with cryptosporidiosis, campylobacteriosis, and cystic fibrosis.

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Address for correspondence: John E. Moore, Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast, BT9 7AD, Northern Ireland, UK; fax: +44-28-2589-2887; email: jemoore@niphl.dnet.co.uk

SARS Exposure and Emergency Department Workers

Wei-Tien Chang,* Chuan-Liang Kao,* Ming-Yi Chung,* Shyr-Chyr Chen,* Shou-Ju Lin,* Wen-Chu Chiang,* Shey-Ying Chen,* Chan-Ping Su,* Po-Ren Hsueh,* Wen-Jone Chen,* Pei-Jer Chen,* and Pan-Chyr Yang*

Of 193 emergency department workers exposed to severe acute respiratory syndrome (SARS), 9 (4.7%) were infected. Pneumonia developed in six workers, and assays showed anti-SARS immunoglobulin (Ig) M and IgG. The other three workers were IgM-positive and had lower IgG titers; in two, mild illness developed, and one remained asymptomatic.

The first case of severe acute respiratory syndrome (SARS) in Taiwan was reported from the National Taiwan University Hospital (NTUH) in mid-March 2003 (1). An infected businessman returning from mainland China was the source of a cluster of infections involving his family and a physician. Thereafter, a number of sporadic cases or small outbreaks emerged in the following month, mostly imported from abroad.

A tertiary university medical center in metropolitan Taipei, NTUH was responsible for most SARS screening during this time. Many patients with symptoms or signs of SARS were transferred to the emergency department of NTUH for evaluation and management. After April 20, 2003, the number of SARS patients increased markedly because of outbreaks in two hospitals in Taipei. During the epidemic in Taiwan, >2,000 febrile patients visited the emergency department of NTUH, and laboratory-confirmed SARS was diagnosed in 79 of them. All 79 patients tested positive for anti-SARS immunoglobulin (Ig) G by using a commercial immunofluorescent assay (IFA) (EUROIMMUN Anti-SARS-CoV-IIFT, Lübeck, Germany), and 25 of them also had positive results of reverse transcriptase–polymerase chain reaction on two separate respiratory samples (sputum or throat swabs) or one respiratory sample and one nonrespiratory (urine or stool) sample. After exposure to SARS, fever or diarrhea occurred in many emergency department workers, and 13 of them were admitted to the hospital. On May 12, the emergency department of NTUH was closed; it was reopened on May 26, when all personnel had no indications of disease after >10 days of isolation.

The Study

Clinical symptoms and signs (fever, cough, headache, sore throat, rhinorrhea, and diarrhea), which developed in the 193 healthcare workers working at the emergency department of NTUH from March 30 to June 30, were retrospectively evaluated through a formal questionnaire. Two IFA methods (in-house and EUROIMMUN) for detecting IgG, IgM, and IgA, and a direct enzyme-linked immunosorbent assay (ELISA) (SARS-96[TNB], General Biologicals Corp, Hsin-Chu, Taiwan) for detecting IgG against the SARS–associated coronavirus (SARS-CoV) were performed on serum specimens from all of these workers. For in-house IFA, whole-cell lysate of infected Vero E6 cells was used as an antigen. Spot slides were prepared by applying the suspension mixed with Vero E6 cells infected with SARS-CoV (TW1 strain, GenBank accession no. AY291451) and uninfected cells. Slides were dried and fixed in acetone. The conjugates used were goat antihuman IgG, IgM, and IgA conjugated to fluorescein isothiocyanate (Organon Teknika-Cappel, Turnhout, Belgium). The starting dilution of serum specimens was 1:25 for the in-house IFA and 1:10 for the EUROIMMUN kit. Before IgM and IgA were determined by IFA, antibodies of class IgG were removed from the patient's serum by antihuman IgG by using two immunoabsorption kits: EUROSORB (EUROIMMUN) for commercial IFA and GULLSORB (Meridian Bioscience Inc., Cincinnati, OH) for the in-house assay. The cutoff values for a positive result for IgG, IgM, and IgA were all 1:25 by in-house IFA and 1:10 by the commercial IFA kit. IgG against SARS-CoV by an indirect ELISA was also performed by using recombinant nucleocapsid as the coated antigen. The cutoff value of IgG by ELISA was 0.26.

As control sera, we used 200 paired samples from patients with community-acquired pneumonia seen at NTUH from October 2001 to December 2002, 70 serum samples from hospitalized patients with acute respiratory distress syndrome treated in 2002 at the hospital, and 10 serum samples obtained from 10 pregnant women during routine pre-labor check-ups in 2002. All control serum specimens were negative for IgG by ELISA and IgG, IgM, or IgA by two IFA methods.

Reverse transcriptase–polymerase chain reaction (RT-PCR) assays (nested and real-time) for respiratory specimens (throat swabs and sputum) and serum were performed for the six workers in whom pneumonia developed. Cases of laboratory-confirmed SARS in workers were further classified as asymptomatic, mild, or severe, according to the criteria provided by the Centers for Disease Control and Prevention (2).

The 193 workers included 54 men and 139 women, with a mean age of 32.7 ± 8.2 years. They included 33 physicians, 95 nurses, 17 radiology technicians, 16 clerks,

13 sanitation workers, 13 administrative personnel, and 6 ambulance drivers. From March 30 to June 30, 2003, 45 (25.4%) of these workers reported feeling feverish, 51 (26.4%) had cough, 47 (24.4%) had myalgia, 60 (31.6%) had headache, 54 (28.0%) had sore throat, 41 (21.2%) had rhinorrhea, and 52 (26.9%) had diarrhea. From May 8 to May 20, 13 of these workers were consecutively admitted and isolated; these included 3 physicians, 7 nurses, 2 sanitation workers, and 1 clerk. Six (3.1%, 6/193) of these 13 patients, including 3 nurses, 2 sanitation workers, and 1 clerk, met the criteria for severe SARS caused by pneumonia, positive RT-PCR results for respiratory specimens and serum samples (5 patients), and positive antibody responses. These six patients had a date of disease onset between May 10 and May 17. They were also positive for IgM and IgA and had high titers of specific IgG (1:800 to 1:3,200) against SARS-CoV (Figure part A). The IgG antibody titers remained high for ≥150 days after illness onset. Two of the three physicians (patients A and B) had positive results for both IgM and IgG, although they both had only transient fever (38°C for <1 day) and chills without any respiratory illness (rhinorrhea, sore throat, cough, or dyspnea) or gastrointestinal symptoms (nausea, vomiting, or diarrhea); transient fever developed on May 11 in patient A and on May 12 in patient B. Their leukocyte and lymphocyte counts and liver function were normal. The IgG antibody titers in these two patients were low (1:25 to 1:100) and disappeared rapidly (52 days and 148 days, respectively, after onset of illness; Figure part B) compared with those of patients with severe SARS, in whom IgG is still present as of this writing. RT-PCR studies (Artus, Roche Diagnostics, Hamburg, Germany) of these two patients' serum samples were negative for SARS-CoV. The other five workers who were admitted had negative antibody results; however, one of them (a nurse) was positive (1:640) for antibodies against *Mycoplasma pneumoniae* when the particle agglutination method was used.

All but one (an ambulance driver, patient C) of the 180 workers who were not admitted had negative ELISA and IFA test results on serum samples collected in late June 2003. This worker had normal leukocyte and lymphocyte counts and did not show any signs or symptoms of respiratory illness. However, he had elevated IgM and IgG titers by IFA, which were both at detectable levels (1:10 and 1:50, respectively) on May 17. The IgM titer remained positive in serum samples collected on June 9 and then became negative on June 30 and was still negative on October 6. Results of RT-PCR studies (Artus, Roche Diagnostics) of the serum, throat swab, and stool specimens from this patient were negative.

Overall, the incidence of SARS-CoV infection among the emergency department workers in this hospital was 4.7% (9/193), including 6 (3.1%) with severe SARS, 2 (1.0%) with mild SARS, and 1 (0.6%) who was asymptomatic. The incidence of SARS-CoV infection was highest in ambulance drivers (16.7%), followed by sanitation workers (15.4%), clerks (6.3%), physicians (6.1%), and nurses (3.2%).

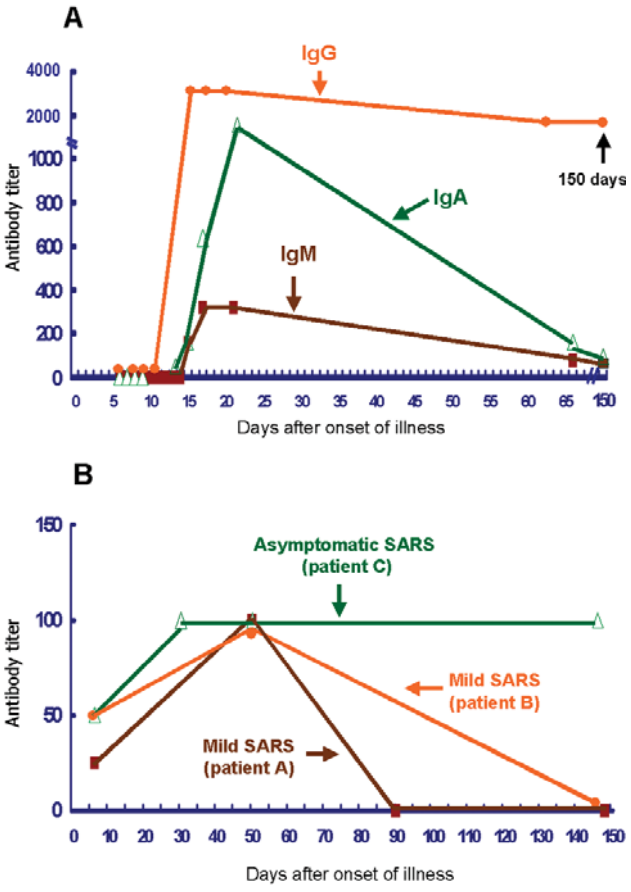

Figure. Changes over time in levels of antibodies against severe acute respiratory syndrome–associated coronavirus (SARS-CoV) in patients with laboratory-confirmed SARS. A denotes the changes in immunoglobulin (Ig) G, IgM, and IgA titers for a representative patient with severe SARS. B denotes the changes in IgG for two patients with mild SARS and one asymptomatic worker with SARS-CoV infection. The date of illness onset for patient C was assumed to be May 12, 2003 (the mean date of eight other SARS patients during the outbreak).

Conclusions

This study illustrates three key aspects of the spread of SARS in an emergency department setting. First, not only the medical personnel but also the paramedical workers were at risk for SARS-CoV infection. Although universal precautions should be strictly followed when staff encounter patients with a variety of symptoms and signs, implementing infection-control measures is more difficult in the emergency department than in the wards or intensive care units, after patients' conditions have been identified.

In fact, emergency department medical staff members have been reported to be at a higher risk for infection than staff members in other hospital departments (3). Second, persons infected with SARS-CoV might manifest only transient febrile illness and minimal respiratory illness or be completely free of any clinical symptoms or signs suggestive of SARS. These findings highlight the possibility that SARS-CoV might produce only mild or asymptomatic infection, although few previous reports have described this form of infection with SARS-CoV (4,5).

Finally, patients with mild or asymptomatic SARS-CoV infection in this study had lower levels (≤1:100) of IgG antibody and earlier seroconversion than those of patients with severe SARS. This finding partly supports the hypothesis that an upsurge of antibody response is associated with increased severity of pulmonary condition (1). However, Lee et al. reported that a nurse with asymptomatic SARS-CoV infection had an IgG antibody titer as high as 1:400; IgG titers on the follow-up serum samples were not reported (4). Li et al. reported two cases of mild SARS, but antibody titers of these two patients were not reported (5). Serologic study of serial serum samples from more persons with mild illness or no symptoms is needed to confirm our findings of lower levels of IgG and earlier seroconversion.

Approximately 30% of emergency department workers without SARS-CoV infection in this study had clinical symptoms and signs similar to those of SARS during this epidemic. These illnesses might have been due to influenza or other upper airway infections; however, differentiating between SARS and other respiratory tract infections in these patients was difficult.

This study not only highlights the presence of mild and asymptomatic infection in healthcare workers during a SARS epidemic but also indicates lower antibody response and earlier seroconversion. Controlling this highly infective emerging disease requires meticulous preparation and vigilance by every worker in the emergency department.

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Dr. Chang is a senior physician in the Department of Emergency Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine. His primary research interest is the epidemiology and pathogenesis of emerging infections.

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Address for correspondence: Po-Ren Hsueh, Department of Laboratory Medicine or Wen-Jone Chen, Department of Emergency Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine, No. 7 Chung-Shan South Road, Taipei, Taiwan, fax: 886-2-23224263; email: hsporen@ha.mc.ntu.edu.tw or jone@ha.mc.ntu.edu.tw

Antibody Prevalence of West Nile Virus in Birds, Illinois, 2002

Adam M. Ringia,* Bradley J. Blitvich,† Hyun-Young Koo,‡ Marshall Van de Wyngaerde,* Jeff D. Brawn,‡ and Robert J. Novak*

Antibodies to West Nile virus were detected in 94 of 1,784 Illinois birds during 2002. Captive and urban birds had higher seropositivity than did birds from natural areas, and northern and central Illinois birds' seropositivity was greater than that from birds from the southern sites. Adult and hatch-year exposure rates did not differ significantly.

West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) was first identified in the Western Hemisphere in 1999 (1) and had been detected in 27 states of the United States by the end of 2001 (2). Despite abundant evidence of avian, mosquito, and mammalian transmission (2), few reports are available on the exposure of live birds to WNV outside of New York and New Jersey.

WNV activity was first detected in Illinois in September 2001 (3). During 2001, its distribution was limited to seven counties, primarily in northeastern Illinois (3). In 2002, however, Illinois had the greatest number of human WNV cases in the country (884 cases, 66 deaths) as well as reports of WNV infections in mammals, mosquitoes, and dead birds from all but two counties (3). Prior to and concurrent with this outbreak, we collected blood samples from both wild and domestic birds to compare exposure rates among species, geographic regions, and urban and natural habitats.

The Study

Wild birds were collected from 43 study sites in Illinois (Figure 1) from February through December 2002 by using standard methods (4). Sites were classified as urban (agricultural, industrial, and residential), natural (forested areas, woodlots, and wetlands), or captive (locations where birds were confined). All captured birds were identified to species and, when possible, by sex and as adult or hatch-year (5,6). Before release, all birds were marked with fingernail polish on the tarsus and retrices to prevent repeated sampling of the same bird within a short period. Captive birds were collected from six study sites (one northern, three central, and two southern locations).

Serum samples were tested for antibodies to WNV by epitope-blocking enzyme-linked immunosorbent assay (ELISA), according to the protocols of Blitvich et al. (7). ELISAs were performed with two monoclonal antibodies (MAbs), 3.1112G and 2B2. Recent studies have shown that assays performed with these MAbs detect antibodies to WNV in taxonomically diverse North American avian species (7). Furthermore, assays performed with MAb 3.1112G discriminate between WNV and St. Louis encephalitis virus infections in birds. The ability of the Illinois bird sera to block the binding of the MAbs to WNV antigen was compared to the blocking ability of normal chicken serum (Vector Laboratories, Burlingame, CA). The percentage inhibition value was calculated as previously described (7). Any serum sample that blocked the binding of both MAbs by $\geq$30% was considered positive for antibodies to WNV. We required a positive result from both MAbs because of our lack of access to plaque reduction neutralization testing.

Serum samples were collected from 1,784 birds, representing 10 orders and 81 species. In total, 94 birds, representing 5 orders and 19 species, were positive for antibodies to WNV (Table 1). The overall exposure rate for the year was 5.3%. The species with the highest seroposi-

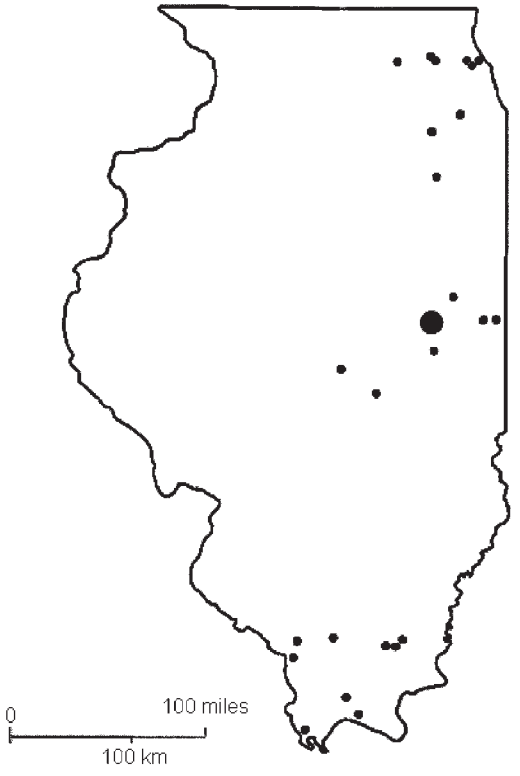

Figure 1. Locations of the study sites in the avian serologic survey for West Nile virus infection, Illinois, 2002.

*Illinois Natural History Survey, Champaign, Illinois, USA; †Colorado State University, Fort Collins, Colorado, USA; and ‡University of Illinois, Champaign, Illinois, USA

Table 1. Birds, listed alphabetically by order, tested for WNV antibody in Illinois in 2002, including number of birds positive and number tested[a]

Order	Common name	No. tested	No. WNV-positive (%)	95% CI
Anseriformes	Canada Goose	253	3 (1.2)	0.3 to 3.4
	Wood Duck	120	3 (2.5)	0.5 to 7.1
	3 additional species	35	0	
Columbiformes	Mourning Dove	11	1 (9.1)	0.2 to 41.3
	Rock Dove[b]	20	11 (55.0)	31.5 to 76.9
Galliformes	Chukar[b]	22	6 (27.3)	10.7 to 50.2
	Domestic Chicken[b]	63	5 (7.9)	2.6 to 17.6
	2 additional species	16	0	
Passeriformes	Cedar Waxwing	5	1 (20.0)	0.5 to 71.6
	Blue Grosbeak	2	1 (50.0)	1.2 to 98.7
	Indigo Bunting	28	1 (3.6)	0.1 to 18.4
	Northern Cardinal	129	16 (12.4)	7.3 to 9.4
	American Crow	157	5 (3.2)	1.0 to 7.3
	Red-winged Blackbird	39	3 (7.7)	1.6 to 20.9
	Brown Thrasher	19	2 (10.5)	1.3 to 33.1
	Gray Catbird	72	6 (8.3)	3.1 to 17.3
	Ovenbird	32	1 (3.1)	0.1 to 16.2
	House Sparrow	185	21 (11.4)	7.1 to 16.8
	American Robin	79	3 (3.8)	0.8 to 10.7
	Swainson's Thrush	32	1 (3.1)	0.1 to16.2
	45 additional species	422	0 (0)	
Strigiformes	Great Horned Owl[b]	9	4 (44.4)	13.7 to 78.8
	2 additional species	3	0	
Other (5 orders)	10 species	31	0	
Total (10 orders)	81 species	1784	94 (5.3)	4.2 to 6.4

[a]WNV, West Nile virus; CI, confidence interval.
[b]Indicates captive specimens.

tivity (>10% and >1 positive sample) were Rock Doves, Great Horned Owls, Chukar, Northern Cardinals, House Sparrows, and Brown Thrashers.

We determined the relative importance of region (north/central/south), habitat (urban/natural), and month of capture to antibody prevalence by using stepwise logistic regression (8; Table 2). This model explains 14% of the variation in antibody prevalence.

Our first seropositive bird was captured on April 26, 2002, in central Illinois. In some birds, immunoglobulin (Ig) M and IgG antibodies are not detectable for 4 to 5 days, and then build to a peak at 7 to 8 days or 3–4 weeks, respectively, before antibody levels start to decline (9). This pattern suggests that transmission in Illinois occurred at least as early as mid-April. However, the specific immune response to WNV is unknown in most bird species.

Avian movement can have a major impact on the measured temporal exposure rates of birds. Although antibody-positive rates increased steadily beginning in August, prevalence decreased during October (Figure 2). This decrease corresponds with the time when many birds are migrating into and through Illinois (10). If these birds are moving from areas of lower transmission, the proportion of antibody-positive birds may have been reduced. Additionally, WNV antibody prevalence was highest in both urban and natural settings in the final collections of 2002, after many of the migrants had moved on. We speculate that the increased rate of seropositive birds in the winter was the result of the changing geographic distribution of birds in the winter rather than continuing winter transmission, despite the rare winter detection of virus in raptors (11).

We compared antibody prevalence by habitat type, geographic region, and age separately using chi-square analysis (Table 3). The prevalence of antibodies to WNV was significantly higher in birds from northern and central Illinois than in those from southern Illinois. The north-south gradient in temperature, vegetation, topography,

Table 2. Logistic regression analysis of Illinois avian WNV antibody prevalence, 2002[a]

Factor	DF	Wald χ^2	p value
Region	2	17.65	< 0.0001
Month	10	44.80	< 0.0001
Habitat (urban/natural)	1	1.29	0.26
Full model	13	78.21	< 0.0001
$r^2 = 0.14$			

[a]WNV, West Nile virus; DF, degrees of freedom.
[b]Dependent variable, presence or absence of WNV antibody.

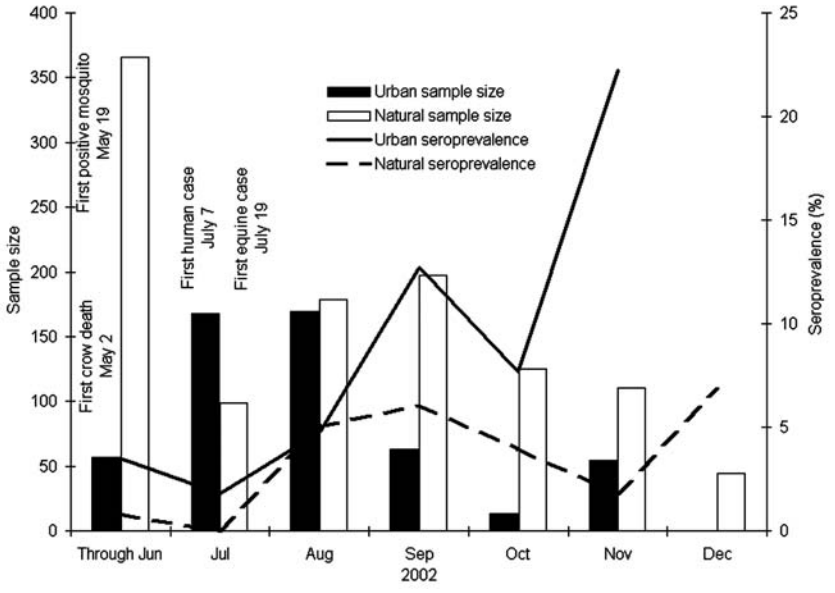

Figure 2. Monthly percentage of West Nile virus antibody–positive birds in Illinois during 2002, with corresponding sample size. First human, mosquito, and equine cases for Illinois are reported for comparison. Bars show the monthly sample size and lines indicate the monthly seroprevalence.

human population density, and land use could all influence regional transmission dynamics. Also, both birds and mosquito species vary across the state (6,12). Any of these factors may contribute to the differences that we report.

Overall, birds from urban areas were more commonly seropositive than birds from natural sites (Table 3). A likely explanation for this result is that *Culex pipiens*, the primary vector of WNV in Illinois, is closely associated with human environments (12). Also, captive species showed higher rates of exposure to WNV than birds in either urban or natural habitats (Table 3). In fact, among the species most frequently infected were Rock Doves, Chukar, and Great Horned Owls, all of which are captive species. Captive birds are housed in unnatural conditions that may facilitate their exposure to WNV infection by increased bird density, increased bird-to-bird transmission from contact with sick or injured birds, or their inability to escape from mosquitoes (13). Many of the serum specimens from Great Horned Owls, for example, were collected from sick birds that had been turned in to wildlife rehabilitators. Therefore, we suspect that the seroprevalence values of WNV in captive birds may not be representative of the infected proportion of those species found in the wild. House Sparrows, Brown Thrashers, and Northern Cardinals, the free-ranging species with the highest antibody prevalence, are all locally abundant birds, which increases the probability of their contact with infected mosquitoes. Although many of the species with high exposure rates are common birds in urban areas (House Sparrows, Cardinals), others (Brown Thrashers, Gray Catbirds) are more frequently associated with natural habitats, which suggests that WNV transmission occurred in both habitat types. Our serologic results and the reservoir competence studies of Komar et al. (14) indicate that members of the families *Cardinalidae* and *Mimidae* are good candidates for reservoir competence testing. We speculate that the variation in seroprevalence is the result of a combination of factors, including defensive behaviors, host preference of mosquitoes, habitat association, and roosting behaviors.

American Crows were rarely seropositive, despite the collection of crows exhibiting WNV symptoms. Several of these crows were subsequently found to be WNV positive on necropsy (RJ Novak, unpub. data), supporting the findings of Komar et al. (14) that American Crows and Blue

Table 3. Differences in West Nile virus seropositivity in birds by age, region, and habitat using chi-square analysis, Illinois, 2002

Comparison		Samples, n (% total); N = 1,784	Antibody + (%)[a]
Habitat	Urban	524 (29.4%)	34 (6.49%)[A]
	Natural	1,121 (62.8)	34 (3.03%)[B]
	Captive	139 (8.2%)	26 (18.71%)[C]
		χ^2=63.06	p < 0.0001
Region	Northern	412 (23.1%)	20 (4.85%)[D]
	Central	796 (44.6%)	62 (7.79%)[D]
	Southern	576 (32.3%)	12 (2.08%)[E]
		χ^2=21.98	p < 0.0001
Age[b]	Adult	455 (25.5%)	10 (2.2%)[F]
	Hatch-year	508 (28.5%)	21 (4.1%)[F]
		χ^2=2.81	p < 0.096

[a]Like capital letter superscripts indicate no difference in pairwise comparisons.
[b]821 of the 1,784 birds had no age recorded at time of collection. The remaining 963 were used for the age analysis.

Jays frequently die 4–6 days postinfection, which is before antibodies are detectable in some species (9). This finding suggests that antibody prevalence may not be correlated with the impact of WNV on population numbers in some species.

We found no significant difference in the proportion of adult and hatch-year birds with antibodies to WNV (Table 3), which supports the finding of Komar et al. (15) that that pattern is normal for virus activity in a new location. We did not detect antibodies to WNV in any birds captured before late April, which suggests that limited or no WNV transmission occurred before or during the winter of 2001 in Illinois.

Although WNV was first reported in Illinois in 2001, statewide WNV activity was not detected until 2002. The mechanisms for both the short- and long-distance dispersal of WNV are not fully understood. Migrating birds are suspected of playing a major role in the long-distance dispersal of WNV into new areas (16). In our collections, we found only one seropositive bird that does not nest or winter in Illinois, a Swainson's Thrush, captured on August 28, 2002.

Conclusions

WNV infections were detected in numerous mosquito pools, dead birds, equines, and >800 humans in Illinois in 2002, with virus activity reported in almost every county (3). However, the overall avian seroprevalence (5.3%) of WNV in the present study was low. Similarly, low WNV infection rates were reported in birds during the New York epizootics of 2000 and 2001 (6.9% and 7.0, respectively; 17,18). However, several species exhibited exposure rates ≥10%.

Our data demonstrate the great diversity of avian species that are susceptible to WNV infection, a finding consistent with earlier studies (19). Although transmission rates and corresponding variation in seroprevalence may be related to defensive behaviors, grouping, or habitat associations, our results show that captive birds and those in urban areas are more likely to be infected than those in natural areas. Dead bird surveillance is typically limited to corvids (Blue Jays and Crows). However, live bird serosurveys clearly demonstrate the broad range of avian species exposed to WNV. The impact of WNV on the illness and death of most of these species remains unknown. Therefore, continued research is required to understand the complex transmission patterns and epidemiologic impact of WNV.

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Mr. Ringia is the avian team leader of the Medical Entomology Laboratory at the Illinois Natural History Survey in Champaign, Illinois. His primary research interests are in the conservation and ecology of birds and reptiles, and, more recently, in the interactions among vectors, hosts, and habitats.

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Address for correspondence: Robert J. Novak, Illinois Natural History Survey, 607 E. Peabody, Champaign, IL 61820, USA: fax: 217-333-2359; email: rjnovak@uiuc.edu

Reference Group Choice and Antibiotic Resistance Outcomes

Keith S. Kaye,* John J. Engemann,* Essy Mozaffari,† and Yehuda Carmeli‡

Two types of cohort studies examining patients infected with methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) were contrasted, using different reference groups. Cases were compared to uninfected patients and patients infected with the corresponding, susceptible organism. VRE and MRSA were associated with adverse outcomes. The effect was greater when uninfected control patients were used.

Although several investigators have performed outcomes studies of patients infected or colonized with antimicrobial resistant bacteria, the design and interpretation of results with various methods has not been discussed (1). Typically, these outcomes studies use a cohort design and study patients infected with resistant bacteria (the exposure of interest for cases), who are compared either to patients without infection selected from a similar population (2–6) or to patients infected with corresponding, susceptible bacteria (e.g., comparing patients with methicillin-resistant *Staphylococcus aureus* [MRSA] to patients with methicillin-susceptible *S. aureus* [MSSA]) (7–13) (Online Appendix, available at http://www.cdc.gov/ncidod/eid/vol10no6/02-0665_app.htm). When cases are compared to an uninfected reference group or "control group," the effect of a new, antimicrobial-resistant bacterial infection is assessed. When case-patients are compared to reference patients or "controls" infected with the corresponding susceptible bacteria, the impact of acquiring a resistance determinant is measured. Both types of comparison are valid and important, but they address different clinical scenarios.

We examined how the choice of the reference group might influence results of outcomes studies pertaining to antimicrobial resistant bacteria. We compared and contrasted the results of outcomes cohort studies for resistant bacteria by using the two different reference groups discussed previously. We used results from original studies of MRSA and vancomycin-resistant enterococci (VRE) (9,10) that initially used one reference group. In our study, we performed additional analyses comparing case-patients to different reference patients and contrasted the results.

*Duke University Medical Center, Durham, North Carolina, USA; †Pfizer, New York, New York, USA; and ‡Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA

The Study

Both MRSA and VRE studies were designed as cohort studies and are discussed in detail elsewhere (9,10) (Online Appendix). Cases were defined as patients with MRSA surgical site infection (SSI) (i.e., the exposure of interest for study 1) and VRE wound infection (i.e., the exposure of interest for study 2). In each study, two different reference groups were used in separate analyses. Control group A included patients who did not have an infection caused by the target pathogen (*S. aureus* or enterococci). Control group B included patients with infection caused by the susceptible phenotype of the target pathogen (i.e., MSSA and vancomycin-susceptible enterococci [VSE]).

In both studies, three outcomes were examined: death, length of hospital stay, and total hospital charges. Hospital charges were variable direct charges obtained from hospital financial databases and are a surrogate for cost. Hospital costs were estimated using a cost-to-charge ratio of 0.7 (14).

Outcomes studies of antimicrobial drug resistance are notoriously hard to perform because of confounding variables related to underlying coexisting conditions (1). To control for confounding, we analyzed several variables, including individual coexisting conditions, the Charlson score, the American Society of Anesthesiologists-Physical Status (ASA) score, and duration of hospitalization before infection (Online Appendix). These variables were analyzed in multivariable analysis. Each of the outcomes was analyzed independently. The inverse log value was calculated for β coefficients of variables included in the predictor models, and these effect measures were described as the odds ratio (OR) for death rate and the multiplicative effects (ME) on length of stay and charges.

In the analysis comparing patients with SSI caused by MRSA to uninfected controls, the study cohort included 314 patients: 121 MRSA SSI cases and 193 uninfected surgical controls (Online Appendix). In multivariable analysis, MRSA SSI was significantly associated with death (OR = 11.4, $p < 0.001$). In the analysis comparing patients with MRSA SSI to patients with SSI caused by MSSA, the same 121 MRSA case-patients were compared to 165 control-patients with MSSA SSI. In multivariable analysis, MRSA SSI was significantly associated with death (OR = 3.4, $p = 0.003$). Additional covariates included in the adjusted models for death are listed in the footnotes of Table 1 and are discussed in the Online Appendix. The

Table 1. Outcomes and adjusted analyses for MRSA for study 1[a]

Outcome	Cases	Controls	Adjusted analyses OR (95% CI)[b]	Attributable to MRSA	p value
Three analyses comparing patients MRSA cases (n = 121) and uninfected controls (n = 193)					
Deaths	20.7%	2.1%	11.4 (2.8 to 34.9)[c]	–	< 0.001
Hospital days after surgery, mean per case	29.1	6.1	3.2 (2.7 to 3.7)[d]	13.4	< 0.001
Charges ($), mean/case	118,414	34,395	2.2 (2.0 to 2.6)[e]	41,274	< 0.001
Three analyses comparing MRSA cases (n = 121) and MSSA controls (n = 165)	(n = 121)				
Deaths	20.7%	6.7%	3.4[f]	–	0.003
Hospital days after infection, mean per case	22.0	13.2	1.2[g]	2.6	0.11
Charges ($), mean per case	118,414	73,165	1.2[h]	13,901	0.03

[a]OR, odds ratio; CI, confidence interval; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*.
[b]Odds ratio for deaths, and multiplicative effect (ME) for continuous outcomes (length of stay and charges).
[c]Adjusted for American Society of Anesthesiologists-Physical Status (ASA) score >3 and age.
[d]Adjusted for ASA score, duration of surgery, hospital, renal disease, diabetes, and length of hospital stay before surgery.
[e]Adjusted for ASA score, hospital, duration of surgery, renal disease, length of hospital stay, and intensive care unit (ICU) stay before surgery.
[f]Adjusted for ASA score >3, duration of surgery, and age.
[g]Adjusted for ASA score, renal disease, diabetes mellitus, hospital, duration of surgery, and length of stay before infection.
[h]Adjusted for ASA score, duration of surgery, length of hospital and ICU stay before infection, hospital, renal disease, and diabetes.

effect of MRSA on deaths was approximately threefold greater for the analysis using uninfected controls than for the analysis using MSSA controls.

In the analysis comparing patients with SSI caused by MRSA to uninfected controls, multivariable modeling demonstrated that MRSA SSI was significantly associated with an increased length of stay (ME = 3.2, $p < 0.001$). Having an MRSA SSI was associated with an average adjusted attributable increase of 13.4 hospital days per case. In the analysis comparing patients with MRSA SSI to controls with SSI due to MSSA, a trend was seen toward an association between MRSA SSI and total hospital days (ME = 1.20, $p = 0.11$). Methicillin resistance was associated with an average adjusted attributable increase of 2.6 days per case, although this did not reach statistical significance. Additional covariates included in the adjusted models for length of stay are listed in the footnotes of Table 1 and are discussed in the Online Appendix. The effect of MRSA on length of stay was approximately threefold greater (11 days) for the analysis using uninfected controls than for analysis B using MSSA controls.

In the analysis comparing patients with SSI due to MRSA to uninfected controls, multivariable modeling showed that MRSA SSI was significantly associated with increased hospital charges (ME = 2.2, $p < 0.001$). MRSA was associated with mean adjusted additional attributable charges of $41,274 per case and an attributable cost of $28,891 per case. In the analysis comparing patients with SSI due to MRSA to controls with SSI due to MSSA, MRSA was significantly associated with increased hospital charges (ME = 1.2, $p = 0.03$). Methicillin resistance was associated with mean adjusted additional attributable charges of $13,901 per MRSA SSI case and an attributable cost of $9,731 per case. Additional covariates included in the adjusted models for cost are listed in the footnotes of Table 1 and are discussed in the Online Appendix. The effect of MRSA on cost was approximately twofold greater ($15,000) for the analysis using uninfected controls than for the analysis using controls with SSI due to MSSA.

In the analysis comparing patients with wound infection due to VRE to uninfected controls, 99 patients with VRE wound infection were compared to 280 matched controls who were not infected with enterococci (Online Appendix). In adjusted analysis, VRE wound infection was not an independent predictor of deaths (OR 2.0, $p = 0.13$). In the analysis comparing patients with wound infection due to VRE to control patients with wound infection due to VSE, the same 99 VRE wound infection cases were compared to 213 control patients with VSE wound infections. In multivariable analysis, VRE was significantly associated with mortality (OR 2.5, $p = 0.04$). Additional covariates included in the adjusted models for death rates are listed in the footnotes of Table 2 and are discussed in the Online Appendix. The magnitude of effect of VRE on deaths was similar for both analyses.

In the analysis comparing patients with wound infection due to VRE to uninfected controls, multivariable modeling showed a significantly longer duration of hospitalization after inclusion in the cohort for VRE cases than for controls not infected with enterococci (ME 1.8, $p < 0.001$, average adjusted attributable increase of 6.2 days in length of stay). In the analysis comparing patients with wound infection due to VRE to control patients with VSE wound infection, length of stay after isolation of enterococci was similar among VRE cases and VSE controls (mean of 15.2 vs. 13.6 days, $p = 0.5$) and the differences in length of stay remained non-significant in multivariate analysis (ME = 1.0, $p = 0.5$). Additional covariates included in the adjusted models for length of stay are listed in the

Table 2. Outcomes and adjusted analyses for vancomycin-resistant enterococci (VRE) in study 2

Outcome	Cases	Controls	Adjusted analyses		
			OR[a]	Attributable to VRE	p value
Three analyses comparing VRE patients (n = 99) and uninfected controls (n = 280)					
Deaths	12.1%	6.1%	2.0[b]	–	0.13
Length of stay (d), mean per case	15.2	8.5	1.8[c]	6.2	<0.001
Charges ($), mean per case	46,660	27,224	1.5[d]	13,884	<0.001
Three analyses comparing VRE patients (n = 99) and vancomycin-susceptible enterococci (VSE) controls (n = 213)					
Deaths	12.1%	6.6%	2.5[e]	–	0.04
Length of stay (d), mean per case	15.2	13.6	1.0[f]	–	0.5
Charges ($), mean per case	46,600	31,915	1.4[g]	12,766	<0.001

[a]Odds ratio for deaths, and multiplicative effect (ME) for continuous outcomes (length of stay and charges).
[b]Adjusted for number of comorbid illnesses and admission to the intensive care unit (ICU).
[c]Adjusted for propensity score (i.e., likelihood of being a VRE case [Online Appendix]), being transferred from another institution, renal disease, malignancy, and admission to the ICU.
[d]Adjusted for propensity score (i.e., likelihood of being a VRE patient), having had surgery before cohort inclusion, and duration of hospitalization before cohort inclusion.
[e]Adjusted for surgery, sex, and admission to the ICU.
[f]Adjusted for duration of hospitalization before cohort inclusion, admission to the ICU, and malignancy.
[g]Adjusted for having had surgery before inclusion in the cohort, and duration of hospitalization before cohort inclusion.

footnotes of Table 2 and are discussed in the Online Appendix. The effect of VRE on length of stay was approximately twofold greater (6 days) for the analysis using uninfected controls than for the analysis that used VSE controls.

In the analysis comparing patients with wound infection due to VRE to uninfected controls, multivariable modeling demonstrated that VRE cases generated significantly greater hospital charges than controls (adjusted ME = 1.5, $p < 0.001$, mean adjusted additional attributable charges of $13,884 per VRE wound infection and attributable cost of $9,719 per infection). In the analysis comparing patients with wound infection due to VRE to controls with VSE wound infection, VRE wound infection was associated with increased hospital charges (ME = 1.4, $p < 0.001$, average adjusted additional attributable charges of $12,766 per infection and attributable cost of $8,936 per infection). Additional covariates included in the adjusted models for cost are listed in the footnotes of Table 2 and are discussed in the Online Appendix. The effect of VRE on cost was similar in both analyses.

Conclusions

We examined how the criteria used to select a reference group (i.e., a comparison or control group for cases) influenced outcomes study results. Two types of control patients were studied, and in both types of analyses, VRE and MRSA were associated with significant, adverse clinical outcomes. In general, the effects (i.e., OR or ME) were of greater magnitude when controls not infected with the target organism (and thus representative of a random sample of the source population) were used. This is logical since analyses using uninfected controls assess the effect of acquiring a new infection and a resistant pathogen. When patients who are infected with a susceptible organism are used as controls, the analysis quantifies only the effect of acquiring a resistance trait.

The differences in results between the two analyses were much greater for the MRSA SSI study than for the VRE wound infection study. The impact on clinical outcomes was two- to threefold greater when patients with MRSA SSI were compared to an uninfected control group as opposed to comparison with control patients infected with MSSA SSI. In contrast, when patients with VRE wound infection were compared to uninfected patients, similar results were obtained as when patients with VSE wound infections were used as controls. We believe that the magnitude of differences in results for the two analyses is directly related to the virulence of the infecting organism (Online Appendix).

The studies were performed in two different geographic locales and by using slightly different analytic methods. While this is a limitation in that cost results are not directly comparable, we feel including these two studies improves the generalizability of our results and strengthens our findings.

For studies of antimicrobial resistance, a reference group must be chosen on the basis of the investigators' objective. From a public health perspective, results from outcomes studies pertaining to antimicrobial resistance are frequently used to help allocate resources for interventions. If the objective of a study is to investigate the independent effects of a resistance trait or phenotype (e.g., methicillin resistance), then the most appropriate control group would consist of patients infected with a susceptible corresponding organism. If the goal is to assess the effect of a new infection caused by a particular pathogen, uninfected control patients would be the preferable comparison group. Alternatively, a complete analysis might include both types of control groups; this analysis would allow the

reader to assess the effect of acquiring a resistance phenotype alone and the impact of acquiring a new infection caused by a resistant bacteria.

Dr. Kaye is an assistant professor of medicine at Duke University Medical Center, where he is director of Hospital Epidemiology and Infection Control and chair of the Antibiotic Evaluation Committee. His research interests include antimicrobial resistance, antimicrobial utilization, selective antimicrobial pressure, surgical site infections, infections in the elderly, and hospital-acquired infections.

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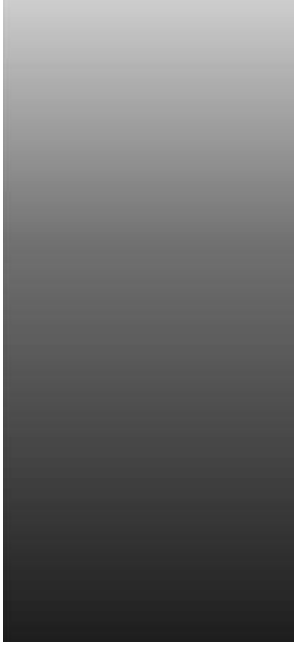
Address for correspondence: Keith S. Kaye, Box 3152, Durham, NC 27710, USA; fax: 919-684-3137; email: kaye0001@mc.duke.edu



Enhancing West Nile Virus Surveillance, United States

John S. Brownstein,* Theodore R. Holford,* and Durland Fish*

We provide a method for constructing a county-level West Nile virus risk map to serve as an early warning system for human cases. We also demonstrate that mosquito surveillance is a more accurate predictor of human risk than monitoring dead and infected wild birds.

The introduction of West Nile virus (WNV) to the Western Hemisphere resulted in a human epidemic in New York City during 1999 (1). By 2002, WNV had spread to 44 states and the District of Columbia, with a total of 4,156 human cases of infection reported by the Centers for Disease Control and Prevention (CDC). Although a nationwide human surveillance system has been established, passive surveillance data are problematic because of variability in disease reporting. The inaccuracies in disease reporting are compounded by random variability inherent in estimating disease incidence rates, a fact that makes interpreting a risk map based on raw data difficult (2). Accounting for these issues should allow for a more precise delineation of spatial risk patterns and for improved targeting of limited prevention resources earlier in the transmission season. In addition to human cases, risk for WNV can be assessed by nonhuman surveillance systems, including infected birds and mosquitoes (3). However, these systems have not been statistically compared for their predictive ability of human risk. A quantitative assessment of the value of the nonhuman surveillance systems would also help direct resources for WNV surveillance. We provide a statistical method to estimate an accurate early assessment of human risk and to determine the predictive capabilities of nonhuman surveillance systems.

The Study

Human Surveillance Model

Human case data were taken from the weekly U.S. Geological Survey West Nile maps for the 2003 transmission season based on county-level data provided by ArboNet through voluntary reporting by state and local health officials to CDC (4). The case numbers comprise reports of mild West Nile fever as well as the more severe West Nile meningitis or encephalitis. Crude county-specific incidence rates were calculated by using the Census 2000 county population totals.

We created a human risk map for WNV based on the crude human incidence early in the transmission season, on August 13, 2003. A disease map that displays observed human incidence will show not only spatial variation in risk but also random variation resulting from low case numbers relative to the base populations. Removing random noise permits improved estimates of disease risk (2). We have approached this procedure by finding the estimates of expected incidence from a conditional autoregressive model (5,6). The model helps remove random variation based on the premise that contiguous regions tend to have similar disease risks, when compared to regions that are far apart. We applied the conditional autoregressive model to calculate expected WNV incidence rates (Appendix).

The first step was to identify the adjacent neighbors of each county by using a geographic information system (GIS, ArcView 3.2, ESRI, Redlands, CA). A data file that included the number of cases, total population, and number and names of neighboring counties for each county was then generated in SAS (SAS Institute Inc., Cary, NC). The file was imported into WinBUGS v1.4 (Imperial College, St. Mary's, UK; and Medical Research Council, Cambridge, UK). This software implements a simulation process to estimate model parameters, including improved estimates of WNV incidence rates. These estimates were then brought back into GIS to display the human WNV risk map.

To verify the method's potential use as an early warning system for human risk, we calculated the validity of the model-estimated risk map versus the raw incidence map from August 13 for predicting the case distribution for October 1, 2003. The two time points represent an ≈14-fold increase in total cases, from 399 to 5,685. For each of the three disease maps, counties were grouped into high- and low-risk classes on the basis of WNV incidence. High risk was defined as human incidence >1 case per 1 million population for the August 13 maps and 1 case per 100,000 for the October 1 map, findings that reflect the change in risk over time. The sensitivity of the method for predicting risk was calculated as the proportion of high-risk counties on October 1 that was correctly identified as such by the model-estimated August 13 risk map. Similarly, specificity was defined as the proportion of low-risk counties on October 1 that was correctly identified as such by the modeled risk map. The sensitivity and specificity values were compared to those obtained when the raw August 13 incidence map was used to predict risk on October 1. Measure of agreement between risk classes of the August 13 map

*Yale University School of Medicine, New Haven, Connecticut, USA

and the October 1 map was assessed by the κ statistic, which accounts for the degree of overlap expected by chance alone; κ has a range of 0 to 1; values of <0.4 represent poor agreement (7).

Nonhuman Surveillance Model

We assessed the quantitative predictive ability of the nonhuman surveillance systems by fitting a regression model to the rate of WNV human cases for counties with the final USGS maps for the 2002 season (4). The model includes covariates for the number of virus-positive tissue samples from dead and diseased wild birds and virus-positive mosquito pools, both provided by state health officials at the county level (Appendix). Each covariate was considered together and separately to determine its contribution for predicting WNV incidence. The model was fitted by using GENMOD in SAS (SAS Institute). The contribution of nonhuman surveillance systems to variability in human risk was determined by calculating the proportion of the deviance explained (R^2).

Conclusions

The maps of Figure 1 show the raw county-specific incidence rates for August 13, 2003 (Figure 1A), the model-estimated risk for August 13 (Figure 1B), and the raw incidence rate on October 1 (Figure 1C). The model-estimated risk surface of August 13 displays a much larger area of high risk than the reported incidence map on the same date, with 930 high-risk counties compared to 128 counties (Figure 1A and 1B). The disease map for October 1 shows a similarly larger high-risk area, with 569 counties classified as high risk (Figure 1C).

The early warning capability of our model was evaluated by comparing the validity of the raw and modeled early season disease maps for predicting the case distribution late in the transmission season (October 1). The raw data on August 13 produced a sensitivity of 19.7% (112/569) for predicting high-risk counties on October 1. In contrast, application of the model allowed for 76.1% (433/569) of the October 1 high-risk counties to be predicted, yielding a fourfold increase. This increase in sensitivity did not have a comparable negative effect on specificity, which decreased from 100% to 80.4% (2,043/2,540). In addition, the August 13 model yielded good agreement with the October 1 data, as shown by a κ statistic of 0.45 (95% confidence interval [CI] 0.42 to 0.49), whereas agreement was poor when the raw August 13 map with a κ statistic of 0.27 (CI 0.23 to 0.31) was used. Accounting for confounding caused by age distribution of WNV patients could further improve overall validity of our model.

This method has the potential to be applied in real-time to identify high-risk counties before the major influx of cases during the transmission season. The model could enable control methods to be implemented early in the season as prevention efforts before the first human case. This time advantage could provide more effective disease prevention efforts.

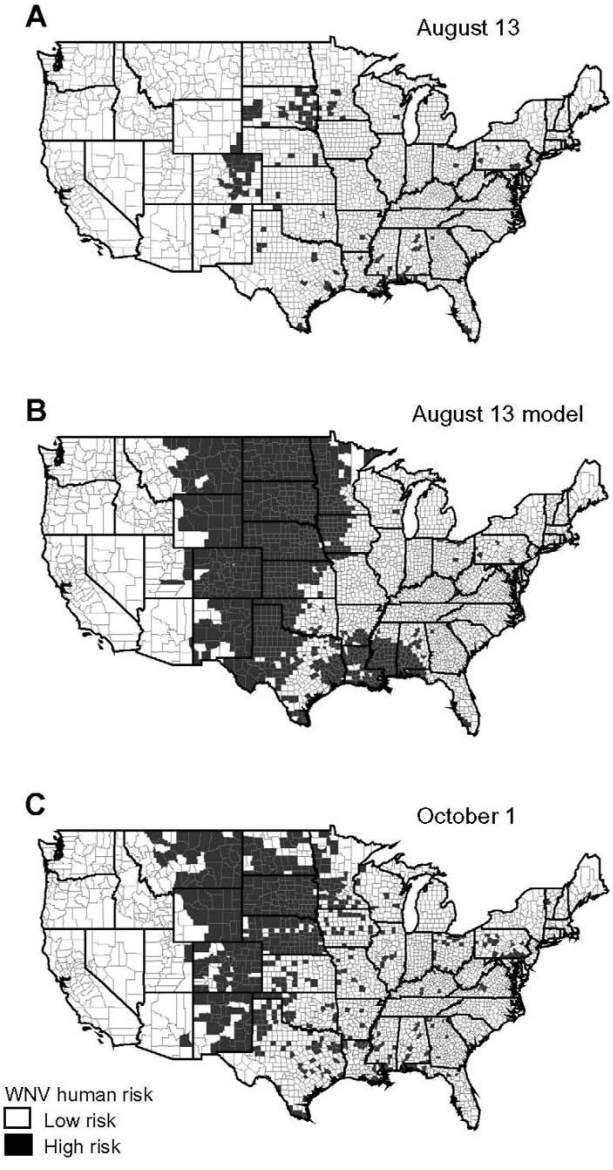


Figure 1. A) Human incidence map for West Nile virus (WNV) early in the transmission season, 2003, based on raw data. Incidence rates were calculated by using the number of new human cases of WNV per county through August 13, 2003, reported to the ArboNet surveillance network. High risk is defined as incidence >1 case per 1 million inhabitants. B) Model-estimated human incidence map for WNV in 2003. Expected risk was derived from the observed incidence rates from August 13, 2003. High risk is defined as incidence >1 case per million persons. C) Observed human risk for WNV late in the transmission season, 2003. Incidence rates were calculated by using the number of new human cases of WNV per county through October 1, 2003. High risk is defined as incidence >1 case per 100,000. This risk surface served to compare the predictive ability of the (A) raw versus (B) modeled early season disease maps.

Risk modeling can also be used to effectively quantify the utility of nonhuman surveillance. Despite support for the use of bird surveillance as an early warning for WNV human risk (8–10), this system has not been statistically compared to active mosquito surveillance. The predictive ability of these surveillance systems for human risk was assessed by their inclusion as quantitative variables in a regression model. Although each variable alone was a significant predictor of human risk (χ^2_{bird} = 138.0, p_{bird} < 0.0001; $\chi^2_{mosquito}$ = 2,605.9, $p_{mosquito}$ < 0.0001), the numbers of WNV-infected dead birds could only explain 2.5% of the deviance, whereas the number of WNV-positive mosquito pools explained 38%. Thus, quantitative mosquito data predict 15 times more of the variation in human cases than quantitative bird data do. The model with both covariates also explained 38% of the deviance by showing that bird data added proportionally less information about human risk (χ^2_{bird} = 5.3, p_{bird}=0.022; $\chi^2_{mosquito}$ = 2,489.0, $p_{mosquito}$ < 0.0001). Plots of the observed and fitted incidence rates, when compared to the covariate alone, showed a much stronger positive relationship between human WNV incidence and the number of WNV-positive mosquito pools than for WNV-positive dead birds (Figure 2).

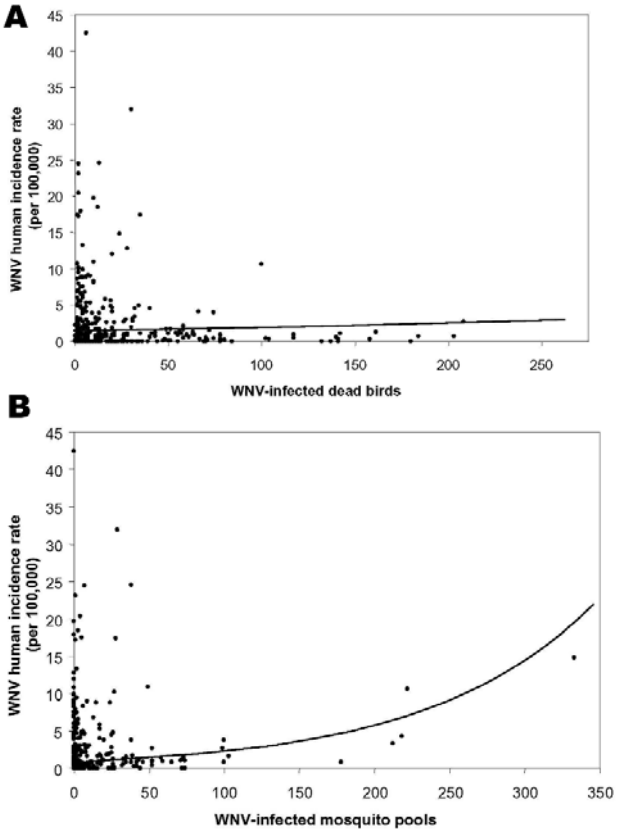


Figure 2. Plots of West Nile virus (WNV) incidence by collections of virus-positive dead birds and virus-positive mosquito pools. Log linear models fit to both surveillance systems considered alone are displayed. WNV-infected dead birds explain 2.5% of the variation in human incidence (A), whereas WNV-infected mosquito pools explain 38% (B).

Our finding that mosquito surveillance is more sensitive to human risk than bird surveillance can be explained by the fact that human infection in the natural WNV cycle is accidental (11–14). Because birds are the zoonotic reservoir host, a WNV-infected bird only indicates enzootic transmission. For human transmission to take place, mosquito species that can act as bridge vectors must be present in sufficient numbers. Therefore, because mosquitoes represent the link to human transmission, mosquito infection prevalence should more accurately predict human risk. Furthermore, once the important human vector species can be clearly identified, the predictive ability of mosquito surveillance should increase. Standard methods for collecting mosquito data applied uniformly would also greatly aid the interpretive value of these data. Our analysis has shown that active mosquito surveillance should be emphasized in WNV surveillance systems, as it is the most sensitive marker of human risk. Surveillance systems based entirely on dead bird reports lack sensitivity for early warning as well as crucial abundance data for targeting effective prevention efforts. Entomologic surveillance should continue to be the keystone for public health programs directed toward preventing WNV infections in humans.

In summary, disease surveillance and prevention efforts could benefit from enhanced risk mapping that draws from corrected human case data and a clear understanding of the predictive ability of nonhuman surveillance.

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Mr. Brownstein is completing a doctorate in the Department of Epidemiology and Public Health at Yale University School of Medicine. His research interests include the application of landscape ecology, spatial statistics, geographic information systems, and remote sensing to the surveillance of infectious diseases.

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Address for correspondence: Durland Fish, Department of Epidemiology and Public Health, School of Medicine, Yale University, 60 College Street, P.O. Box 208034, New Haven, CT 06520-8034, USA; fax: 203-785-3604; email: durland.fish@yale.edu

Appendix

Human Surveillance Model

Because the human West Nile virus (WNV) case number is low relative to the base population, it was assumed to have a Poisson distribution. Under general conditions, the Poisson provides a good description for the distribution of the numerator for an incidence rate (1). However, our model also allowed for the estimation of "extra-Poisson" variation in case it is also needed to provide an accurate description of these data.

The log linear model used for spatial smoothing assumed that the number of disease cases in the *i*-th county, n_i, has a mean, $P_i\lambda_i$, where P_i, is the denominator for the rates and

$$\lambda_i = \exp\{\alpha_o + b_i + h_i\}$$

where α_o is the intercept, b_i is the spatially correlated random variation with mean 0 and variance σ_b^2, and h_i the unstructured extra-Poisson variability with mean 0 and variance σ_h^2. In addition, we assume that both the spatial and the unstructured variability have Gaussian distributions, which are independent in the latter case. On the other hand, the mean for the spatial component, conditional on the means for the contiguous neighbors, is

$$\mathrm{E}\left[\mu_i \middle| \text{mean for all regions}\right] = \frac{\sum_{j \text{ neighbors of region } i} \mu_j}{r_i}$$

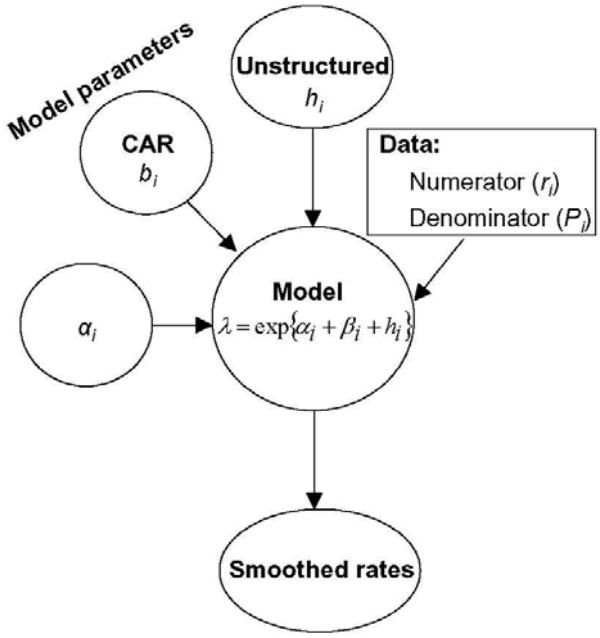
where r_i is the number of neighbors for region *i*. The adjacent neighbors for each county were determined by using a geographic information system (GIS, ArcView 3.2, ESRI, Redlands, CA). Thus, the overall log linear model for the number of cases in the *i* county that incorporates both spatial correlation and unstructured variability is

$$\log n_i = \log P_i + b_i + h_i + \alpha_0$$

The population size for county *i* (P_i) was determined from the Census 2000 data.

Markov Chain Monte Carlo (MCMC) simulation methods were used to find Bayesian estimates of the model parameters as implemented in WinBUGS v1.4 (Imperial College and Medical Research Council) (2,3). Gamma prior distribution parameters were assumed for the variances of the Gaussian distributions, and a plot of the history of the simulation was used to determine the number of iterations required for the process to equilibrate. The approach provides improved estimates of county-specific rates that have been spatially smoothed.

In the MCMC method, parameters estimated from each step are used in turn to determine values for the next step; therefore, a good set of initial values is essential before gleaning the values



Appendix Figure. Diagram of the conditional autoregressive smoothing model.

that will be used in the estimation. To accomplish robust parameter estimates, an arbitrary set of values was chosen, and the number of successive steps taken to stabilize the simulations was noted, which is known as the burn-in. The burn-in period was determined through the use of two chains and the modified Gelman-Rubin convergence statistic. This statistic indicates the point at which the process stabilized by describing how well the chains overlap. Final estimates were obtained by using 1,000 iterations as the burn-in period, and the next 9,000 were used as the sample for deriving the Bayes estimates of the smoothed WNV incidence rates.

Nonhuman Surveillance Model

The quantitative predictive ability of the nonhuman surveillance systems was assessed by once again fitting a log-linear model to the rate of WNV human cases. For this analysis, we instead used a maximum likelihood approach, in which we assumed a Poisson distribution for the number of cases, allowing for extra-Poisson variation by estimating the scale factor. In this model,

$$\log n_i = \log P_i + \beta_A M_i + \beta_M A_i + \alpha_0$$

where P_i is the population offset, A_i is avian mortality attributable to WNV, and M_i is the number of virus-positive mosquito pools. The model was implemented by using GENMOD in SAS (SAS Institute Inc., Cary, NC). Only counties that submitted both mosquito and bird samples were included in the analysis (N = 382) (Appendix Figure).

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Bacteriophages and Diffusion of β-Lactamase Genes

Maite Muniesa,* Aurora García,†‡ Elisenda Miró,† Beatriz Mirelis,†‡ Guillem Prats,†‡[1] Juan Jofre,* and Ferran Navarro†‡

We evaluated the presence of various β-lactamase genes within the bacteriophages in sewage. Results showed the occurrence of phage particles carrying sequences of bla_{OXA-2}, bla_{PSE-1} or bla_{PSE-4} and bla_{PSE}-type genes. Phages may contribute to the spread of some β-lactamase genes.

Bacteriophages provide one of the most efficient vehicles for moving DNA sequences between bacterial cells. One consequence of transduction is disseminating sequences that allow bacteria to become more pathogenic and antimicrobial drug resistant (1–5). In vitro, phages can transduce resistance to imipenem, aztreonam, and ceftazidime in *Pseudomonas aeruginosa* (4), methicillin in *Staphylococcus epidermidis* (5), tetracycline in *S. aureus* (3) and *Actinobacillus actinomycetemcomitans* (2). They can also transduce resistance genes from *Salmonella enterica* serovar Typhimurium DT104 (1).

Increasing levels of resistance to antimicrobial agents in bacteria, particularly in gram-negative rods resistant to β-lactam antimicrobial drugs, have become evident (6,7). The major mechanism of resistance that causes clinically important infection in gram-negative bacteria is the production of β-lactamases, which includes chromosome- and plasmid-encoded enzymes (6,7). Introducing cephamycins and broad-spectrum cephalosporins, such as cefotaxime, ceftazidime, and cefepime, monobactams and carbapenems (7) initially stopped the widespread occurrence of classic plasmid-mediated TEM-1, TEM-2, SHV-1, and OXA-1 β-lactamases. Gram-negative bacteria quickly acquired resistance to these drugs by acquiring plasmid-encoded extended-spectrum β-lactamases (ESBLs), cephamycinases, or carbapenemases, among other mechanisms (7).

Methods for host-independent detection of transducing phage particles have recently been described. These include phages carrying genes linked to specialized transduction (8) and genes likely linked to generalized transduction (9). We evaluated genes that encode resistance to β-lactam agents within phage particles present in sewage samples.

The Study

The study was performed with sewage samples collected during a 6-month period (November 2001 to April 2002). One liter of raw sewage samples was collected monthly from the influent raw urban sewage at three different wastewater treatment plants (Table). Plants 1, 2, and 3 serve populations of 50,000, 400,000, and 1,400,000, respectively. Samples with contamination of animal origin were added to the study to increase information on the presence of phages carrying β-lactamase genes in the environment. Samples were collected from three different abattoirs for poultry, pigs, and cattle.

Samples were evaluated for levels of fecal contamination by using fecal coliforms and somatic coliphages. Fecal coliforms were enumerated by membrane filter procedures. Somatic coliphages are those which infect *Escherichia coli* WG5 through the cell wall and are detected by standardized methods (ISO 10705-2). They comprise a wide range of phages and are always found in sewage. These phages, currently used to indicate viral fecal contamination in environmental samples, were used in this study to indicate the presence of phages in the samples. Somatic coliphages were enumerated according to the standard method. Values of fecal indicators in the samples are the arithmetic mean of three independent replicas and are summarized in the Table. In all samples, different *Enterobacteriaceae* and *Pseudomonadaceae* strains resistant to β-lactams were present (data not shown), although they were not quantified since they were detected after enrichment cultures were taken.

For these experiments, two samples were used in a first attempt to establish the best method to be applied in the remaining samples. For this purpose, 1 L of raw sewage was collected from the influent raw urban sewage at two different wastewater treatment plants (samples 1A and 2A, Table). Samples were collected and processed within 6 hours of sampling. To partially purify bacteriophages, two assay approaches were used to optimize the method. For both approaches, 10 mL of sewage was filtered through 0.22-μm polyether sulfone (PES) low-protein-binding membranes (Millex-GP Millipore, Bedford, MA) to exclude bacteria and other particles present in sewage and to recover viruses. Samples were then purified with Ultrafree-4 centrifugal filter units of Biomax-PB polyethersulfone membranes with a molecular weight cutoff of 100,000 kDa (Catalog number UFV4 BHK 25, Millipore), recommended by the manufacturer for protein isolation,

*University of Barcelona, Barcelona, Spain; †Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; and ‡Autonomous University of Barcelona, Barcelona, Spain

[1]Current affiliation is Hospital Vall d'Hebrón, Universitat Autònoma, Barcelona, Spain.

Table. Levels of fecal indicators; fecal coliforms and somatic coliphages in the samples used in this study[a]

Sample[a]	Fecal coliforms (CFU/mL^{-1})[b]	Somatic coliphages (PFU/mL^{-1})[b]	Somatic coliphages in the purified fraction (PFU mL^{-1})[c]	*bla*$_{OXA}$ PCR	*bla*$_{PSE}$ PCR	Other *bla* genes[d]
Plant 1A (10 mL)	9.7 x 10^4 (2.2 x 10^4)	5.1 x 10^4 (3.6 x 10^3)	2.0 x 10^6	+[e]	+	–
Plant 1A (10 mL + CsCl)	9.7 x 10^4 (2.2 x 10^4)	5.1 x 10^4 (3.6 x 10^3)	7.5 x 10^4	+	+	–
Plant 1A (100 mL)	9.7 x 10^4 (2.2 x 10^4)	5.1 x 10^4 (3.6 x 10^3)	1.0 x 10^7	+	+	–
Plant 2A (10 mL)	9.2 x 10^4 (7.2 x 10^3)	5.1 x 10^4 (4.4 x 10^3)	1.3 x 10^6	+	+[e]	–
Plant 2A (10 mL + CsCl)	9.2 x 10^4 (7.2 x 10^3)	5.1 x 10^4 (4.4 x 10^3)	8.5 x 10^4	+	+	–
Plant 2A (10 mL)	9.2 x 10^4 (7.2 x 10^3)	5.1 x 10^4 (4.4 x 10^3)	1.3 x 10^6	+	+	–
Plant 2B (10 mL)	2.0 x 10^4 (9.6 x 10^3)	8.3 x 10^4 (4.6 x 10^3)	2.5 x 10^6	+	+	–
Plant 2C (10 mL)	9.0 x 10^4 (5.0 x 10^3)	6.2 x 10^4 (1.7 x 10^3)	1.5 x 10^6	+	+	–
Plant 3A (10 mL)	2.5 x 10^5 (6.0 x 10^4)	8.9 x 10^4 (3.0 x 10^3)	3.0 x 10^6	+	+	–
Plant 3B (10 mL)	3.0 x 10^5 (1.7 x 10^4)	9.3 x 10^4 (2.0 x 10^3)	2.5 x 10^6	+	+[e]	–
Poultry (10 mL)	1.8 x 10^6 (7.2 x 10^5)	7.9 x 10^4 (1.5 x 10^3)	1.0 x 10^6	+	+	–
Pigs (10 mL)	3.6 x 10^5 (1.3 x 10^5)	1.8 x 10^5 (1.7 x 10^4)	6.1 x 10^6	+	+[e]	–
Cattle (10 mL)	2.3 x 10^4 (7.2 x 10^3)	8.5 x 10^2 (2.7 x 10^2)	3.5 x 10^4	+	+	–

[a]Results of nested polymerase chain reaction (PCR) amplification of the β-lactamases *bla*$_{OXA}$ and *bla*$_{PSE}$. Parentheses indicate the protocol used (phage DNA obtained from 10 mL and 100 mL before purification or from 10 mL after CsCl purification).
[b]Arithmetic mean of three independent replicas. Standard deviation in parentheses.
[c]Bacteriophage enumeration of 10 μL of the purified fraction of the sample used for DNA extraction and PCR.
[d]The studied *bla* genes were: *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CMY}$, *bla*$_{LAT}$, *bla*$_{MOX}$, *bla*$_{FOX}$, and *bla*$_{VIM}$ genes family; *bla*$_{OXA-1}$-, *bla*$_{PSE-1}$-, *bla*$_{CTX-M}$-, and *bla*$_{OXA-2}$-related genes.
[e]Sequenced PCR fragments (see Figure).

purification, and concentration of virus. Suspensions containing virus were concentrated at 3,000 *g* for 30 min. Phages in the column were recovered with 300 μL phosphate-buffered saline (PBS). After purification, samples were supposedly free from other microorganisms other than viruses that could interfere with the results (first approach). Ten microliters was used for bacteriophage enumeration, as described previously for somatic coliphages to verify the presence of phages in the purified fraction (Table).

Results of phage enumeration of the fraction obtained after concentration confirmed the presence of bacteriophages (Table). To avoid other nonviral particles at the final stage, which could interfere with results, a second approach was performed on samples 1A and 2A (Table). The bacteriophages present in the 300-μL sample at this stage were purified by CsCl centrifugation at 60,000 x *g*. The band in which we expected a broad range of bacteriophages (10), corresponding to a density of 1.46 ± 0.5 g mL^{-1}, was collected and dialyzed to remove the CsCl. A final volume of 300 μL was adjusted with PBS. Ten microliters was used for bacteriophage enumeration, as described above for somatic coliphages, to verify that phages were present in the purified fraction. Results of phage enumeration of the fraction obtained with the CsCl densities confirmed the presence of bacteriophages in the purified fraction (Table). Values in PFU mL^{-1} in the purified fraction were lower than those observed in samples without CsCl purification because of some loss of phages in different gradient densities and after the dialysis step. Samples obtained with or without CsCl gradient purification were then processed for DNA extraction and amplification as described below. However, no variation was observed in either sensitivity results or the kind of β-lactamase genes detected because of the purification with CsCl densities (Table), and since CsCl purification implied a reduction in the number of phages detected and used for the polymerase chain reaction (PCR) analysis, this step was not applied in the remaining samples.

To evaluate whether we could detect other β-lactamase genes in a lower concentration of a larger volume of sewage, we also tested for bacteriophages from 100 mL of sewage in samples 1A and 2A (Table). Bacteriophages partially purified from sewage were concentrated by ultracentrifugation, and the pellet, resuspended in 300 μL of PBS, was treated for DNA purification and PCR as described below. Again, no differences were observed concerning the kind of β-lactamase genes detected when testing in parallel 10 mL or 100 mL (Table). The protocol of purification from 10 mL of samples was thus applied in the remaining samples.

In all cases, samples were then treated with an extra amount of DNase to a final concentration of 1,000 U/mL of the water sample, and incubated for 1 h at 37°C to inactivate any free DNA. Previous work performed by our research group to isolate phage particles from sewage (8), as well as well-known methods for phage isolation from bacterial strains, showed that lower amounts of DNase (10 U/mL of sample) were sufficient to eliminate any traces of chromosomal DNA in the sample. However, since the concentration of β-lactam genes in sewage is not established, in these experiments we increased the DNase concentration 100-fold, and we performed extra controls with chromosomal DNA to exclude any possibility of contamination.

A 0.1-mL aliquot of each sample was used for direct PCR reaction. Samples containing the virus particles were identified as nondecapsidated samples. DNase was heat

inactivated at 95°C for 5 min before PCR amplification. Additionally, sewage samples previously sterilized at 121°C for 15 min were used as negative controls.

Bacteriophage DNA was then extracted with the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen, Inc., Valencia, CA). We used 200 μL of each sample for DNA extraction, and DNA was finally diluted in 100 μL of double-distilled water (decapsidated samples). Excess DNase was removed by following the steps in the washing section of the Qiagen protocol and by heat inactivation as described previously.

We used 5 μL of phage DNA (≈5 ng/μL) for subsequent PCR reactions. The detection of bla_{TEM} (expected size of the amplified fragment was 951 bp), bla_{SHV} (expected size of 1,016 bp), bla_{CMY} (461 bp), bla_{LAT} (461 bp), bla_{MOX} (408 bp), bla_{FOX} (407 bp), and bla_{VIM} (801 bp) genes family and bla_{OXA-1}-related (694 bp), bla_{PSE-1}-related (420 bp), $bla_{CTX-M-9}$-related (857 bp), and other bla_{CTX-M}-related (394 bp), enzymes was accomplished by PCR as previously described (6,11,12). The bla_{OXA-2}-related (701 bp) gene was amplified by using the primers OXA2/3 (5′-GCC AAA GGC ACG ATA GTT GT-3′) and OXB2/3 (5′-GCG TCC GAG TTG ACT GCC GG-3′), submitted by D. Sirot, in the same conditions as for bla_{SHV}. The full size of all *bla* genes was 801–1,146 bp. Finally, a DNA fragment of the gene that encodes the 16S rRNA was also amplified (≈909 bp) as previously described (13). Nested PCR using the same pair of primers and conditions was done in all cases.

All decapsidated samples, independently of their origin, gave positive amplification (Table) when primers for bla_{OXA-2}-related and bla_{PSE-1}-related β-lactamases and 16S rRNA from eubacteria as positive controls were used (9,13). None of the DNA sequences of the remaining β-lactamases studied were amplified in any of the samples, indicating no presence or a very low concentration of any other β-lactamases. DNA amplification was not observed in any nondecapsidated samples, a finding that shows that no free DNA was present in the sample after DNase treatment. Despite efforts to increase the sensitivity of the method by increasing the sample volume, we were unable to detect bla_{TEM}, and bla_{SHV}, the most frequent enzymes among *Enterobacteriaceae*. The sewage samples studied differed in the presence of phage particles carrying DNA fragments coding for various β-lactamases.

Although the calculations of the number of phages carrying the β-lactamases in the samples could not be determined, a rough estimation based on the minimal number of phages necessary to obtain a positive PCR result in the volume tested assumed that 1–10 phages per milliliter carry a β-lactamase gene in these samples.

The positive amplification of 16S rDNA used as a control of bacterial DNA contamination was useful to confirm the lack of bacterial contamination in the nondecapsidated samples, since results were negative. Moreover, these results confirm the validity of the DNase concentration used. However, in the decapsidated samples, we obtained positive amplification of 16S rDNA. This finding could be explained by the presence of bacterial 16S rDNA in phage DNA attributable to generalized transduction, as previously described by other authors (9).

The amplified products of bla_{OXA-2}-related and bla_{PSE-1}-related genes of several of the samples were sequenced by the dideoxy method by using fluorescent terminators and an automatic laser fluorescent DNA sequencer (Pharmacia Biotech, Uppsala, Sweden) (Table) (6). The deduced amino acid sequence was identical to OXA-2, PSE-1 or PSE-4, and *bla*P from *Proteus* plasmid pCS229 (accession no. JS0755) (Figure). The bla_{OXA} and bla_{PSE} occur predominantly in *Pseudomonas* isolates, although they have also been reported in members of *Enterobacteriaceae* and *Vibrionaceae*. The β-lactamases most frequently associated with *Enterobacteriaceae*, such as TEM and SHV enzymes, were not detected, whereas OXA and PSE, significantly less frequent, were recovered. OXA and PSE enzymes have often been associated with integron structures, a finding that may play some role in these results. Nevertheless, integrons usually occur on broad host-range plasmids, which may be transferred not only among

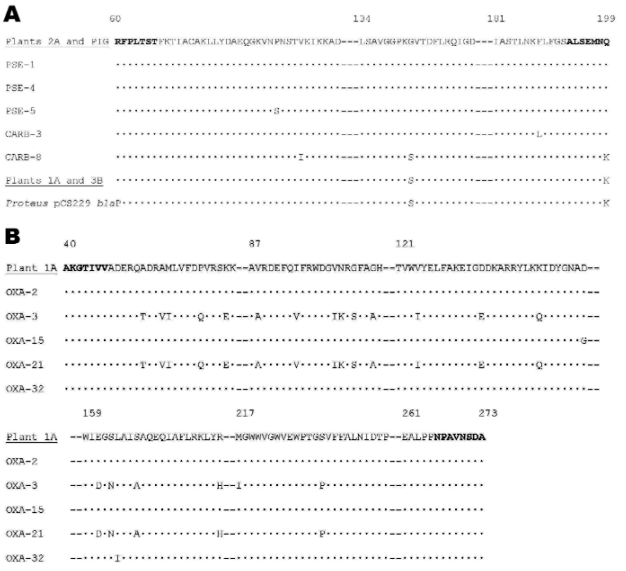

Figure. Deduced amino acid sequence of PSE-1 (A), and OXA-2 (B) related enzymes that could be detected by polymerase chain reaction. Amino acids in boldface correspond to the regions of the primers. Dots indicate identical amino acids, and dashes indicate omitted regions with identical amino acids. Numbers correspond to amino acid position. Sequences obtained are underlined. GenBank accession no. of sequences: PSE-1: Q03170, PSE-4: P16897, PSE-5: AAG23870, CARB-3: P37322, CARB-8: AAM92465, *Proteus* pCS229 *bla*P: JS0755, OXA-2: CAA30246, OXA-3: AAC41449, OXA-15: AAB05874, OXA-21: CAA71699, and OXA-32: AAK58418.

Enterobacteriaceae but also to *Pseudomonas* and *Acinetobacter*. These species may have more transducing phages than *E. coli*. While TEM, encoded by *Tn*3, has been found on IncP plasmids, it is more frequently found on plasmids of a more restricted host range, such as IncF.

Conclusions

Results reported here show that sewage carries a substantial number of phage particles with various β-lactamase genes. These represent a potential for transduction, and according to the number of particles, the probability of occurrence cannot be overlooked. Our results cannot determine whether generalized or specialized transduction is involved. For this purpose, isolation and characterization of the phage particles would be necessary. Neither can our results show whether the β-lactamase genes detected in phage particles are part of a gene cassette. However, the role of gene cassettes in the emergence and spread of antimicrobial drug resistance has been well-established.

Phages can incorporate genes or groups of genes in their genomes. Their genomic structure is usually modular, with genes of related functions clustered in the genome with their sites of action. This structure allows these genes to be exchanged between related phages by co-infection of host cells and recombination between phage genomes. Some bacteriophages have a broad host spectrum that taxonomically includes very distant species, such as *Sphaerotilus natans*, *E. coli*, and *P. aeruginosa* (14). Consequently, infection of these bacteria by phages could be the way the genes, or groups of genes, are able to move over great phylogenetic distances (15). A sequence of events in which a phage is infective for two different hosts (A and B) will transfer genes between these hosts. Recombination of these phages in host B with another phage able to infect hosts B and C may facilitate transfer of DNA sequences from host A to host C. Host C could be phylogenetically distant from host A and through this mechanism would acquire the DNA sequences from host A.

Our results indicate β-lactamase genes in naturally occurring phage particles. These genes have been described in several bacteria able to share phages (7,14) and have also been detected in the sewage samples tested. Therefore, we cannot rule out that phages potentially contribute to the spread of chromosomal genes bla_{OXA} and bla_{PSE} among *Pseudomonadaceae*, *Enterobacteriaceae*, and *Vibrionaceae*, and the posterior emergence of plasmid-linked antimicrobial-drug resistance cannot be ruled out and is worthy of further investigation. Futures perspectives will be focused on isolating and characterizing single phage particles encoding β-lactamase from genes from sewage and their ability to transduce the character to diverse host strains.

Dr. Muniesa works in the Department of Microbiology at the University of Barcelona. Her research is focused on bacteriophages in the environment and their role in the mobility and transfer of virulence genes between bacteria.

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Address for correspondence: Ferran Navarro, Departament de Microbiologia, Hospital de la Santa Creu i Sant Pau, Av. Sant Antoni Mª Claret, 167, E-08025 Barcelona, Spain; fax: 34 93 2919070; email: fnavarror@hsp.santpau.es

Predominant Tuberculosis Spoligotypes, Delhi, India

Urvashi Balbir Singh,* Naga Suresh,* N.Vijaya Bhanu,* Jyoti Arora,* Hema Pant,* Sanjeev Sinha,* Ram Charan Aggarwal,* Sushma Singh,* Jitendra Nath Pande,* Christophe Sola,† Nalin Rastogi,† and Pradeep Seth*

One hundred five *Mycobacterium tuberculosis* clinical isolates from the Delhi area were typed by spoligotyping; 45 patterns were identified. Comparison with an international spoligotype database showed type 26, Delhi type (22%), type 54 (12%), and type 1, Beijing type (8%), as the most common. Eighteen spoligotypes did not match any existing database pattern.

India accounts for 30% of tuberculosis (TB) cases worldwide. Each year, this disease develops in approximately 2 million people in India. Approximately 0.5 million people die, a figure likely to increase with emergence of multidrug-resistant tuberculosis (MDR-TB) and the HIV epidemic (1). Delhi alone has an annual risk for infection of 2.4% (state TB officer, Delhi, pers. comm.). Traditional methods for tracing transmission of TB are imprecise and ineffective in controlling the disease. The impact of control programs is often difficult to assess in high-incidence areas, where infection and disease patterns are highly heterogeneous. This difficulty can be overcome by an alternative approach in which molecular strain-typing techniques are used. Spoligotyping detects the presence and absence of nonrepetitive unique spacer sequences (36–41 bp in length) in the direct repeat region of *Mycobacterium tuberculosis*. Its utility as an initial screening method is well documented (2–4).

The aim of our study was to identify predominant spoligotypes with an international designation responsible for transmission and prevalence of TB in Delhi. The spoligotypes obtained were compared with an international spoligodatabase, spolDB3.0 (5,6).

The Study

The study included patients with culture-confirmed TB whose cases were reported to a district TB center, a primary health center, and an outpatient department, All India Institute of Medical Sciences (AIIMS), Delhi, the coordinating center. AIIMS is the top tertiary-care hospital with referrals from the entire city of Delhi, as well as from other states, with a daily outpatient attendance of 4,000 to 5,000. The district TB and primary health centers serve a well-defined population, including all TB patients in their areas.

Patients with a diagnosis of new, smear-positive pulmonary TB or those with a high suspicion for TB on clinical or radiologic grounds were included in the study. In all, 1,500 patients who met inclusion and exclusion criteria were recruited from the Delhi area over 2 years. Demographic data were collected on the patient's sex, age, present address, employment, economic status, literacy, living conditions, household contacts, chest radiologic findings, and HIV infection. Three sputum specimens were collected in the early morning on consecutive days and transported to the AIIMS TB laboratory. Sputum specimens were processed by Petroff's method and plated on duplicate Lowenstein-Jensen slants. A smear was examined after Ziehl-Neelsen staining. Species confirmation was followed by tests for drug susceptibility (proportion method) for rifampicin (RIF, 40.0 μg/mL), ethambutol (ETH, 2.0 μg/mL), streptomycin (STR, 4.0 μg/mL), and isoniazid (INH, 0.2 μg/mL) (7). The isolates were processed on the respective days of collection with the date and other details recorded. During the initial 3 months, January 2001–March 2001, 105 (55%) of 190 patients enrolled were culture-positive. The 105 individual *M. tuberculosis* isolates from different patients (34 from the district center, 12 from the primary health center, and the rest from AIIMS), both smear-negative and smear-positive samples, were spoligotyped. Work on the isolates collected later in the study is ongoing.

Spoligotyping to detect 43 known spacers in the direct repeat locus was performed with a commercially available kit, according to the instructions supplied by the manufacturer (Isogen Bioscience B.V., Maarsen, the Netherlands). To avoid any possibility of artifactual hybridization spots on the commercial membranes, appropriate controls included DNA from *M. bovis* and *M. tuberculosis* H37Rv and autoclaved purified water for adequate number of negative controls in each experiment. Reproducibility of spoligotyping was confirmed by repeating the test with the DNA extracted again from a few isolates (data not shown). None of the negative controls demonstrated carryover DNA.

Results were doublechecked visually by an experienced operator to eliminate any systematic artifact caused by using commercial membranes. The results obtained were entered in a binary format as Excel spreadsheets (Microsoft, Redmond, WA) and compared to the

*All India Institute of Medical Sciences, New Delhi, India; and †Institut Pasteur de Guadeloupe, Pointe-à-Pitre, Guadeloupe

spolDB3.0 of the Pasteur Institute of Guadeloupe. At the time of the matching analysis, spolDB3.0 contained 13,008 patterns distributed into 813 shared types (patterns reported at least twice that grouped 11,708 clinical isolates) and 1,300 orphan patterns from >90 countries (6). The results were also computed into Recognizer files of the Taxotron package (P.A.D. Grimont, Taxolab, Institut Pasteur, Paris) to calculate the 1-Jaccard Index (8) and to allow the construction of dendrograms by using the unweighted pair-group method with arithmetic averages (UPGMA [9]). Odds ratios for clustering with 95% confidence intervals were calculated to compare characteristics of clustered and nonclustered patients. Differences were considered significant if values were <0.05 (Table 1).

Table 1 shows the detailed demographic data of the study population. None of the patients' families had symptoms suggestive of TB during the study period. In 2% of families, death attributable to pulmonary TB was reported.

A total of 45 distinct spoligopatterns were obtained from the study population of 105 isolates (Figure). Twenty-nine (28%) clinical isolates were represented by a unique pattern, whereas 76 (72%) isolates were clustered in 16 clusters, i.e., 2 predominant clusters of 23 (22%) and 13 (12%) isolates (ST26 and ST54), followed by 1 cluster of 9 isolates (ST1), 2 clusters of 4 isolates each (ST11 and ST119), 1 cluster of 3 isolates (ST1088), and 10 clusters of 2 isolates (ST100, ST276, ST1089-1092 and ST1094-1097). The isolates in all the clusters had different drug-resistance profiles and had been collected, processed, and amplified into separate batches on different days; hence, carryover contamination was ruled out.

Spoligotypes were compared with those in spolDB3.0. The three most prevalent spoligotypes from this study were type 26, 54, and 1. ST26 (22%) was initially described in 1997 in a study performed in the United Kingdom (4). It was later shown to belong to the major genetic group I of *M. tuberculosis* complex organisms (10,11), i.e., an ancestral group of human TB, as evidenced by the presence of the TbD1 region in these strains (12). Until now, this family of strains has been reported in 11 countries of the Middle East (Iran, Pakistan, and India), Oceania (Australia), the United States, and Europe (United Kingdom, the Netherlands, France, Sweden, Italy, and Austria; [5]). In Europe and Australia, these strains were regularly found to be linked with immigrants from the Middle East and Central Asia, hence the name of Central Asian 1 or CAS1 family, which was recently given to all isolates characterized by the absence of spacers 4–7 and 23–34 (6). Indeed, a total of 20 shared-type variants linked to this family of strains have been found to date (5). In a recent article, this predominant clade of bacilli and some of their variants were called the Delhi type (13). For spoligotype patterns, see Table 2.

Type 54 (12%) is also likely to belong to group I organisms, as suggested by the existence of a closely linked profile, found within the Houston study (clinical isolate S179; [10]). This shared-type is less widespread, and its distribution is different. It has been reported to be

Table 1. Clinical and epidemiologic characteristics of patients harboring clustered versus nonclustered strains[a]

Parameters	No. (%) of patients in Clustered group	Nonclustered group	OR for clustering (95% CI)	p value
Age, y				
15–45	63 (81)	15 (19)	4.52 (1.6 to 12.96)	0.001
≥46	13 (48)	14 (52)		
Sex				
Male	53 (72)	21 (28)	0.88 (0.30 to 2.49)	NS
Female	23 (74)	8 (26)		
HIV status				
Seropositive	2 (100)	0 (0)	UD	
Seronegative	74 (72)	29 (28)		
Previous history of TB				
No previous therapy	52 (80)	13 (20)	2.53 (0.89 to 7.22)	0.05
Previously treated	19 (61)	12 (39)		
Drug resistance				
Drug resistance[b]	22 (71)	6 (29)	1.56 (0.51 to 4.97)	NS
Susceptible to all drugs	54 (70)	23 (30)		
Radiologic findings				
Extensive cavitary	12 (76)	5 (24)	1.6 (0.33 to 11.22)	NS
Limited cavitary	6 (60)	4 (40)		
Sputum smear positive				
1–10 AFB/10–100 fields	30 (73)	11 (27)	0.96 (0.34 to 2.70)	NS
>1 AFB per field	37 (74)	13 (26)		

[a]OR, odds ratio; CI, confidence interval; TB, tuberculosis ; NS, not statistically significant; UD, undefined ; AFB, acid-fast bacilli.
[b]Resistance to one or more drugs.

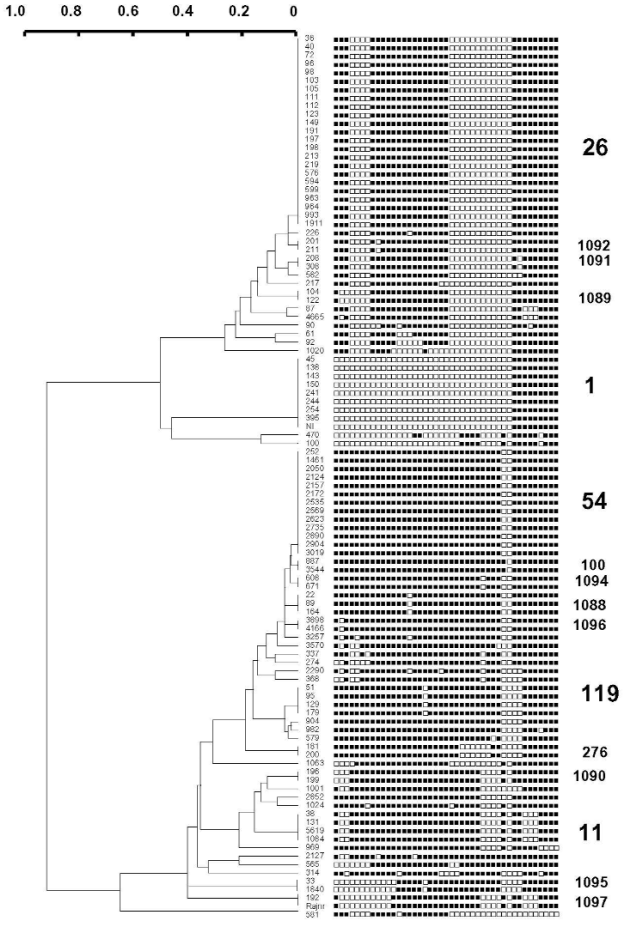


Figure. Dendrogram built on 105 *Mycobacterium tuberculosis* clinical isolates from Delhi based on spoligotyping results using the Taxotron software package (PAD Grimont, Taxolab, Institut Pasteur, Paris). This phylogenetic tree, based on the 1-Jaccard Index (8) and drawn using the unweighted pair group method with arithmetic averages (UPGMA), shows the presence of five major shared types (ST) of spoligotypes in Delhi; ST26 (Central Asian Family 1 or CAS1), ST1 (Beijing Family), ST54 (also newly designated as T1 ancestor), ST119 (X1 family) and ST11 (East African Indian 3 or EAI3 family). The strain designation appears left of the binary spoligotyping profile, and shared-type designations appear on the right of the spoligotype. Orphan patterns (unique isolates) have not been provided with a spoligotype designation on this tree. A print-ready Adobe Acrobat file of this dendrogram is available at http://www.cdc.gov/ncidod/EID/vol10no6/ 03-0575-G.htm

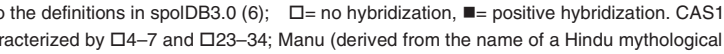
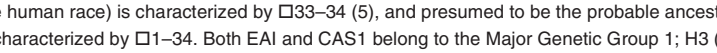
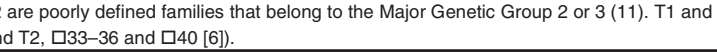
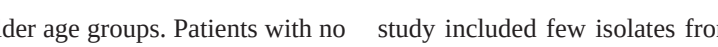
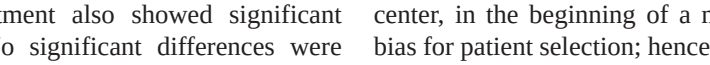
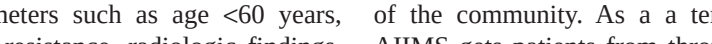
present in Africa (Guinea-Bissau and Senegal) and in Europe (France, United Kingdom, and the Netherlands). This type may be an ancestor of both the CAS and the Beijing family. It is characterized by the absence of spacers 33 and 34, two spacers likely to be of high phylogenetic importance in group I organisms since they permit distinguishing between 1) East African Indian (EAI) superfamily (presence of spacer 33, absence of 34), 2) CAS family (absence of 33 and 34), and 3) *M. bovis* (presence of 33 and 34). Combined prevalence of these two spoligotypes (34%) indicates that these two families are highly prevalent in our high-incidence area and may play an important role in disease transmission in Delhi (Table 2).

The third most prevalent type is the Beijing type shown in nine isolates (8%). This type was originally described by van Soolingen et al. in China and is highly prevalent throughout Asia and Eurasia (15), with a reported prevalence of approximately 3% in India (13). Among these isolates, seven were resistant to ETH, STR, and INH, and one isolate (strain 45) was resistant to all four drugs tested. An epidemiologic link could be established for eight patients who resided in one area (Faridabad) and were referred to the district TB center. Two shared types (ST11 and ST119) belong to the EAI and X families, respectively (14). The presence of the X family in India could be linked to the past British history in this region.

Spoligotypes that did not match any existing pattern in the database were defined as orphans. Of 45 patterns observed in this study, 18 (observed for isolates 4665, 90, 92, 1020, 470, 100, 3257, 3570, 337, 274, 2290, 368, 1063, 1024, 2127, 565, 314, 581) were true orphans (no counterpart in the database). This percentage referred to patterns (40%) is high and reflects both the current absence of knowledge on the genetic diversity of Indian *M. tuberculosis* strains and the microevolutionary genetic driving forces active in TB-epidemic dynamics in India. Of 105 isolates, 18 (17%) had orphan spoligopatterns. New shared types were also created for newly identified types (ST1088–1092 and 1094–1097), which may either reflect homoplasia (creation of common genetic structures without common ancestor, also called convergence; [16]) or true synapomorphy (common ancestors). Except for one clinical isolate belonging to ST1092, isolated in 2002 in New York (S863, J. Driscoll, unpub. data), all the shared types mentioned above have not been reported elsewhere in the world. A total of 10 isolates unique to this study (Table 2) did match with strains already reported elsewhere (ST50, 52, 53, 138, 141, 357, 381, 427, 458, and 1093). The origin of their counterparts from different parts of the world is described in spolDB3.0. However, many of these patterns (particularly ST 138, 381, 1093) were originally reported in the United Kingdom by Goyal et al. in 1997 (4), who described many strains harboring Indian genetic characteristics.

Little information is available from India or the neighboring countries on the molecular epidemiology of TB. Our study has demonstrated that the epidemiology of TB in India is much different than TB epidemiology elsewhere (5). Our results showed significant clustering in the 15- to 45-year age group ($p = 0.001$) and significant

Table 2. Predominant spoligotype prevalent in Delhi and shared type designations for 10 unique isolates, clade designation, and initial description (reference)

Spoligotype designation	Spoligotype pattern	Clade[a]	Reference
Predominant spoligotype			
Type 26		CASI (Delhi)	13
Type 54		Manu	5
Type 1		Beijing	10
Shared spoligotype			
Type 50		H3	4
Type 52		T2	4
Type 53		T1	4
Type 138		EAI	4
Type 141		CAS1	5
Type 357		CAS1	5
Type 381		CAS1	4
Type 427		CAS1	5
Type 458		EAI	5,14
Type 1093		CAS	4

[a]Clades were defined according to the definitions in spolDB3.0 (6); □= no hybridization, ■= positive hybridization. CAS1 (Central Asian 1) family (also termed as the "Delhi type") is characterized by □4–7 and □23–34; Manu (derived from the name of a Hindu mythological figure supposed to be the world's first king and father of the human race) is characterized by □33–34 (5), and presumed to be the probable ancestor of both the CAS and EAI (East African Indian). Beijing family is characterized by □1–34. Both EAI and CAS1 belong to the Major Genetic Group 1; H3 (Haarlem 3) belongs to the Major Genetic Group 2; and T1 and T2 are poorly defined families that belong to the Major Genetic Group 2 or 3 (11). T1 and T2 need other markers for a better characterization (T1, □33–36 and T2, □33–36 and □40 [6]).

lack of clustering in the older age groups. Patients with no history of previous treatment also showed significant clustering ($p = 0.05$). No significant differences were observed for other parameters such as age <60 years, gender, HIV status, drug resistance, radiologic findings, and sputum smear positivity.

Conclusions

Spoligotyping is based on the variability in the direct repeat locus of *M. tuberculosis*, which most likely occurs by one of three mechanisms—homologous recombination between neighboring or distant direct variable repeats, IS-mediated transposition, and DNA replication slippage (17). Spoligotyping is useful for tracking TB epidemics, detecting new outbreaks, and better defining high-risk populations to focus prevention strategies (6,18). Spoligotyping may also constitute a potential tool for global TB epidemiology, population genetics, and phylogeny, although it should be used with another independent genotyping method in many settings to prove clonality (5,6,10). Another poorly investigated theoretical limitation issue is the level of convergence that may jeopardize phylogenetic reconstruction when spoligotyping is used (16).

To have a better knowledge of moving and expanding clones of *M. tuberculosis* within the Indian subcontinent, we attempted to identify the predominant spoligotypes prevalent in Delhi and determine their specific signature. Comparison with the spolDB3.0 database enabled us to compare spoligotypes generated in a New Delhi laboratory with different laboratories around the world. The present study included few isolates from patients enrolled at one center, in the beginning of a multicentric study, with no bias for patient selection; hence, it was truly representative of the community. As a a tertiary-care referral center, AIIMS gets patients from throughout Delhi. In addition, isolates from the district TB and the primary health centers (which cover all the patients in their respective areas) were also included. It seemed imperative to study the population structure of Indian *M. tuberculosis* strains by using additional genetic markers to better comprehend the origin and evolutionary genetics of TB in this geographic region. The study strains have been further subjected to IS*6110*-RFLP and double repetitive element PCR. Also the spoligo clusters are being subjected to mycobacterial interspersed repetitive units—variable number of DNA tandem repeats. Investigation of strains from Delhi and those collected from the other centers from all over the country is ongoing.

Clustering was much higher within the age group of 15 to 45 years but decreased with increasing age and was lowest in patients >60 years (odds ratio for clustering 4.52, $p = 0.001$). This finding may suggest active transmission of TB among younger persons as opposed to possible reactivation of disease in the older age group. A high rate of clustering in a population with no history of TB treatment further corroborated the notion of active transmission of certain prevailing genotypes, despite the existence of genetically diverse strains in our high-incidence community. These prevailing genotypes may play an important role in the propagation of the TB epidemic in Delhi and surrounding regions in coming years. Extensive cavitary disease and degree of

infectiousness (smear positivity) indicative of large bacterial populations did not affect clustering.

Studies focusing on the polymorphism of *M. tuberculosis* isolates from developing countries, where TB is highly prevalent, would provide new insights into epidemiology, transmission dynamics, phylogenetic analysis, and virulence. Similar studies with detailed epidemiologic data that would reflect on the TB control programs are needed to understand the current epidemic in India.

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Dr. Singh is assistant professor and chief of the Tuberculosis Laboratory, Department of Microbiology, All India Institute of Medical Sciences, New Delhi, where she established molecular epidemiology and molecular resistance detection techniques for *Mycobacterium tuberculosis*. Her research interests include molecular diagnostics and molecular epidemiology of tuberculosis for clinical applications.

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Address for correspondence: Urvashi B. Singh, Tuberculosis Laboratory, Department of Microbiology, All India Institute of Medical Sciences, New Delhi-110 029, India; fax: 91-11-26588663; email: urvashi00@hotmail.com

SARS and Common Viral Infections

**Janice K. Louie,*†[1] Jill K. Hacker,*[1]
Jennifer Mark,* Shilpa S. Gavali,* Shigeo Yagi,†
Alex Espinosa,† David P. Schnurr,†
Cynthia K. Cossen,† Erin R. Isaacson,†
Carol A. Glaser,† Marc Fischer,‡
Arthur L. Reingold,* and Duc J. Vugia,*†
for the Unexplained Deaths and
Critical Illnesses Working Group**

In California, molecular testing was useful in decreasing suspicion for severe acute respiratory syndrome (SARS), by detecting common respiratory pathogens (influenza A/B, human metapneumovirus, picornavirus, *Mycoplasma pneumoniae*, *Chlamydia* spp., parainfluenza virus, respiratory syncytial virus, and adenovirus) in 23 (45%) of 51 patients with suspected SARS and 9 (47%) of 19 patients with probable SARS.

Severe acute respiratory syndrome (SARS) has become the new paradigm for the global havoc that can be produced by an emerging infectious disease (1). As of July 31, 2003, a total of 8,096 probable SARS cases had been reported to the World Health Organization from 29 countries or areas, with 774 deaths and a case-fatality ratio of 9.6% (2). California was particularly affected by the SARS outbreak, reporting one fifth of suspect or probable cases in the United States (15% of suspect SARS cases and 26% of probable SARS cases), with two serologically confirmed cases. In 2002, the California Unexplained Pneumonia (CUP) Project, a respiratory surveillance project that uses enhanced laboratory techniques to identify etiologic agents of severe pneumonia, was initiated at the California Department of Health Services (CDHS) in collaboration with the Centers for Disease Control and Prevention (CDC) Emerging Infections Program. The CUP project's extensive diagnostic testing algorithm was applied to specimens submitted to CDHS for SARS testing.

The Study

From March 12, 2003, through July 30, 2003, cases of possible SARS reported to the CDHS were classified as suspect, probable, or laboratory-confirmed, according to CDC criteria (3). Extensive diagnostic testing was performed at the CDHS Viral and Rickettsial Disease Laboratory on specimens from 165 patients, including those with conditions that did not meet strict CDC case criteria (Table 1).

Submitted specimens were transported on cold pack and either frozen at –70°C or processed immediately. A total of 281 respiratory specimens and 78 serum specimens were analyzed, including 210 nasopharyngeal swabs, 23 nasal swabs, 17 throat swabs, 15 nasal washes, 11 sputum specimens, 5 endotracheal aspirates, 39 single acute-phase serum specimens, and 39 acute- and convalescent-phase paired serum specimens. Convalescent-phase serum specimens were collected at least 28 days after symptom onset. Because of difficulties obtaining convalescent-phase sera, specimens from only 32 case-patients underwent combined testing by polymerase chain reaction (PCR), culture, and serologic methods.

Total nucleic acid was extracted from all respiratory specimens for reverse transcriptase (RT)-PCR by using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Technologies, Madison, WI). RT-PCR assays were performed according to Erdman et al. (5) with primers for respiratory syncytial virus (RSV), parainfluenza virus (PIV) types 1–3, and influenza A and B; PIV 4 (6); coronavirus (CoV) 229E reverse primer (7), and human metapneumovirus (HMPV) (8). Primers for CoV OC43 and CoV 229E forward primer were modified from Myint et al. (9). We used adenovirus and picornavirus primers (adenovirus: forward primer 5′- CCC(AC)TT(CT)AAC-CACCACCG -3′ and reverse 5′- ACATCCTT(GCT)C-(GT)GA AGTTCCA -3′; picornavirus: forward primer 5′-GGCCCCTGAATG(CT)GGCTAA-3′ and reverse 5′-GA-AACACGGACACCCAAAGTA-3′). Reaction products were visualized on ethidium bromide–stained agarose gels with ultraviolet illumination. RT-PCR for SARS-CoV was performed on an iCycler (BioRad Laboratories, Inc., Hercules, CA) by using the TaqMan One-step RT-PCR Master Mix (Applied Biosystems, Foster City, CA) with primers and probes developed at CDC.

Based on β-actin gene amplification, specimens from 151 patients were suitable for molecular testing. In 63 (42%) of these, RT-PCR detected a respiratory pathogen (Table 2). No patient had more than one agent identified by molecular methods. In addition, respiratory samples with adequate volume were added to Vero E6, primary rhesus monkey kidney cells, and human fetal diploid lung cells, according to standard diagnostic procedures. Viruses were isolated from 16 (10%) of 154 patients (Table 2). All specimens yielding positive viral cultures were also positive by RT-PCR. Overall, RT-PCR assays were more sensitive than culture; for example, of the 26 patients who had influenza A detected by RT-PCR, 9 were culture-positive.

*California Emerging Infections Program, Berkeley, California, USA; †California Department of Health Services, Berkeley, California, USA; and ‡Centers for Disease Control and Prevention, Atlanta, Georgia, USA

[1]J.K. Louie and J.K. Hacker authors contributed equally to this work.

Table 1. Respiratory testing algorithm[a]

Respiratory specimens	Serologic testing[b]
Viral culture	Acute-phase serum specimens: IgM
Polymerase chain reaction	*Chlamydia* spp.
Influenza A	*Mycoplasma pneumoniae*
Influenza B	Paired serum specimens: IgG
Respiratory syncytial virus	*Chlamydia* spp.
Parainfluenza virus types 1–4	*M. pneumoniae*
Human metapneumovirus	Influenza A and B
Coronavirus OC43 and 229E	Respiratory syncytial virus
Adenovirus	Parainfluenza virus types 2–4
Picornavirus	Adenovirus
SARS-CoV	SARS-CoV

[a]Ig, immunoglobulin; SARS-CoV, severe acute respiratory syndrome–associated coronavirus.
[b]All serologic assays were in-house enzyme immunoassays (4), except for the Meridian IgM assay for *M. pneumoniae* (Meridian Bioscience, Inc., Cincinnati, OH) and enzyme-linked immunosorbent assay of the Centers for Disease Control and Prevention for SARS-CoV

Serologic testing was performed on specimens from 78 patients (Table 2). A significant rise in immunoglobulin (Ig) G was seen in 10 of the 39 patients who had paired acute- and convalescent-phase serum specimens (*M. pneumoniae* [2 patients], influenza A [4 patients], 1 each of *Chlamydia* spp., RSV, influenza B, and adenovirus). Of the 39 patients from whom a single serum sample was collected, two patients had detectable IgM (one each of *Chlamydia* spp. and *M. pneumoniae*). The patient with *M. pneumoniae* also had influenza A detected by PCR and culture; this patient was the only one with evidence of possible co-infection. Seven putative causal agents were identified by serologic testing alone without corresponding positive findings by PCR or culture, including one each of influenza A, influenza B, RSV, and adenovirus. PCR assays performed retrospectively on specimens from patients with positive serologic results for *M. pneumoniae* and C*hlamydia* spp were negative for those organisms. Specimens from an additional two patients showed rises in IgG to multiple antigens, consistent with a nonspecific immune response. No respiratory specimens were positive for SARS-CoV by RT-PCR, although serologic tests of samples from two patients were positive for SARS-CoV antibody.

Sequence analyses confirmed the identity of HMPV and influenza A RT-PCR amplification products. A BLAST (available from: www.ncbi.hlm.nih.gov/BLAST) comparison of five putative HMPV specimens showed identity to HMPV sequences in GenBank. Similarly, influenza A amplification products from 12 patients identified solely by RT-PCR showed homology to known influenza A viruses.

Of the 165 patients tested, 51 (31%) met the criteria for suspect SARS, 19 (12%) met the criteria for probable SARS, and 2 had serologically confirmed SARS (Table 3). A likely pathogen was detected in 23 (45%) of the 51 suspect and 9 (47%) of the 19 probable SARS patients (Table 3). RT-PCR was the most sensitive diagnostic method, identifying a likely causal agent in 21 (41%) suspect SARS patients (influenza A [10 patients], HMPV [4 patients], picornavirus [4 patients], influenza B [1 patient], RSV [1 patient], and PIV3 [1 patient]) and 6 (32%) probable SARS patients (influenza A [4 patients] and HMPV [2 patients]). Serologic testing identified a likely etiologic agent in specimens from an additional 6 patients who met CDC criteria: 3 (16%) for suspect SARS (influenza A [1

Table 2. Detection of respiratory pathogens by polymerase chain reaction (PCR), culture, and serologic testing for cases tested at the California Department of Health Services[a]

	PCR (N = 151); n (%)	Culture (N = 154); n (%)	Serologic testing[b] (N = 78); n (%)
Influenza A	27 (18)[c]	9 (6)	4 (5)[d]
Influenza B	1 (1)	1 (1)	1 (1)[d]
Respiratory syncytial virus	5 (3)	1 (1)	1 (1)[d]
Parainfluenza virus types 2–4	6 (5)	5 (4)	0
Human metapneumovirus	11 (7)	0	ND
Coronavirus OC43	1 (1)	0	ND
Coronavirus 229E	0	0	ND
Parainfluenza virus type 1	0	0	ND
Adenovirus	0	0	1 (1)[d]
Picornavirus	12 (8)	0	ND
Mycoplasma pneumoniae	0[e]	ND	3 (4)[c]
Chlamydia spp.	0[e]	ND	1 (1)
SARS-CoV	0	0	2 (3)
Total positive	63 (42)	16 (10)	13 (17)

[a]ND, not done; SARS-CoV, severe acute respiratory syndrome–associated coronavirus.
[b]Measured as a significant rise in immunoglobulin (Ig) G in paired serum samples for all specimens except one positive for *M. pneumoniae* IgM.
[c]Specimens from one case-patient positive for influenza A (by PCR and culture) were also positive for *M. pneumoniae* IgM.
[d]One specimen negative by culture.
[e]*Mycoplasma pneumoniae* and *Chlamydia pneumoniae* PCRs were performed retrospectively only on specimens from patients with serologic evidence of *M. pneumoniae* (n = 3) and *Chlamydia* spp. (n = 1) infection.

Table 3. Summary of positive laboratory results at the California Department of Health Services by CDC case criteria[a,b]

Pathogen	Total (N = 165) n (%)	Suspect (N = 51) n (%)	Probable (N = 19) n (%)	Confirmed (N = 2) n (%)	Non-SARS cases (N = 94) n (%)
Influenza A	28 (17)[c]	11 (22)	4 (21)	0	13 (14)
Influenza B	2 (1)	1 (2)	0	0	1 (1)
Respiratory syncytial virus	6 (4)	1 (2)	0	0	5 (5)
Parainfluenza virus types 2–4	6 (5)	1 (2)	0	0	5 (5)
Human metapneumovirus	11 (7)	4 (8)	2 (11)	0	5 (5)
Coronavirus OC43	1 (1)	0	0	0	1 (0)
Coronavirus 229E	0	0	0	0	0
Parainfluenza virus type 1	0	0	0	0	0
Adenovirus	1 (1)	1 (2)	0	0	0
Picornavirus	12 (7)	4 (8)	0	0	8 (9)
Mycoplasma pneumoniae	3 (2)[c]	0	2 (11)	0	1 (1)
Chlamydia spp.	1 (1)	0	1 (5)	0	0
SARS-CoV[d]	2 (1)	0		2 (100)	0
Total positive	73 (44)	23 (45)	9 (47)	2 (100)	39 (42)

[a]Using criteria for suspect, probable, or laboratory-confirmed severe acute respiratory syndrome (SARS) recommended by the Centers for Disease Control and Prevention (CDC).
[b]Includes polymerase chain reaction (PCR), culture, and serologic tests.
[c]One patient had evidence of co-infection with both influenza A (PCR and culture) and *M. pneumoniae* (immunoglobulin [Ig] M detection).
[d]SARS-CoV, SARS-associated coronavirus.

patient], influenza B [1 patient] and adenovirus [1 patient]) and 3 (16%) for probable SARS (*M. pneumoniae* [2 patients] and *Chlamydia* spp. [1 patient]). HMPV, whose role in SARS-CoV infection remains undefined, was detected by PCR in four patients with suspect SARS and 2 patients with probable SARS.

Discussion

From March to July 2003, California reported more patients who met criteria for suspect and probable SARS than any other state. Many emergency rooms, hospitals, and public health offices were overwhelmed. Hundreds of persons were evaluated by local counties before being reported to CDHS, where they were classified as having suspect or probable SARS. Of these patients, more than one-third had a pathogen detected that was considered a likely cause of their condition based on their clinical features and course of illness. Twenty-one (81%) of these pathogens were identified by RT-PCR within an average of 4 days. In California, determining a commonly recognized cause for an influenzalike illness allowed cases to be removed from the suspect or probable SARS categories. The resultant removal of a SARS designation alleviated the required epidemiologic investigation, hospitalization or isolation, strict infection control precautions, and additional specimen collection and contact tracing.

Although viral or bacterial co-infection with SARS remains possible, only a few SARS-CoV–infected case-patients worldwide have had documented evidence of dual infection, including *C. pneumoniae*, *M. pneumoniae*, and HMPV (10–13). Most case reports of laboratory-confirmed SARS are noteworthy for the lack of other viral agents (11,14,15). Accordingly, SARS can be ruled out when common culprit viral pathogens are detected in areas without known community transmission of SARS. However, several factors should be considered before discontinuing further evaluation for possible SARS when a likely alternative cause has been identified, including the following: 1) the strength of the epidemiologic link to SARS, 2) specificity of the diagnostic testing performed, and 3) the clinical features and course of illness for the alternative diagnosis. Should SARS become reestablished, these exclusion criteria may need to be reevaluated and applied with particular caution in patients with strong epidemiologic exposure in the context of community transmission.

Applying molecular techniques to outbreak investigations is a relatively recent approach. The limitations of molecular testing include the possibility of false-positive results caused by specimen contamination during processing and false-negative results from primer mismatch or inhibitors in the specimen. Ideally, in the diagnostic setting, positive results by molecular techniques should be confirmed by either testing another specimen by the same method or the same specimen by another method, such as immunoassays or culture. Also, detecting an agent by PCR does not always indicate the true cause of infection; it may instead signify nasopharyngeal carriage or simply be an "innocent bystander."

Nevertheless, given the nonspecific initial signs and symptoms of patients with SARS, the capacity to rapidly diagnose common respiratory infections by using sensitive PCR methods offers advantages in the context of a respiratory outbreak. We found that applying a broad diagnostic molecular panel during the SARS outbreak enabled timely identification of agents of common respiratory viral infections in more than one third of patients with suspect or probable SARS cases. Although serologic testing aided

identification of selected atypical pathogens, the requirement for paired serum specimens did not allow timely removal of a SARS designation. Even when a rapid and definitive diagnostic test for SARS becomes available, laboratories capable of performing molecular-based diagnostic testing, especially for influenza, should be maintained and strengthened.

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Dr. Louie is project director for the California Unexplained Critical Illnesses and Deaths Project, a joint collaborative project of CDHS and CDC. Her research interests include emerging viral infections that cause respiratory illness, particularly the epidemiology, public health aspects, and application of molecular diagnostic testing.

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Address for correspondence: Janice K. Louie, California Emerging Infections Program, California Department of Health Services, 2151 Berkeley Way, Berkeley, CA 94704, USA; fax: 510-883-6015; email: JLouie@dhs.ca.gov

Multistate Shigellosis Outbreak and Commercially Prepared Food, United States

Akiko C. Kimura,* Kammy Johnson,† Mary S. Palumbo,‡ Jacqueline Hopkins,§ Janice C. Boase,¶ Roshan Reporter,# Marcia Goldoft, Karen R. Stefonek,†† Jeff A. Farrar,‡ Thomas J. Van Gilder,† and Duc J. Vugia‡‡**

In 2000, shigellosis traced to a commercially prepared dip developed in 406 persons nationwide. An ill employee may have inadvertently contaminated processing equipment. This outbreak demonstrates the vulnerability of the food supply and how infectious organisms can rapidly disseminate through point-source contamination of a widely distributed food item.

Shigella infects an estimated 450,000 people annually in the United States and is usually transmitted through person-to-person spread (1,2). During January 18–21, 2000, California, Oregon, and Washington reported cases of gastroenteritis that developed in several persons after they ate a commercially prepared (brand X), five-layered bean dip; stool cultures yielded *S. sonnei*. A cohort study of an outbreak at a party implicated this dip; five of six ill attendees had eaten brand X dip compared with none of six well attendees (relative risk = 7.0; 95% confidence interval 1.1 to 42.9). On January 21, the manufacturer began a voluntary recall of the bean dip. We conducted a nationwide investigation to determine the magnitude and severity of the outbreak, confirm its source, and identify the mechanism of contamination.

The Study

A case was defined as *S. sonnei* gastroenteritis that developed within 5 days of a person's consuming brand X five-layered dip. Case-patient follow-up varied by health jurisdiction. In California, detailed interviews were systematically attempted with all case-patients by using a standardized questionnaire.

An environmental investigation of the brand X dip-production facility was completed. It included a review of production procedures and product distribution as well as collection of environmental samples for culture. Employees were interviewed, and stool specimens were collected. Pulsed-field gel electrophoresis (PFGE) patterns of isolates were compared by using PulseNet (3). Antimicrobial susceptibility patterns were obtained from clinical laboratories when possible.

We identified 406 cases in 10 states. Fourteen persons were hospitalized; no deaths were reported. Cases were primarily from the western United States: 217 (53%) from California, 132 (33%) Washington, and 31 (8%) Oregon (Table).

Details of the California outbreak were used to characterize the larger multistate outbreak. Illness onsets ranged from January 8 through February 2, 2000 (Figure). The median incubation period was 2 days (range 1–5 days). The median age of confirmed case-patients was 35 years (range 1–79 years); 65% were female. In addition to diarrhea, the most commonly reported signs and symptoms were abdominal cramps (96%), fever (92%), vomiting (51%), and bloody diarrhea (46%). Most (93%) patients were seen by a physician; 82% were prescribed antimicrobial agents, usually a fluoroquinolone. The median duration of diarrheal illness was 7 days (range 2–21 days).

The dip was a refrigerated product consisting of five layers: beans, salsa, guacamole, nacho cheese, and sour cream. Each layer was prepared and placed in cold storage before manual assembly of the finished product. Preservative (sodium benzoate, 0.1%), when used, was added to individual layers. The beans were the only ingredient cooked during processing. The guacamole and salsa layers contained fresh, raw ingredients and were sold as

Table. Culture-confirmed *Shigella sonnei* cases associated with five-layered bean dip, by patients' state of residence, January 2000

State	No. of cases
Alaska	1
Arizona	1
California	217
Idaho	13
Illinois	2
Minnesota	1
Oregon	31
New Mexico	2
Nevada	6
Washington	132
Total	406

*California Department of Health Services, Gardena, California, USA; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; ‡California Department of Health Services, Sacramento, California, USA; §San Diego County Health and Human Services Agency, San Diego, California, USA; ¶Public Health-Seattle and King County, Seattle, Washington, USA; #Los Angeles County Department of Health Services, Los Angeles, California, USA; **Washington State Department of Health, Shoreline, Washington, USA; ††Department of Human Services, Portland, Oregon, USA; and ‡‡California Department of Health Services, Berkeley, California, USA

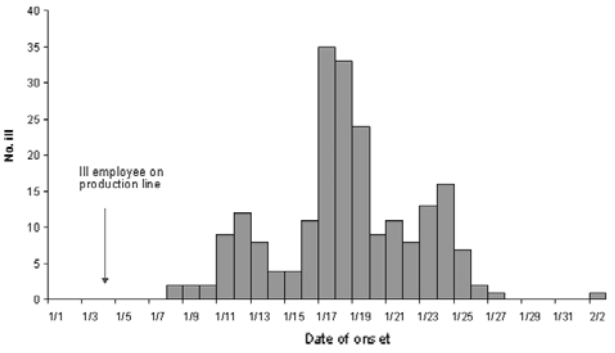

Figure. Illness onset dates of persons who ate brand X five-layered bean dip, California, January–February, 2000 (N = 217).

individual products with and without preservative in addition to being used in the dip. The remaining layers were prepared solely for the dip.

The cheese layer was prepared in large batches by the same employee once or twice a week. Blocks of cheese were cut into chunks with a knife, broken into pieces by hand, and placed into a colloid mill, which sheared the mixture into a paste. When disassembled during the environmental investigation, the colloid mill had a build-up of residue on the shearing mechanism, a part reported as being difficult to clean properly. In addition, the facility had numerous violations of good manufacturing practices (4), including lack of standard operating procedures (4), inadequate refrigeration of the product, and inadequate cleaning and sanitation of processing equipment.

Product distribution was nationwide through a number of vendors but primarily through grocery chain A (17%) and warehouse store B (76%). Both facilities sold the dip without preservatives only in their West Coast stores. Brand X dip production and distribution records were incomplete; however, on the basis of recalled expiration dates, estimated production dates were December 28, 1999–January 18, 2000. All employees working on the dip production line were questioned about gastrointestinal illness just before and during the suspected production period of the contaminated dip. Only one employee reported having gastroenteritis during that period. He went home ill with diarrhea on January 3 and returned to work on January 5 (the plant was not in operation January 4). Stool cultures were not taken at the time of his illness, and he was not given antimicrobial agents; diarrhea reportedly lasted 1 day. He was an hourly employee and had no paid sick leave. Breaking the cheese up by hand and feeding it through the colloid mill were solely the responsibility of this employee.

The PFGE patterns of the outbreak isolates were either indistinguishable (pattern A) or differed by only 1–2 bands. *S. sonnei* with PFGE pattern A was isolated from an unopened container of brand X five-layered bean dip. Antibiograms of *S. sonnei* isolates from 22 case-patients were reviewed; all but one were resistant to both ampicillin and trimethoprim-sulfamethoxazole (TMP-SMZ).

Stool cultures from all employees on the dip production line were negative. Environmental and fresh produce cultures also tested negative for bacterial pathogens; however, cultures of the colloid mixer residue collected on February 2 grew 410,000 coliforms/g but were negative for *Shigella*.

Conclusions

In January and February 2000, we identified 406 cases of drug-resistant shigellosis in 10 states in persons who had eaten brand X five-layered dip. This dip was epidemiologically implicated as the outbreak source through a cohort study and supported by these findings: 1) patients in several states whose only reported common exposure was the dip and who had PFGE-matched *S. sonnei* infections; 2) isolation of *S. sonnei* PFGE pattern A from brand X dip; and 3) outbreak termination after the product was recalled.

Numerous problems in manufacturing practices were noted at the dip-production facility, which suggests that the contamination occurred there, probably by an infected employee. Large-scale shigellosis outbreaks caused by infected food handlers are not uncommon (5,6) and are frequently attributed to poor food-handler hygiene (7). The employee who broke up the cheese by hand reported illness consistent with mild shigellosis during the suspected production period of the implicated product. Though culture-negative, his stool specimens were collected >3 weeks after his symptoms resolved. If this worker inadvertently contaminated the cheese, S*higella* may then have propagated in the colloid mixer, which was not cleaned regularly and was stored in a non–air-conditioned room. The wide range of illness onsets and product expiration dates suggests that contamination occurred on more than one production date, a fact that further supports this hypothesis.

The possibility that this outbreak is produce-related cannot be discounted, particularly since several shigellosis outbreaks have been due to fresh produce (8,9). PFGE pattern A was seen in a parsley-associated outbreak of *S. sonnei* in the summer of 1998 (8). However, despite enhanced surveillance, no illness in persons who ate brand X guacamole or salsa—the only layers containing fresh produce—as stand-alone products was reported. These products were not part of the recall.

The *S. sonnei* isolates from this outbreak were resistant to both ampicillin and TMP-SMZ. This finding has clinical implications because TMP-SMZ has been the treatment of choice for shigellosis acquired in the United States (2). This resistance pattern is common in developing countries, where antimicrobial use is relatively unrestricted, but it has been seen with increasing frequency in the United States

(10,11). Most patients in this outbreak were treated with fluoroquinolones, to which the organism was sensitive.

Most cases occurred in western states, where the dip without preservative was distributed. The antimicrobial effects of the food preservative sodium benzoate have been well-documented (12,13). Having a preservative-containing alternative may have averted a more extensive outbreak of disease.

The evolving epidemiology of foodborne outbreaks reflects changes in the way that food is processed and distributed (14). The consumer can be educated to cook or wash minimally processed products such as raw meats, eggs, and fresh produce thoroughly before eating. However, in the case of a ready-to-eat product such as this dip, the responsibility to ensure safety of the product before opening rests with the growers, manufacturers, distributors, and retailers. Increasing emphasis is being placed on improving food safety through identifying and controlling potential hazards. These establishments also need to provide frequent, linguistically appropriate food safety training for all employees and remove financial disincentives for employees with gastrointestinal illnesses.

In this outbreak, a drug-resistant, virulent organism was rapidly disseminated through a commercially processed product. Although this outbreak was likely unintentional, it illustrates the vulnerability of the food supply, which is increasingly characterized by centralized production and broad distribution, and the potential for commercially produced food to be used in an act of bioterrorism. Intentional contamination of a ready-to-eat, widely distributed food product with an organism that has a low infectious dose (e.g., *Shigella*) can cause considerable illness and can be extremely costly in terms of personal, medical, and public health resources (15). Whether intentional or unintentional, early, open lines of communication between local and state public health departments, the Centers for Disease Control and Prevention, regulatory agencies, industry, clinicians, and consumers are critical in identifying and terminating a widely disseminated outbreak. Continued preparedness of the public health community at all levels to respond to foodborne events through protocol development and exercises designed to test their adequacy is also needed.

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Dr. Kimura is a medical epidemiologist with the Infectious Diseases Branch, California Department of Health Services. She is board-certified in internal medicine and was previously an Epidemic Intelligence Service Officer with the Foodborne and Diarrheal Diseases Branch, Centers for Disease Control and Prevention.

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Address for correspondence: Akiko C. Kimura, Infectious Diseases Branch, California Department of Health Services, 19300 S. Hamilton Ave. Ste 140; Gardena, CA 90248, USA; fax: 310-217-6911; akimura@dhs.ca.gov

Antibacterial Resistance, Wayampis Amerindians, French Guyana

Karine Grenet,* Didier Guillemot,† Vincent Jarlier,‡ Brigitte Moreau,§ Stéphane Dubourdieu,* Raymond Ruimy,* Laurence Armand-Lefevre,* Pierre Bau,* and Antoine Andremont*

Drug resistance in fecal bacteria was high in Wayampis Amerindians who did not take antibacterial agents and were not hospitalized for 1 year. In the Wayampis Amerindians, an isolated traditional community in French Guyana, antibacterial use was 0.64 treatments per person per year. Hospitalization rate was 6.1% per year. Antibacterial drug–resistant bacteria can spread in persons who are not taking antibacterial agents.

Antibacterial-resistant bacteria can spread in persons not taking antibacterial agents. This resistance results from contaminated food, antibacterial drug exposure, and cross-contamination from humans or animals. Antibacterial resistance is high in developing countries (1) because of self-medication, the suboptimal quality of antibacterial drugs, and poor community and patient hygiene (2).

To analyze the role of cross-transmission on the resistance of fecal commensal enterobacteria, we conducted a study from October 1 through 15, 2001, among Wayampis Amerindians who lived in the most southern part of French Guyana, in an isolated, ethnically homogeneous, traditional community. The community was made up of 184 males and 204 females (193 children <15 years and 195 adults), who were evenly distributed in three villages (Z, n = 248; TS, n = 85; and YP, n = 55). Access to the villages was restricted to residents, and the sites were isolated, 100 km south of the closest village. Villagers shared large huts (13.9 + 8.6 inhabitants per hut [range 4–38]) with no latrines or hygienic facilities, and used a single spot on the river for drinking, bathing, and disposal of human waste. They ate only local food (crops grown in a traditional manner and meat from fishing or hunting). They did not raise farm animals except a few free-running chickens. A paramedic officer permanently residing in Z provided the only antibacterial agents in the vicinity and recorded their dispensation. When necessary, the villagers were hospitalized in Cayenne, the capital of French Guyana. Medical care was free.

The Study

Rates of antibacterial exposure were calculated as the ratio of the number of treatments prescribed divided by the number of villagers and compared by using analysis of variance. Chi-square-tests or Fisher exact tests were used for binary variables. During the year preceding the study, 24 (6.1%) of 388 villagers had been hospitalized; 235 (60.6%) had received no treatment with antibacterial agents. One hundred fifty-three (39.4%) of the villagers had received 250 courses of antibacterial treatment. Of the therapeutic agents used, 72 (28.8%) were aminopenicillins, 111 (44.4%) were metronidazole, 36 (14.4%) were macrolides, and 31 (12.4%) were different antibacterial agents (17 penicillin M, 2 penicillin G, 7 cotrimoxazole, 3 cyclines, and 2 first-generation cephalosporins). Ninety-eight (25.3%) villagers had received one course of treatment, 30 (7.7%) received 2 courses of treatment, 13 (3.4%) received 3 courses of treatment, and 12 (2.1%) received ≥4 courses of treatment. Overall antibacterial and aminopenicillin exposures were significantly higher in village Z, where the paramedical officer resided (Table 1), and in children.

In October 1999, one of the investigators (V.J. or A.A.) asked each adult to participate in the study. Children were recruited with the help of the teachers at school. Exclusion criteria were fever, diarrhea, or acute infection, and a stay outside the study zone, a history of hospitalization (verified by Cayenne's hospital records), or treatment with an antibacterial agent (verified by records of the paramedical officer) during the preceding year. We chose this period for surveillance because it was the longest period for which information was available. Information was verified by one of the investigators.

The study was approved by the Comité Consultatif de Protection des Personnes se prêtant à des Recherches Biomédicales (CCPPRB) of Cayenne and by the administrative authorities of French Guyana and was authorized by the French Ministry of Health. Before participants were included, the study was presented to the villagers, with the aid of the chief of the village who explained that some of them would be asked to participate. Consent was obtained before participants were included in the study.

Study participants were asked to bring fresh stool samples (unformed samples were excluded) to the paramedic officers. The samples were then mixed into brain-heart infusion broth with 10% glycerol and frozen in liquid

*Assistance Publique-Hôpitaux de Paris, Paris, France; †Pharmaco-Épidémiologie Institut Pasteur, Paris, France; ‡Groupe Hospitalier Pitié-Salpêtrière, Paris, France; and §Centre Hospitalier de Cayenne, French Guyana

nitrogen. Upon harvesting, 25 μL of broth was added to 2 mL of brain-heart infusion broth, incubated for 4 h, and plated on cetrimide, Chapman, and bile-esculin-acid agar containing 10 mg/L of vancomycin (after an enrichment step of 18 h in broth containing 1 mg/L of vancomycin) to detect *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and vancomycin-resistant enterococci, respectively. Antibacterial-resistant gram-negative bacteria were detected by using two separate methods. The first method explored the predominant flora. Drigalski agar plates were plated with the fecal dilutions; after 48 h growth, five colonies were randomly selected, identified, and tested for antibacterial susceptibility as described (http://www.sfm.asso.fr). A participant was defined as colonized in the predominant flora with gram-negative bacteria resistant to a given antibacterial agent when at least one strain resistant to this antibacterial agent was isolated. The second method explored the subdominant flora. Drigalski agar plates were supplemented with either ampicillin (10 mg/L), ceftazidime (2 mg/L), streptomycin (20 mg/L), kanamycin (20 mg/L), chloramphenicol (20 mg/L), tetracycline (10 mg/L), or nalidixic acid (50 mg/L) (3), used within 24 h after preparation, and kept at 4°C until used. The plates were plated with the fecal dilutions, incubated for 48 h, and inspected for lactose-positive and lactose-negative colonies. A participant was defined as colonized in the subdominant flora with gram-negative bacteria resistant to a given antibacterial agent when at least one colony grew on agar containing the corresponding antibacterial agent. No further identification was performed; for quality control purposes, some positive plates were randomly selected for confirmation of antibacterial susceptibility of the isolates. Because the number were few and unexpected, colonies that grew on agar containing ceftazidime were all identified by conventional methods or 16S RNA gene sequencing (4), when needed; their antibacterial susceptibility was tested; and genes encoding for extended spectrum β-lactamase were characterized, when needed, by polymerase chain reaction and sequencing (5). When needed, the clonality of isolates was determined by using pulsed-field gel electrophoresis (PFGE) (6).

The fecal flora was thus analyzed in a subgroup of 93 volunteers (39 men and 54 women; 41.2% from village TS, 18.1% from village Z, and 23.6% village YP [$p < 0.001$]) who met the inclusion criteria cited above, representing 93 (23.9%) of 388 villagers and 93 (39.6%) of 235 who had not received antibacterial agents for 1 year. Carriage of resistant gram-negative bacteria in subdominant flora of these 93 volunteers ranged from ≥90% for those resistant to ampicillin, streptomycin, and tetracycline to 7% for those resistant to nalidixic acid (Table 2), with no significant difference for sex or age. We found no association between the rate of resistance in the study participants and the number persons who used antibacterial agents or of children in the hut. Fourteen participants (all living in village Z but not in the same hut) were colonized by gram-negative bacteria resistant to ceftazidime, including three *Escherichia coli* strains with a similar pattern by PFGE that produced Bla_{TEM-52} extended spectrum β-lactamase. Nine participants (three, one, and five living in villages TS, YP and Z, respectively, and only two in the same hut) were colonized by strains of *Acinetobacter baumannii* sharing the same susceptibility pattern. Two participants (one in village TS and one in village Z) were colonized by strains of *Ochrobactrum* spp. Neither *S. aureus* or *P. aeruginosa* were isolated. One participant from village Z was colonized by a vancomycin-resistant strain of *Enterococcus gallinarum*.

Resistance rates in the predominant flora were from 95% to tetracycline to 0% to ceftazidime and nalidixic acid, with no significant differences between adults and children, men and women, or villages. Although they were not chosen randomly, approximately 40% of the untreated villagers were included, which suggests that the group was representative of the whole community.

Recently, high resistance rates were also reported in remote populations from Bolivia (7) and Nepal (2). Here, however, we have provided additional information on exposure to antibacterial drugs and hospitalizations of the study participants. In the villages studied, the global antibacterial exposure (0.64 treatments/person/year) was roughly half that of France (8), thus close to the mean rate

Table 1. Antibacterial exposure in 388 Wayampi Amerindians from three villages in southern French Guyana during the year preceding the study[a]

Antibacterial agents	Frequency of antibacterial exposure in (%)				p value[b]
	TS (n = 85)	YP (n = 55)	Z (n = 248)	Total (N = 388)	
Aminopenicillins	5.9	14.6	23.8	18.6	0.007
Metronidazole	16.5	30.9	32.3	28.6	0.15
Macrolides	8.2	1.8	11.3	9.3	0.2
Other	5.8	7.3	8.9	8.0	0.7
Overall	36.5	54.6	76.2	64.4	0.007

[a]Frequency of antibacterial exposure was calculated by dividing the number of all antibacterial courses during the year preceding the study by the number of persons in each village.
[b]Analysis of variance (ANOVA).

Table 2. Prevalence of carriage of gram-negative bacteria resistant to various antibacterial agents in the predominant and the subdominant fecal flora of the 93 study participants[a]

	Predominant flora[b]					Subdominant flora		
Antibacterial agent	*Escherichia coli* (%)	*Klebsiella* sp. (%)	*Enterobacter* sp. (%)	Other (%)	Any (%)	Lactose positive (%)	Lactose negative (%)	Any (%)
Ampicillin	51 (55)	19 (21)	5 (5)	2 (2)	77 (83)	89 (96)	31 (33)	93 (100)
Ceftazidime	0	0	0	0	0	3[c] (4)	11[d] (12)	14 (15)
Nalidixic acid	0	0	0	0	0	7[e] (8)	0	7 (8)
Pefloxacine	0	0	0	0	0	ND	ND	ND
Streptomycin	42 (45)	2 (2)	0	0	44 (47)	83 (90)	19 (21)	86 (93)
Kanamycin	5 (5)	0	0	0	5 (5)	45 (49)	4 (5)	47 (51)
Tetracycline	59 (63)	14 (15)	0	2 (2)	75 (81)	86 (93)	25 (27)	86 (93)
Cotrimoxazole	27 (29)	0	0	0	27 (29)	ND	ND	ND
Chloramphenicol	22 (24)	0	0	0	22 (24)	48 (52)	27 (29)	60 (67)

[a]Accounting for 23.9% (93 of 388) of the global population of the villages studied and not significantly different from it for male/female ratio or age distribution.
[b]Bacteria with intermediate susceptibility were categorized as resistant.
[c]Extended spectrum β-lactamase *E. coli.*
[d]*Acinetobacter baumannii*: 9 (all with the same susceptibility pattern including susceptibility to ureidopenicillins, third -generation cephalosporins, aminoglycosides, and quinolones) and *Ochrobactrum* spp. (identified by 16s RNA genes sequencing): 2.
[e]*E. coli.*

of European Union countries (9), which stresses the indirect impact of antibacterial use on persons who do not use them. Unrecorded antibacterial drug use in the participants was unlikely because no alternate sources of antibacterial agents existed, and free antibacterial agents were provided when needed, decreasing the likelihood of nonprescribed use or sharing of antibacterial agents by family members.

Our results can be compared only to those of studies performed with similar methods and in persons who also had not taken antibacterial drugs. For instance, resistance rates of predominant *E. coli* in the Wayampis population to ampicillin, tetracycline, and streptomycin were higher than that observed in French bank workers (10) or Bostonian children (1), but close to that reported in children from Venezuela and China (1). That the resistance rate was higher in the Wayampis Amerindians than in the French workers was unexpected, considering the higher levels of antibacterial drug use in the French community. These data suggested frequent cross-transmission likely attributable to poor hygiene (1,2). Cross-transmission is common among households, even in industrialized countries (11). Its role in the spread of drug-resistant bacteria among the Wayampis Amerindians, even in adults, was further indicated by the lack of difference in resistance rates according to age.

Resistance to quinolones, which were not used in this community, was lower than in Europe (12), which illustrates the specificity of selective pressure. Third-generation cephalosporins were also not used. Thus, how samples from three participants were colonized by an *E. coli*–carrying Bla_{TEM-52} (13), an extended-spectrum β-lactamase-gene found only in hospitals, is difficult to explain. However, enterobacteria that produced an extended spectrum β-lactamase were prevalent at the time in the Cayenne hospital (B.M., unpub. data). Since the three persons with the Bla_{TEM-52} -colonized samples had not been hospitalized during the previous year, possibly this strain, or a different one, carrying Bla_{TEM-52} was acquired and spread in the community by one of the 24 villagers hospitalized during the year preceding the study. Extended-spectrum β-lactamase can disseminate in the community (14). We cannot exclude that the three Bla_{TEM-52} carriers had been hospitalized earlier. If they were hospitalized and colonized during hospitalization, this colonization would not have been likely to have persisted for so long; indeed, carriage of resistant nasal staphylococci can last for months, but resistance in intestinal enterobacteria decreases within 10–20 days after selective pressure ends (15).

Conclusions

Antibacterial agents in the food chain are a source of resistance in industrialized countries (16). In our study, food was strictly local but may have been the source of the wild-type naturally resistant *A. baumannii* and *Ochrobactrum* spp. strains that we isolated. Environmental species have also been isolated in Amerindians living in nearby (formerly Dutch) Guyana (17).

Because data confirmed the lack of direct antibacterial drug exposure in our study participants, the results demonstrate that, once resistance elements are introduced into a population, moderate use of antibacterial drugs in the environment is enough to maintain them in intestinal bacteria when sanitary conditions are poor.

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Dr. Grenet is a pharmacist and clinical biologist. She is currently working in the Laboratory of Bacteriology, Groupe Hospitalier Pitié-Salpêtrière, Paris, France. Her main research insterest is bacterial resistance in humans.

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Address for correspondence: Antoine Andremont, Laboratoire de Bactériologie, Hôpital Bichat Claude Bernard, 46 rue Henri-Huchard, 75018 Paris, France; fax: 331- 40-25-85-81; email: antoine.andremont@bch.ap-hop-paris.fr

Global Genetic Diversity of Human Metapneumovirus Fusion Gene

Guy Boivin,* Ian Mackay,† Theo P. Sloots,† Shabir Madhi,‡ François Freymuth,§ Dana Wolf,¶ Yonat Shemer-Avni,# Herbert Ludewick,‡ Gregory C. Gray, and Éric LeBlanc***

We analyzed 64 human metapneumovirus strains from eight countries. Phylogenetic analysis identified two groups (A and B, amino acid identity 93%–96%) and four subgroups. Although group A strains predominated, accounting for 69% of all strains, as many B as A strains were found in persons ≥3 years of age.

Studies from various parts of the world have identified human metapneumovirus (HMPV) as one of the leading causes of hospitalization for acute respiratory tract infections in young children (1–5). Severe respiratory infections associated with HMPV have also been reported in elderly and immunocompromised persons (6,7). Studies from our group (6,8) and others (2,5) have identified two major lineages of HMPV, with some studies indicating the splitting of those groups into subgroups. Recently, the complete genomic sequence of a representative strain from each of the two groups was determined (9). In this study, our objective was to analyze the fusion (F) gene sequences of a large set of HMPV strains collected from various countries over several years and to identify sequence signatures in different HMPV subtypes.

The Study

HMPV sequences included were from isolates grown in LLC-MK2 (monkey kidney) cells or polymerase chain reaction (PCR)-amplified products from nasopharyngeal aspirates (NPA) (1,3,5,6,10). Viral RNA was extracted from 200 µL of cell culture supernatants or NPA specimens by using the QIAamp viral RNA Mini Kit (QIAGEN, Inc., Mississauga, ON, Canada). For phylogenetic studies, nucleotide sequences were obtained from amplified HMPV F-gene products as previously described (6). The sequence region comprising nucleotides 60–708 of the F gene was entered into a multiple alignment generated by Clustal-W and corrected through final visual inspection with the SeqLab application (Wisconsin package version 10.3, Accelrys Inc., San Diego, CA). Phylogenetic analysis was performed by using distance methods with the PAUP 4.0b10 program (Sinauer Associates, Inc., Sunderland, MA) for the Macintosh. The parameters for the distance method were Kimura 2-parameters, using the neighbor-joining algorithm. Five hundred additional bootstrap analyses were performed on those phylogenetic trees.

A total of 64 HMPV sequences were analyzed, 34 from Canada (years 1993–2002) and 30 from various other countries (years 2000–2002): Peru (n = 2), the United States (n = 2), France (n = 6), Israel (n = 5), Republic of South Africa (n = 7), Australia (n = 7), and the prototype strain 001 (GenBank accession no. AF371337) from the Netherlands. Phylogenetic analysis of the HMPV F gene showed the existence of two main groups (A and B) that could be further subdivided into two subgroups (1 and 2; Figure 1). Bootstrap analysis strongly supported dividing

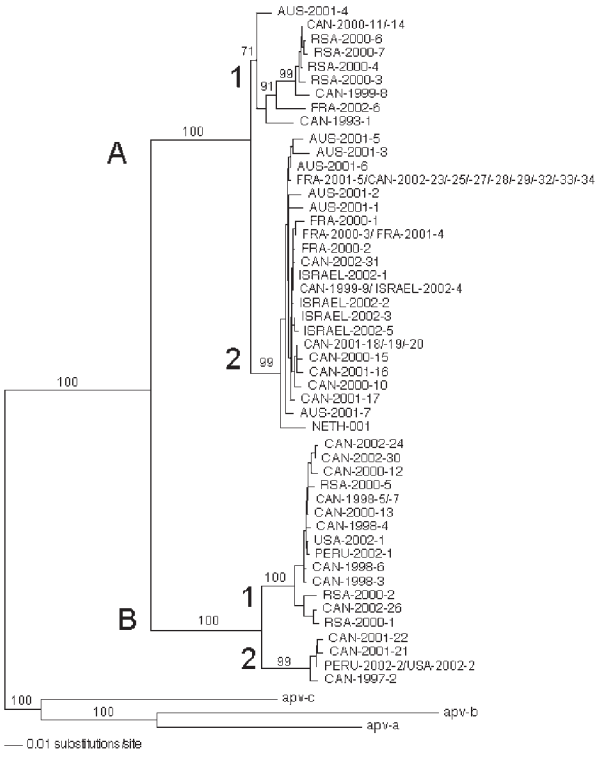

Figure 1. Phylogenetic analysis of the fusion (F) gene of 64 human metapneumovirus (HMPV) strains recovered from various countries (CAN, Canada; RSA, Republic of South Africa; FRA, France; AUS, Australia; NETH, the Netherlands). Neighbor-Joining consensus tree was obtained from the nucleic acid alignment representing nucleotides 60–708 of the HMPV prototype sequence NETH-001. Numbers represent the frequency of occurrence of nodes in 500 bootstrap replicas.

*Centre Hospitalier Universitaire de Québec and Laval University, Québec City, Canada; †Royal Children's Hospital, Herston, Queensland, Australia; ‡University of Witwatersrand, Bertsham, South Africa; §Centre Hospitalier Universitaire de Caen, Caen, France; ¶Hadassah University Hospital, Jerusalem, Israel; #Soroka Medical Center, Beer Sheva, Israel; and **University of Iowa, Iowa City, Iowa, USA

HMPV into the A and B clusters (100% of bootstrap replicas) as well as subdividing them into B1, B2, and A2 (99%–100% of bootstrap replicas). However, the topology of the tree regarding the A1 subgroup was supported by only 71% of the bootstrap replicas. This result may be explained by the strain AUS-2001-4, which could not be firmly assigned to any of the two A subgroups. In addition, even though the strain CAN-1993-1 was strongly supported as a member of the A1 group by 91% of the bootstrap replicas, it was clearly the most divergent, as can be expected from an older strain.

Identical F-gene sequences (648 nt) were found for some strains isolated in the same year from the same country (CAN-1998-5/7, CAN-2000-11/14, CAN-2001-18/19/20) but also for strains isolated in different years from different countries (ISRAEL-2002-4 and CAN-1999-9, FRA-2001-5 and CAN-2002-23/25/27/28/29/32/33/34). Topology of the phylogenetic tree was also supported by analysis of homology between sequences. Nucleotide identity between groups A and B was 81.5%–85.3%, whereas it was 91.6%–95.3% and 92.0%–94.1% between subgroups A1–A2 and B1–B2, respectively. The sequences within the three subgroups A2, B1, and B2 shared a nucleotide identity of 96.3%–99.0%, 96.0%–99.9%, and 97.2%–99.4%, respectively. The A1 group was the most divergent; sequences shared 94.4%–99.9% of nucleotide identity, which was consistent with phylogenetic data. AUS-2001-4 and CAN-1993-1 strains shared only 94.2% and 96.5% of nucleotide identity with other members of the A1 subgroup. At the amino acid level, the identity was 93.1%–96.3% between groups A and B, whereas it was 96.3%–99.1% and 98.1%–99.1% between subgroups A1–A2 and B1–B2, respectively. Within subgroups, amino acid identity was 98.2%–100%, 96.8%–100%, 99.5%–100%, and 99.1%–100% for subgroups A1, A2, B1, and B2, respectively. Based on phylogenetic analysis, the distance between the two HMPV groups was slightly smaller than between prototypes from the two respiratory syncytial virus (RSV) groups (14.5 versus 16.5 substitutions/100 residues, data not shown).

An amino acid alignment of all distinct HMPV F sequences (representing amino acids 20–233 of the prototype strain NETH-001), as well as those of other metapneumoviruses and pneumoviruses, is shown in Figure 2. Cysteine residues were conserved at positions 28, 60, and 182 in all HMPV strains. Analysis of this multiple sequence alignment showed six amino acid substitutions found at positions 61, 122, 135, 167, 175, and 233 that may be used as signature sequences to differentiate group A and B strains. In addition, we also identified an amino acid substitution at position 185 that may allow us to differentiate the A1 subtype from others, whereas substitutions specific to B2 subtypes were located at positions 143 and 179. Some of these substitutions were located in potential functional domains, such as the fusion domain (codon 122) and the heptad repeat A (HRA) region (codons 135, 143, and 167). Two potential N-glycosylation sites were found in all HMPV sequences.

We investigated associations between HMPV genotypes and demographic or clinical data, although the small number of non-Canadian strains limited analyses. Based on phylogenetic analysis, a total of 44 (68.8%) HMPV strains were classified in group A (9 [14.1%] A1 and 35 [54.7%] A2), whereas 20 (31.2%) belonged to group B (15 [23.4%] B1 and 5 [7.8%] B2). Group B strains accounted for 38.2% and 23.3% of Canadian and non-Canadian strains, respectively ($p = 0.20$). No B strains were recovered from France, Australia, and Israel as part of this study. Cocirculation of both A and B strains was found in Canada during 2000, 2001, and 2002 as well as in South Africa during 2000. Among the 26 HMPV strains recovered before 2001, 57.7% were from group A compared to 42.3% from group B, whereas the ratios were 76.3% and 23.7% for the 38 strains analyzed in 2001 and 2002 ($p = 0.12$). When analysis was restricted to Canadian strains, group A strains accounted for 46.7% of all HMPV recovered before 2001 and 73.7% of HMPV recovered after 2001 ($p = 0.11$). All but one of the recent (year 2001 or later) A strains were from the A2 subgroup.

Subsequent analyses were performed without the prototype strain NETH-001, for which no detailed information was available. Most strains from the Northern Hemisphere (41 [87.2%] of 47) and the Southern Hemisphere (11 of [68.8%]of 16) were recovered over the typical respiratory virus season, spanning a 5-month period during winter and spring. Most (69.8%) of the HMPV strains were recovered from young children ≤3 years, whereas only 25.6% were from adults ≥18 years of age, although this finding may only reflect more intense investigation into viral cases in children. Group B strains accounted for 25% of total HMPV strains from study participants ≤3 years of age compared to 47.4% of those from patients >3 years of age ($p = 0.08$). The ratios of group A/group B strains were 62.5%/37.5% for the 32 male and 74.2%/25.8% for the 31 female patients ($p = 0.32$). Groups A and B strains were associated with a similar proportion of cases of pneumonitis (12 [27.9%] of 43 vs. 7 [35%] of 20, $p = 0.57$). Finally, although group B strains were found in approximately half of the positive LLC-MK2 cultures (14 [53.8%] of 26), such strains only represented 16.2% of total HMPV strains detected by reverse transcription–PCR from NPA samples ($p = 0.002$).

Conclusions

Our results, based on a large dataset of viral strains collected over several years from the Northern and Southern

Hemispheres, confirm two main HMPV groups and at least four minor subgroups. One strain (AUS-2001-4) could not be ascribed to one of the two A subtypes and may constitute a third subtype. We have further demonstrated that both HMPV genotypes could circulate in a single year during a typical respiratory virus season, i.e., over the winter and early spring months in countries in both hemispheres. Our phylogenetic data were based on analysis of the HMPV F protein, one of two viral glycoproteins considered the major antigenic determinant in RSV (11). Our data confirm that HMPV is more closely related to avian pneumovirus type C than to RSV, which is classified in a separate genus (*Pneumovirus*) within the same subfamily.

Overall, the amino acid sequence identity of the two HMPV F groups was slightly higher than that calculated for the two RSV groups (93%–96% vs. 89%) (11).

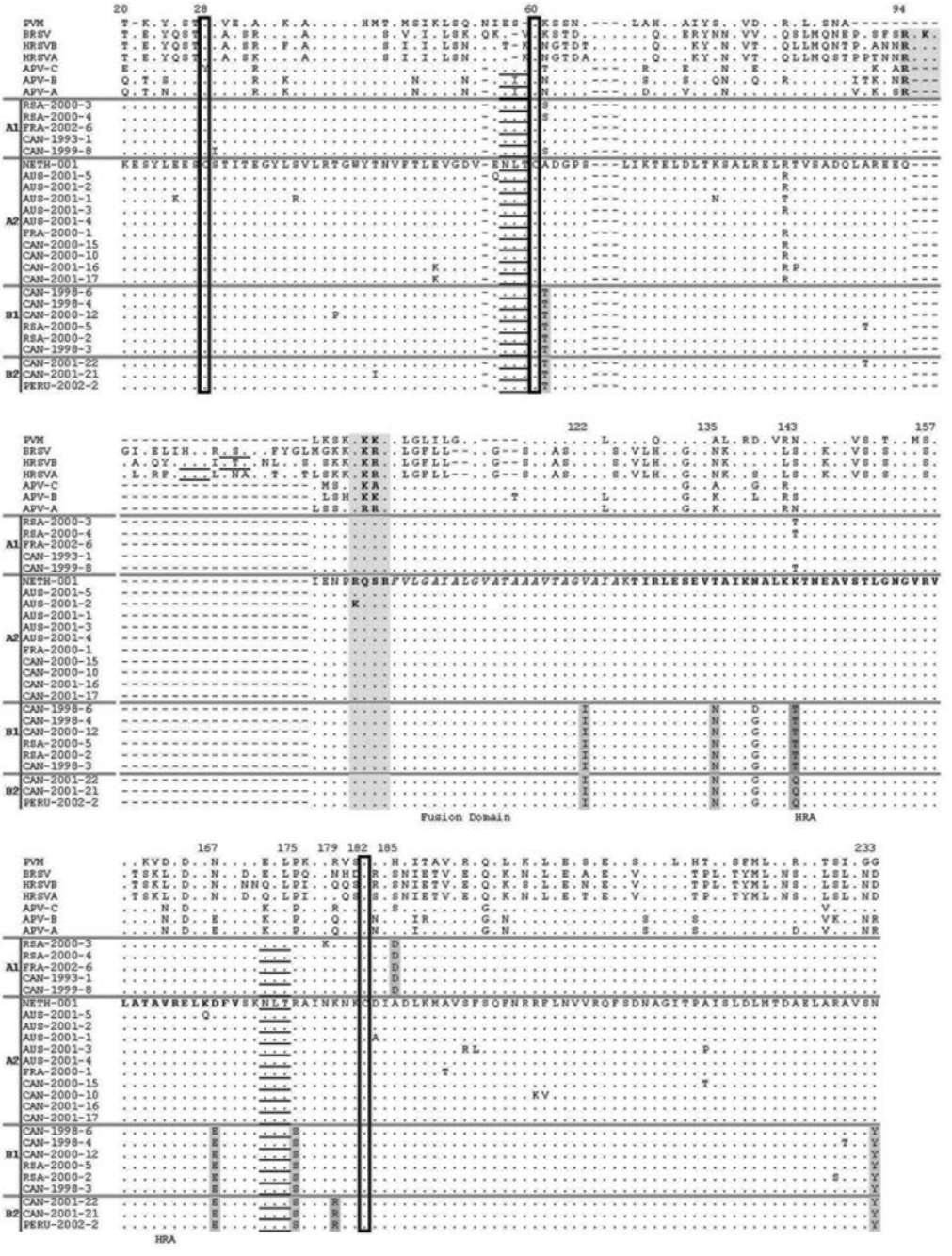

Figure 2. Amino acid sequence alignment of the fusion (F) protein of various human metapneumovirus (HMPV) strains and other paramyxoviruses. For full reproduction of this image, please see http://www.cdc.gov/ncidod/EID/ 03-1097-G2.htm Note that only distinct HMPV strains were included in the alignment.

Although only the first half of the F gene was sequenced in this study, our data indicate that all A and B strains can be easily differentiated on the basis of unique amino acid changes, some of which are located in functional regions of the protein, e.g., the putative fusion domain and the HRA region implicated in paramyxovirus fusion to their cell receptors. Some of the latter amino acid changes were nonconservative. Of note, the sequence signatures reported here were also present in two other recent Canadian isolates representing the two major HMPV groups (12). Additional studies will be required to evaluate the impact of such changes on pathogenesis and immune response.

Close to 70% of all HMPV strains belonged to group A with a possible shift towards more A2 strains in recent years. However, more young children were evaluated in our study, and the group A genotype infects three times as many children ≤3 years of age. In addition, group B strains, which occurred more frequently in adults, could have been underestimated, as suggested by the detection of fewer group B strains in clinical samples (16%) than in infected LLC-MK2 cells (54%). In that regard, most HMPV PCR primers designed so far have been selected from the sequence of strain NETH-001 (which belongs to group A2 in this study), and consequently, they may not have been optimal to detect group B strains. Alternatively, isolating HMPV group A strains may be less efficient in LLC-MK2 cells. Additional studies are required to validate these hypotheses. In RSV, group A strains are often thought to be associated with more severe disease than are group B strains (13). However, we found no differences in severity between the two HMPV genotypes when we used pneumonitis as the clinical endpoint in this small, retrospective study.

Our study has some limitations. We analyzed relatively few non-Canadian strains over a short period of time, consistent with the recent description of this viral pathogen in 2001. Also, much more genetic variability could have been observed by sequencing the gene encoding for the attachment glycoprotein (G gene) as suggested by RSV and limited HMPV sequences (14,15). Using different PCR primers to initially identify HMPV and studying different populations in the various centers may have also introduced bias in interpreting our results. Nevertheless, our study confirms the worldwide distribution of HMPV and provides initial insights into the epidemiology of the two main viral genotypes.

Dr. Boivin is an infectious disease specialist and virologist at the Centre Hospitalier Universitaire de Québec. He is also an associate professor of microbiology at Laval University, Québec City, Québec, Canada. His main research interests include the characterization of old and emerging respiratory viruses and the mechanisms of antiviral drug resistance for herpesviruses.

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Address for correspondence: Guy Boivin, CHUL, Room RC-709, 2705 Blvd Laurier, Sainte-Foy, QC, Canada GIV 4G2; fax: 418-654-2715; email: guy.boivin@crchul.ulaval.ca

Antibiotics in Animal Feed and Spread of Resistant *Campylobacter* from Poultry to Humans

Nicole M. Iovine* and Martin J. Blaser*†

Contamination of food with potentially dangerous human pathogens has been recognized since the time of Pasteur (1) and is well-documented in the modern era (2), but the development of antimicrobial agents has helped limit the consequences of such infections. Concomitantly, the widespread use of antimicrobial agents has also led to the emergence of antimicrobial drug–resistant organisms (3,4). Gupta et al. demonstrate the increasing prevalence in the United States of ciprofloxacin-resistant *Campylobacter* species isolated from humans and poultry from 1990 to 1997, and their studies implicate the prophylactic treatment of poultry with fluoroquinolones in this emerging problem (5). Their report indicates that the source of fluoroquinolone-resistant *Campylobacter* infections was consuming poultry colonized with resistant strains (Figure), rather than selection for *Campylobacter* resistance in the human gut after clinical fluoroquinolone use to treat the diarrheal illness (5). This work provides further evidence that fluoroquinolone use in poultry promotes the emergence of resistant *Campylobacter* strains that subsequently infect humans (6). That persons infected with these fluoroquinolone-resistant strains had 3 additional days of illness and were more likely to be hospitalized demonstrates the harm caused by such resistant stains (5).

Since campylobacters are normal enteric flora in many avian species, poultry represents a model system to test the hypothesis that prophylactic and growth-promoting use of antimicrobial agents in food animals selects for the emergence of antimicrobial drug–resistant organisms. In one study, chickens that were naturally colonized with fluoroquinolone-susceptible *Campylobacter* strains began to excrete resistant strains after 2 days of doses of enrofloxacin (7), which is commonly used for prophylaxis in the poultry industry. A single point mutation in gyrA encoding the bacterial DNA gyrase was sufficient to confer high-level resistance (7,8). This small genetic change apparently has a low "fitness cost" to the organism, as evidenced by fluoroquinolone-resistant strains' rapidly replacing susceptible *Campylobacter* in treated chickens (7). Developing an animal reservoir of fluoroquinolone-resistant *Campylobacter* has been the major factor behind transmission of quinolone resistance to humans (8,9).

In contrast, among poultry treated therapeutically with enrofloxacin, no resistance was observed in the 13 *C. jejuni* isolates tested (9). Similarly, after the prophylactic and growth-promoting uses of macrolides in swine were banned in Denmark, the prevalence of macrolide-resistant *C. coli* declined (10). Thus, the major determinant of developing resistance appears to be use of subtherapeutic antimicrobial doses. The antimicrobial drug ban in Denmark did not decrease the amount of meat produced by the poultry and pig production industries, which removed a major concern (10). Evidence suggests that restricting fluoroquinolone use to therapeutic indications only in food animals could decrease rates of fluoroquinolone-resistant *Campylobacter*, and the Danish experience with macrolide

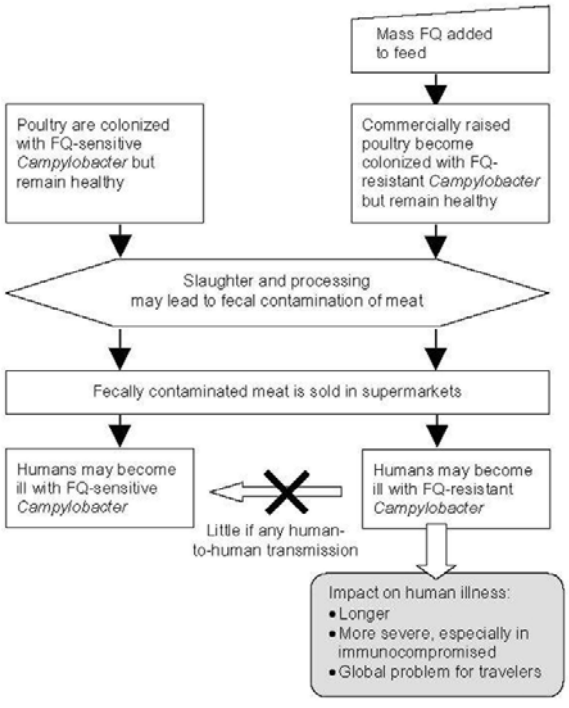

Figure. Acquisition of fluoroquinolone (FQ)-resistant *Campylobacter* from poultry.

*New York University School of Medicine, New York, New York, USA; †New York Harbor Department of Veterans Affairs Medical Center, New York, New York, USA

restriction proves that such limitations need not harm the husbandry of food animals.

The increased likelihood of foreign travel in persons infected with ciprofloxacin-resistant strains (5) illustrates the global threat posed by resistant strains. Appreciating such realities favors concerted efforts to limit use of fluoroquinolones (and other antimicrobial drugs) to therapy only in food animals. This view was supported by a recent (March 2004) landmark decision by Federal Drug Administration Administrative Law Judge Daniel J. Davidson, withdrawing approval for the new animal drug application to use enrofloxacin for prophylaxis or growth-promotion in poultry (11). This decision was the first occasion that a previously approved antimicrobial agent was removed from the U.S. veterinary market because of concerns about antimicrobial drug resistance. With this decision as precedent, we should follow the examples set in Europe and ban use of all antimicrobial agents in food animals, except when necessary for therapy of ill animals.

Dr. Iovine is a fellow in the Division of Infectious Disease and a postdoctoral fellow in the Department of Microbiology at New York University. She studies the interactions between human enteric pathogens and innate host immunity.

Dr. Blaser is the Frederick H. King Professor and chair of the Department of Medicine, and professor of microbiology at New York University. He has been studying *Campylobacter* infections since 1977.

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Address for correspondence: Martin J. Blaser, Department of Medicine, NYU School of Medicine, 550 First Avenue, OBV-606, New York, NY 10016, USA; fax: 212-263-3969; email: martin.blaser@med.nyu.edu

Emerging Issues in Infective Endocarditis

Beverley C. Millar* and John E. Moore*

Infective endocarditis, a serious infection of the endocardium of the heart, particularly the heart valves, is associated with a high degree of illness and death. It generally occurs in patients with altered and abnormal heart architecture, in combination with exposure to bacteria through trauma and other potentially high-risk activities involving transient bacteremia. Knowledge about the origins of endocarditis stems from the work of Fernel in the early 1500s, and yet this infection still presents physicians with major diagnostic and management dilemmas. Endocarditis is caused by a variety of bacteria and fungi, as well as emerging infectious agents, including *Tropheryma whipplei*, *Bartonella* spp., and *Rickettsia* spp. We review the evolution of endocarditis and compare its progression with discoveries in microbiology, science, and medicine.

Endocarditis is a noncontagious chronic infection of the valves or lining of the heart, mainly caused by bacteria, although fungi can also be associated with this infection (1). The risk of infection of heart valves in persons predisposed to acquiring infective endocarditis increases with the following conditions: congenital heart disease, rheumatic fever, major dental treatment, open heart surgery, and genitourinary procedures. New evidence is growing that changes in social behavior, such as an increase in the incidence of body piercing, excessive alcohol consumption, and the use of intravenous self-administered illicit drugs may also predispose a susceptible person to an increased risk of acquiring endocarditis. The patient may exhibit any of the following signs and symptoms: fatigue and weakness; weight loss; fever and chills; night sweats; heart murmur; aches and pains; painful nodes in the pads of fingers and toes; red spots on skin of palms and soles; nail abnormalities; swelling of feet, legs, and abdomen; shortness of breath with activity; and blood in the urine. A medical history, physical examination, and echocardiogram are usually performed. Blood samples are usually taken, and the physical and biochemical properties of the blood are investigated. Endocarditis is usually curable provided an early diagnosis is made, and the patient receives the appropriate antimicrobial treatment; the time needed for recovery is approximately 6–8 weeks. The patient generally requires long-term antimicrobial drugs (4–6 weeks), hospitalization, and in some cases, valve replacement. A number of complications may be associated with the disease such as blood clots, stroke, heart rhythm problems, abscesses, and other infections. Infective endocarditis is associated with severe illness and death and generally occurs in patients with altered and abnormal heart architecture who have been exposed to bacteria through trauma and other potentially high-risk activities.

In 1885, Sir William Osler presented three Gulstonian Lectures on the topic of malignant endocarditis, which gave a comprehensive account of the disease and outlined the difficulties in its diagnosis (2). The disease had, in fact, been described by a French Renaissance physician, Jean François Fernel, approximately 350 years previously (3). More than 100 years after Osler's lectures, this serious infection can still remain a diagnostic and therapeutic dilemma. Its name has been changed several times, first to "bacterial endocarditis" and subsequently to "infective endocarditis" after the observation that microbiologic agents other than bacteria may cause the disease. In the early years of the new millennium, infective endocarditis still proves to be difficult to diagnose and is associated with a high death rate (21%–35%). Although many developments have taken place with respect to antimicrobial drug therapy in the treatment of the disease, its incidence is continuing to rise, with 3.3 cases per 100,000 population per year in the United Kingdom, with similar figures for the United States and 1.4–4.0 cases per 100,000 population per year in Europe as a whole (4). The reasons for this rise are the following: 1) longer survival of patients with degenerative heart diseases, 2) increased use of antibiotics, 3) increased incidence of prosthetic heart valves, 4) congenital heart disease in younger children, 5) increase in bicuspid valve disease, 6) advances in medical and surgical treatments, 7) increase in the number of injection drug

*Belfast City Hospital, Belfast, Northern Ireland, United Kingdom

users, and 8) more sensitive and specific diagnosis. Generally, the incidence is higher in men than in women (2:1), and the average age group affected is in the fifth decade (2).

Historical Perspective

A historical description of developments in endocarditis closely reflects concurrent developments in laboratory medicine, particularly microbiology. Much of the innovations and developments relating to infective endocarditis were made by physicians in Europe, particularly in France (Appendix). Important contributions were, however, made by several German physicians, particularly in association with the birth of bacteriology (Appendix). More recently, the United States has played a strong role in helping define guidelines and diagnostic criteria that facilitate diagnosing infective endocarditis, including the Beth Israel (5), Duke (6) (Table 1), and modified Duke criteria (7,8) (Table 2). In addition, the American Heart Association has published several seminal articles on the antibiotic treatment and prevention of infective endocarditis (9).

For approximately the first 200 years after the disease was initially described, the anatomy of the heart and heart valves in the diseased state of infective endocarditis was comprehensively elucidated in medical anatomical sketches made after postmortem examination. (For a comprehensive account of the early description of endocarditis, see Contrepois [10].) Not until the early to mid-1800s were descriptions recorded of the medical signs and symptoms of the disease in live patients. Such descriptions included the detection of cardiac murmurs, after percussion and auscultation. Detection of such murmurs was aided by the development of the stethoscope in 1816. From 1830 to 1840, elevated body temperature was recorded as an important symptom of the disease. However, not until the late 1800s and early 1900s was a comprehensive synthesis of information formed by various scholars in Europe and North America, including Sir William Osler in Canada (2) and Thomas Horder in England (11) (Appendix). Osler and Horder were instrumental in establishing fundamental mechanisms regarding the pathophysiology of infective endocarditis and are, to a large degree, responsible for how

Table 1. Original Duke criteria for the diagnosis and classification of infective endocarditis[a]

Major criteria	Minor criteria	Diagnosis
1. **Positive blood culture** i) Typical organism in ≥2 blood cultures in the absence of a primary focus (*Staphylococcus aureus*, enterococci, viridans streptococci, *Streptococcus bovis*, HACEK) ii) Persistently positive blood culture drawn more than 12 h apart or all ¾ drawn at least 1 h apart between first and last	1. **Predisposition** Heart condition Drug abuse	1. **Definite** 2 Major 1 Major and 3 minor 5 Minor pathologic/histologic findings
2. **Evidence of endocardial involvement** i) Positive echocardiogram (TOE) Oscillating intracardiac mass on valve, implanted material or supporting structures in path of regurgitant jets Abscess New partial dehiscence of prosthetic valve ii) New valvular regurgitation	2. **Fever** >38°C	2. **Possible** Findings fell short of the definite but not rejected categories
	3. **Vascular phenomena** Major arterial emboli Janeway lesions Septic pulmonary infarcts	3. **Rejected** Alternate diagnosis Resolution of the infection with antibiotic therapy for ≤4 days No pathologic evidence after antibiotic therapy
	4. **Immunologic phenomena** Osler's nodes Roth spots Rheumatoid factor Glomerulonephritis	
	5. **Microbiologic evidence** Positive blood culture not meeting major criteria Positive serologic finding	
	6. **Endocardiographic evidence** Consistent with infective endocarditis but not meeting the major criteria	

[a]Source: (6); HACEK, *Haemophilus aphrophilus*, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae* group; TOE, transesophageal echocardiogram.

Table 2. Recent suggested modifications to the Duke criteria for the diagnosis of infective endocarditis (IE)[a]

Microbiologic	Biochemical	Clinical
Blood culture Bacteremia due to *Staphylococcus aureus* should be considered a major criterion regardless of whether the infection is nosocomially acquired or whether a removable source of infection is present Serology Positive for *Coxiella burnetii* (major criterion) Positive for *Bartonella* spp. Positive for *Chlamydia* spp. Molecular Evidence for the presence of bacterial or fungal DNA in blood or valve material (major criterion)	Elevated level of CRP >100 mg/L Elevated ESR defined as more than one and a half times higher than normal, i.e. , >30 mm/h for patients <60 years of age >50 mm/h for patients >60 years of age	Possible endocarditis now defined as one major and one minor criterion or three minor criteria Omission of criterion "echocardiogram consistent with IE but not meeting major criterion" Newly diagnosed clubbing Evidence of splinter hemorrhages Petechiae Microscopic hematuria (disregarded for patients with positive urine cultures, menstruating women, patients with end-stage renal disease and patients with urinary catheters) Presence of central nonfeeding venous lines or peripheral venous lines (minor) Purpura

[a]Sources: (7,8); CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

we view endocarditis today. The Figure (online only; available at: http://www.cdc.gov/ncidod/EID/vol10no6/03-0848-G..htm) and Appendix chronologically map the history of infective endocarditis, including diagnostic developments, treatment, and prevention, and emerging causal agents.

The birth of bacteriology as a separate discipline of pathology gave rise to the introduction of the important description of microbiology in the etiology of infective endocarditis. With the early technical innovations of Pasteur in France in the 1880s, routine blood cultures were introduced in the late 19th century as an important part of laboratory investigation into the microbiologic causes of infective endocarditis. Although causal agents of infective endocarditis could now be detected and clearly described, little could be achieved in terms of their eradication because the existence of antibiotics was as yet unknown. However, in Germany, Gerard Domagk, bacteriologist and pathologist, was appointed as director of the I.G. Farbenindustrie (Bayer) Laboratory for Experimental Pathology and Bacteriology in Wuppertal in 1925. Domagk was innovative in that he began to experiment with dyes, looking for their possible effects against various infections. He described the effect of prontosil red against streptococcal infections in mice; the active component of prontosil was later described as sulfanilamide. At approximately the same time, Sir Alexander Fleming discovered the antibacterial effects of a secondary metabolite (penicillin), produced from a filamentous fungus. Such discoveries were revolutionary because medicine now had an effective means of treating bacterial infections, including infective endocarditis, caused by a wide variety of bacterial pathogens, most notably *Streptococcus* species. Since wild-type pathogens had not had sufficient time to develop resistance to these newly described antimicrobial agents, treatment failures due to resistance were infrequent. Fleming did observe, however, that some organisms were resistant to penicillin and suggested that the phenomenon be followed up. Approximately 60 years later, the marked increase in resistance to antimicrobial agents is cause for concern on all continents. The tangible consequence is that clinicians may have fewer antimicrobial agents to treat both benign and serious infections, including infective endocarditis. To combat the threat of such a "postantibiotic era," the global pharmaceutical industry has responded by producing novel antimicrobial agents, including the carbapenems (imipenem and ertapenem), the oxozolidones (linezolid), and improved antifungal agents (caspofungin and voriconazole), which prolong antimicrobial effectiveness before the problem of resistance evolves with such new agents.

Over the past century, streptococci and staphylococci have remained the main causative organisms associated with infective endocarditis, with an increase in cases due to staphylococci associated with injection drug users and HIV patients. With substantial advances made in the isolation and identification of microorganisms, scientists now recognize a wide spectrum of causal organisms. Although rare, infective endocarditis is caused by gram-negative organisms such as the HACEK (*Haemophilus aphrophilus*, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, *Kingella kingae*) group, *Bartonella* spp., and *Coxiella burnetii*. More recently, cases of fungal endocarditis have increased, particularly in postoperative patients, injection drug users, and immunocompromised patients (4).

A history of rheumatic fever can serve as a risk factor for acquiring infective endocarditis. The incidence of rheumatic fever, which was common as recently as a century ago, is relatively rare today (12). This decline in the incidence of rheumatic fever has not been mirrored by a pro rata decrease in the incidence of infective endocarditis,

which suggests that additional etiologic factors are becoming more important in acquiring endocarditis.

Current Trends and Future Concerns

Although endocarditis has been documented for approximately 450 years, the diagnostic challenges and treatment dilemmas are as real today as they were in the time of Fernel (3). Major advances have been made in the diagnosis of endocarditis, in both laboratory and clinical (imaging) parameters, but we are witnessing the emergence of several newly described causal bacterial species, such as *Tropheryma whipplei* and *Bartonella* spp., as well as sporadic case reports of unusual and uncommon causal organisms, including *Finegoldia* sp., *Gemella* spp., and *Abiotrophia defectiva*. In addition, since diagnostic methods, mainly 16S rDNA polymerase chain reaction (PCR) and sequencing, are now beginning to identify such infections, no evidence base exists to help determine effective antimicrobial drug regimens to successfully treat endocarditis caused by such organisms. Furthermore, as specimens from many of these infections are culture-negative, conventional antibiotic susceptibility testing does not help the cardiologist decide on the most suitable antimicrobial drug regimens. Another current concern is that we may be returning to a time in which we are largely unable to successfully treat simple infections from panresistant organisms, a scenario that some have described as the postantibiotic era. Indeed, in Northern Ireland, we have now witnessed our first cases of penicillin-resistant pneumococcal meningitis and endocarditis. The increasing incidence of congenital heart disease in children and changing social trends accentuate risk factors for endocarditis.

Endothelial cell dysfunction, resulting from a combination of atypical mechanical forces due to altered cardiac architecture and microbial infection, may lead to an episode of infective endocarditis. Because the endothelium helps regulate vascular tone, inflammation, thrombosis, and vascular remodeling, any insult to the host endothelium may result in infective endocarditis, in which the valves may show changes in the synthetic, morphologic, and metabolic functions of the valvular endothelial cells (13).

Cases in Well-known Persons

Although a relatively uncommon infection, infective endocarditis has been the primary cause of death of several well-known persons, particularly those involved with the arts. One of the late 19th and early 20th century's most influential composers, Gustav Mahler (1860–1911), died from streptococcal endocarditis (10,14). The first sign of valvular problems was observed in 1907, where a compensated mitral contraction was noted. For the next 3 years, he showed little evidence of symptoms of valvular disease until late 1910, when he spent Christmas and the New Year's holiday nursing a sore throat. He was in New York City where he conducted a Philharmonic Orchestra concert on January 17, including the first performance of a revised version of his fourth symphony. On February 24, he became ill with endocarditis, initially diagnosed as influenza. He was attended by one of the most prominent physicians in the city, Emanuel Libman, an important exponent of the value of bacterial blood cultures. Libman demonstrated the presence of viridans streptococci in a large volume (200 mL) of blood drawn from Mahler. Mahler's initial treatment consisted of a "serum treatment" of the times, as well as Metchnikoff's Bulgarian Milk. The latter treatment appeared to work, until early May when blood cultures returned positive with viridans streptococci. The endocarditis was now very marked, with septic abscesses beginning to appear in other parts of his body. On May 18, Mahler died. His untimely death prevented society from hearing him conduct a completed version of his tenth symphony as well as his own opportunity to hear the first public performance of his ninth symphony, which took place on June 26, 1912, by the Vienna Philharmonic Orchestra.

Ottorino Respighi (1879–1936) was an Italian composer who died at the age of 57 from endocarditis. The first signs of Respighi's endocarditis were noted in late 1935, when he was working on his opera Lucrezia; at that time, he was observed to be extremely fatigued, but the cause was unknown (14). In January 1936, *S. viridans* endocarditis was noted when this organism was isolated from his blood. Although sulfonamide drugs were dispatched from Berlin for his treatment, the treatment was unsuccessful, possibly due to the advanced stages of sepsis.

One of Scotland's most famous poets, Roberts Burns (1759–1796), perhaps best known for writing Auld Lang Syne, also had infective endocarditis. He died in July 1796 at the age of 37 years (15). Some historians claim that Burns's work in his teenage years on his father's tenant farm in southwest Scotland did the primary damage to his health. However, Burns's history of rheumatic fever likely predisposed him to infective endocarditis. Burns was attended medically by William Maxwell (1760–1834), who described Burns's symptoms as "flying gout" and prescribed sea-bathing in country quarters and horse riding, so-called cures that probably hastened Burns's death. However, Burns's affinity for alcohol may have contributed to the suppression of his immune system, thus hastening the illness and ultimately his death.

One of the most famous physicians to die of endocarditis was Alois Alzheimer (1864–1915). Alzheimer is most widely known for his description of an "unusual disease of the cerebral cortex," which affected a woman in her fifties, causing memory loss, disorientation, hallucinations, and

ultimately her death at age 55. The disease was named after him by his senior mentor at the Munich Medical School, Emil Kraepelin. Alzheimer was also cofounder and copublisher of the journal Zeitschrift für die gesamte Neurologie und Psychiatrie. Alzheimer's last position was professor of psychiatry at the University of Breslau (now Wroclaw, Poland), which he held for the last 3 years of his life. Historians report that a severe cold was the beginning of Alzheimer's final illness, but endocarditis was responsible for his death at the age of 51 years (16).

Orville Gibson, guitar manufacturer (1856–1918), was another musician who died from endocarditis (17). Gibson's patent contained his ideas for the construction of a mandolin with a carved top and back and with sides, which were constructed from a solid section of wood rather than from thin strips. In 1902, Gibson's physical and mental health began to fail, and he had a history of poor health until 1911. He returned to the St. Lawrence State Hospital, Ogdensburg, New York, in August 1916, a psychiatric center. On August 21, 1918, Gibson died of endocarditis while a patient in the institution.

Rudolph Valentino (1895–1926), a famous actor of the silent screen, also had endocarditis, which also led to his death (18). Valentino had a perforated gastric ulcer closed on August 15, 1926; however, he died from endocarditis on August 23, 1926, at the age of 31 years.

More recently, endocarditis has been described as the cause of death for John Glascock (1951–1997), the recording bass player with the rock band Jethro Tull. Glascock had a tooth abscess, which was believed to be the site of entry for an infectious agent that caused endocarditis. Endocarditis developed in Brian Littrell (1975–), singer with the Backstreet Boys, at the age of 5 years (he was born with a ventricular septal defect, although surgery was not recommended at the time) (19). Brian was admitted to St. Joseph's Hospital, Lexington, Kentucky, where he received extensive intravenous therapy. Endocarditis also developed in a young American actor, Sebastian Hitzig, after he accidentally stepped on a toothpick contaminated with *Staphylococcus aureus*.

In conclusion, considering infective endocarditis to be an "emerging" problem in the 21st century may seem unusual, given that the illness has been well documented over the last 450 years. However, such emergence can be attributed to several factors: 1) the emergence of antimicrobial resistance in classic infective endocarditis microflora, namely, the gram-positive cocci; 2) the existence of antimicrobial resistance in complex ecologic biofilms; 3) the changing pattern of causal agents now regarded as important pathogens of infective endocarditis, e.g., *Bartonella* spp., *T. whipplei*, and fungi; and 4) changing epidemiologic trends of persons who acquire infective endocarditis, including injection drug users, persons with HIV/AIDS, children with congenital heart defects, and persons undergoing body piercing. Furthermore, the way we provide inpatient medical care has also been associated with the emergence of nosocomial infective endocarditis, which can result from invasive procedures such as catheterization, although no cardiac surgery has been performed. The next 100 years will likely witness the emergence of even more changing trends of infective endocarditis, which as yet have not been well recognized.

Although this "old" disease has evolved over the last 450 years, diagnostic and treatment options have developed in tandem, and the prognosis of this disease has markedly improved. However, the emergence of novel etiologic agents, changing social trends, and increased antimicrobial resistance have allowed this disease to remain evasive, which will require new approaches, particularly relating to treatment options in the future.

Appendix. Chronology of important scientific and medical events in the history of infective endocarditis[a]

Year	Scientist/physician, Country	Major findings
1554	Jean François Fernel, France	Earliest report of endocarditis in book Medicini
1669	Richard Lower, England	Accurately described tricuspid valve endocarditis
1646	Lazarus Riverius, France	Described unusual "outgrowths" from autopsy of patient with endocarditis; detected murmurs by placing hand on patient's chest
1708	Giovanni Maria Lancisi, Italy	Described unusual structures in entrance of aorta
1715	Raymond Vieussens, France	Described abnormality in aortic mitral valve
1749	Jean-Baptiste Sénac, France	Described valvular lesions
1769	Giovanni Battistu Morgagni, Italy	Linked infectious disease and endocarditis; observed association with the spleen
1784	Eduard Sandifort, France	Accurately drew intracardiac abnormalities
1797	Matthew Baillie, England	Showed relationship between rheumatism and heart disease
1799	Xavier Bichat, France	Described inflammatory process associated with endocarditis
1806	Jean Nicholas Corvisart, France	Described unusual structures in heart as "vegetations," syphilitic virus as causative agent of endocarditis, and theory of antiviral treatment of endocarditis
1809	Allan Burns, England	Indicated vegetations were not "outgrowths" or "buds" but particles adhering to heart wall
1815	Friedrich Kreysig, Germany	Elucidated inflammatory processes associated with endocarditis
1816	Théophile Laënnec, France	Invented cylindrical stethoscope to listen to heart murmurs; dismissed link between venereal disease and endocarditis

Appendix continued. Chronology of important scientific and medical events in the history of infective endocarditis

Year	Scientist/physician, Country	Major findings
1832	James Hope, England	Confirmed Laënnec's observations
1835–40	Jean-Baptiste Bouillaud, France	Named endocardium and endocarditis; described symptoms; prescribed herbal tea and bloodletting as treatment regimen; described link between acute rheumatoid arthritis and endocarditis
1852	William Senhouse Kirkes, England	Described consequences of embolization of vegetations throughout body. Described cutaneous nodules (named "Osler's nodes" by Libman)
1858–71	Rudolph Virchow, Germany	Examined fibrin vegetation associated with endocarditis by microscope; coined term "embolism;" discussed role of bacteria, vibrios, and micrococci in endocarditis
1861	Jean-Martin Charot, France	Confirmed Virchow's theory on emboli
1861	Alfred Vulpian, Germany	Confirmed Virchow's theory on emboli
1862	Etienne Lancereaux, France	Described granulations or foreign elements in blood and valves, which were motile and resistant to alkalis
1868–70	Samuel Wilks, England	Described infected arterial blood as originating from heart; proposed scarlet fever as cause of endocarditis
1869	Emmanuel Winge, Norway	Established "parasites" on skin transported to heart and attached to endocardium; named "mycosis endocardii"
1872	Hjalmar Heiberg, Norway	Detected microorganisms in vegetations of endocarditis
1878	Edwin Klebs, Germany	All cases of endocarditis were infectious in origin
1878	Ottomar Rosenbach, Germany/ Poland	Combined experimental physiology and infection to produce animal model of endocarditis in rabbit; noted valve had to be damaged before bacteria grafted onto valve
1878	Karl Koester, Germany	Micrococci enter vessels that valves were fitted into; valves exposed to abnormal mechanical attacks over long period created favorable niche for bacterial colonization
1879	Joseph Hamburg, Germany	Virchow's student; employed early animal model of endocarditis
1879	Germain Sée, France	Proposed etiology of endocarditis was based on infectious model and treatment should focus on eliminating "parasitic infection"
1880	Jacques Doleris, France	Working with Pasteur, proposed use of routine blood cultures
1881–86	Arnold Netter, France	Believed endocarditis could appear during various infections; noted translocation of respiratory pathogen from pulmonary lesion to valve through blood
1883	Michel Peter, France	Believed microorganisms were result, not cause, of endocarditis
1884	Joseph Grancher, France	Named disease "infective endocarditis"
1886	Valimir Wyssokowitsch and Johannes Orth, Germany	Demonstrated various bacteria introduced to bloodstream could cause endocarditis on valve that had previous lesion
1885	Sir William Osler, Canada	Synthesized work of others relating to endocarditis
1899	Hermann Lenhartz, Austria	Described streptococcal, staphylococcal, pneumococcal, and gonococcal endocarditis
1903	Hugo Schottmüller, Germany	First described "endocarditis lenta"
1909	John Alexander Mullen, Canada	Credited by Osler as first to observe cutaneous nodes (named "Osler's nodes" by Libman) in patients with endocarditis
1909	Sir Thomas Horder, England	Analyzed 150 cases of endocarditis and published diagnostic criteria relating to signs and symptoms
1910	Emmanual Libman, USA	Described initial classification scheme to include "subacute endocarditis," with clinical signs/symptoms; absolute diagnosis required blood cultures
1981	Von Reyn, USA	Described Beth Israel criteria based on strict case definitions
1994	David Durack, USA	New criteria utilizing specific echocardiographic findings
1995	American Heart Association, USA	Antibiotic treatment of adults with infective endocarditis caused by streptococci, enterococci, staphylococci, and HACEK[a] microorganisms
1996	Pierre Fournier, France	Modified Duke criteria to allow serologic diagnosis of *Coxiella burnetii*
1997	American Heart Association, USA	Guidelines for preventing bacterial endocarditis
1997	Lamas and Eykyn, UK	Suggested modifications to Duke criteria for clinical diagnosis of native valve and prosthetic valve endocarditis: analysis of 118 pathologically proven cases
1998	Working Party of the British Society for Antimicrobial Chemotherapy, UK	Guidelines for antibiotic treatment of streptococcal, enterococcal, and staphylococcal endocarditis
1998	Endocarditis Working Group of the International Society for Chemotherapy, Europe	Antibiotic treatment of infective endocarditis due to viridans streptococci, enterococci, and other streptococci; recommendations for surgical treatment of endocarditis
2000	Jennifer Li, USA	Updated and modified Duke criteria
2002	Beverley C. Millar, UK	Modified Duke criteria to include a molecular diagnosis of causal agents (20)
2001–2003	Didier Raoult, France	Described etiology of *Bartonella* spp., *Tropheryma whipplei*, and *Coxiella burnetii* in endocarditis

[a]HACEK, *Haemophilus aphrophilus, Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens, Kingella kingae* group, *Bartonella* spp., and *Coxiella burnetii*.

Dr. Millar is a clinical scientist in molecular medical microbiology at the Northern Ireland Public Health Laboratory, Belfast City Hospital. She has an active research interest in the molecular diagnosis of infectious diseases, in particular, endocarditis.

Dr. Moore is a clinical scientist in medical microbiology at the Northern Ireland Public Health Laboratory, Belfast City Hospital. His research interests include the application of molecular techniques to aid in patient management, particularly of those with cryptosporidiosis, campylobacteriosis, and cystic fibrosis.

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Address for correspondence: John E. Moore, Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast, BT9 7AD, Northern Ireland, UK; fax: +44-28-2589-2887; email: jemoore@niphl.dnet.co.uk

CTX-M–Type β-Lactamases Affect Community *Escherichia coli* Treatment, Greece

To the Editor: In recent years, a new group of extended-spectrum β-lactamases (ESBLs), the CTX-M–type enzymes, has emerged among *Enterobacteriaceae* (1). These enzymes are much more active against cefotaxime than against ceftazidime and exhibit greater susceptibilty to tazobactam than to clavulanate. Currently, the CTX-M family includes at least 30 alleles, which may be clustered on the basis of sequence similarity into four major evolutionary lineages (2). These enzymes were first detected in South America, Germany, and France, and subsequent reports found them to be in several European countries as well as in the Far East and North and South America (1–5). All of these studies have described the production of CTX-M–type β-lactamases in *Enterobacteriaceae* that have been isolated from patients with hospital infections, whereas surveys assessing countrywide prevalence of CTX-M clusters in community-acquired infections have not been carried out. We report the dissemination of various CTX-M–type enzymes in clinical isolates of *Escherichia coli* recovered from community-acquired infections in two large regions of Greece.

The microbiology databases of two healthcare systems in Greece that serve as tertiary care centers for their region (University Hospital of Larissa and Hippokration University Hospital of Thessaloniki) were prospectively searched from January to September 2003. From almost 75,000 outpatient visits, we tested *E. coli* isolates that were recovered from patients with community-acquired infections and classified as ESBL producers by the E-test ESBL screen method with cefotaxime and ceftazidime plus clavulanate. Community-acquired *E. coli* infections were defined as those contracted outside a hospital environment for persons with no history of hospitalization, surgery, or outpatient care during the previous 30 days.

ESBL-positive isolates for which the cefotaxime MICs were at least eightfold higher than those of ceftazidime by agar dilution were saved and stored at –70°C. Results of antimicrobial susceptibility tests and ESBL screening and confirmatory tests were used to characterize the phenotypes of the isolates. Molecular analysis included polymerase chain reaction (PCR) detection with primers producing an 873-bp amplicon of the $bla_{\text{CTX-M}}$ gene (6), sequencing on both strands of PCR products, pulsed-field gel electrophoresis of *Xba*I chromosomal digests, plasmid analysis, and transferability experiments of cefotaxime resistance.

Treatment outcomes were assessed by reviewing the medical records of all patients for whom a culture yielded a strain of *E. coli* producing a CTX-M–type ESBL. Patients' data included demographic details, presence of existing illness, symptoms, laboratory test results, history of surgery, and exposure to extended-spectrum cephalosporins <30 days before the positive culture. The antimicrobial treatment regimen was recorded, including the agent or agents administered, the duration of treatment, and clinical response.

During the study period, 14 community-acquired *E. coli* isolates (10 in the region of Larissa and 4 in the region of Thessaloniki) were recovered; the E-test ESBL screen test confirmed that these isolates were ESBL producers, and cefotaxime MICs were at least eightfold higher than those of ceftazidime. A CTX-M–type determinant was detected by PCR in 10 isolates (6 from Larissa and 4 from Thessaloniki). Sequencing their amplicons revealed that three of them were CTX-M-1 producers, and seven were CTX-M-3 producers. Genotyping showed that all of these isolates were unrelated. MICs of cefotaxime were always >128 μg/mL, whereas MICs of ceftazidime ranged from 0.5 μg/mL to 16 μg/mL. All isolates were sensitive to cefoxitin and piperacillin/tazobactam. However, the two laboratories reported them as being ESBL producers and recommended that β-lactam antibiotics, with the exception of carbapenems, not be used in their treatment. Several isolates exhibited additional resistance to co-trimoxazole, ciprofloxacin, tetracycline, or gentamicin, but all were susceptible to the other aminoglycosides. The $bla_{\text{CTX-M}}$ determinants were transferable to *E. coli* by conjugation in all but one case, along with other antimicrobial resistance determinants, with transfer frequencies that ranged from 8.3×10^{-5} to 2.2×10^{-2}. Transconjugants contained one to three plasmids of varying size, ranging from 10 to 130 kb.

CTX-M–positive isolates were recovered from five children and five adults. Patients did not have typical risk factors, except for a chronic hematologic malignancy in one patient. Nine of the patients had severe urinary tract infections and received courses of amikacin or ciprofloxacin. One female patient had a purulent perianal infection. Results of blood and wound cultures from samples obtained at admission yielded the CTX-M–positive *E. coli* strain. She was given amikacin and clindamycin, and her condition gradually improved.

β-lactam antibiotics are the most common antimicrobial agents used in the community setting. The documented CTX-M–positive isolates exhibited plasmid-mediated resistance that affected the antimicrobial activity of all penicillins and cephalosporins as well as of several alternative antimicrobial agents used to treat community-acquired *E. coli*

infections. The spread of CTX-M–positive bacteria considerably changes the way we think about treating community-acquired infections and limits the oral antibiotics that may be administered. This finding has major implications for treating children, who should not be given fluoroquinolones and tetracyclines.

The observation that different bla_{CTX-M} alleles, located on plasmids of different sizes, were involved in clinical infections caused by distinct *E. coli* clones implies that CTX-M enzymes may become widespread in the community. A possible association of bla_{CTX-M} genes with insertion sequences like IS*Ecp1B* might have contributed to the enhanced expression and mobilization of bla_{CTX-M} genes among *E. coli* isolates (7). The apparent dissemination of CTX-M producers could represent a substantial barrier in the treatment of community-acquired infections. Additionally, severely ill patients treated in the outpatient setting may transmit such resistant organisms to hospitalized patients.

Spyros Pournaras,*
Alexandros Ikonomidis,*
Danai Sofianou,†
Athanassios Tsakris,‡
and Antonios N. Maniatis*

*University of Thessalia, Larissa, Greece; †Hippokration University Hospital, Thessaloniki, Greece; and ‡University of Athens, Athens, Greece

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Address for correspondence: Athanassios Tsakris, Department of Microbiology, Faculty of Nursing, School of Health Sciences, University of Athens, 123 Papadiamantopoulou Street, 115 27 Athens, Greece;.fax: +30-210-7461489; email: atsakris@med.uoa.gr

Age and Transmissible Spongiform Encephalopathies

To the Editor: Bacchetti (1) notes "Our findings suggest that the possibility should not be discounted that biological factors peaking in the third decade of life may promote variant Creutzfeldt-Jakob disease (vCJD) prion replication and consequent development of disease." Such age specificity of disease risk may be a general feature of transmissible spongiform encephalopathies, which suggests that a general mechanism should be sought. A likely candidate for this mechanism is senescence-related immune system defects.

In a study of scrapie outbreaks in four sheep flocks, the incidence of clinical cases peaked in sheep 2–3 years of age, despite very different forces -of- infection at work and very large differences in disease incidence (2). Similar age specificity has been observed in cattle infected with bovine spongiform encephalopathy (3), which is believed to be the causal agent of variant Creutzfeldt-Jakob disease. There is evidence that an age-specific peak in prevalence also occurs in chronic wasting disease, a laterally transmitted spongiform encephalopathy of North American cervids, specifically elk, mule deer, and white-tailed deer. For example, data on prevalence of chronic wasting disease in mule deer (Figures 4B and 4A of [4]) suggest the existence of age-specific peaks. In aggregate, these observations suggest that a general mechanism might produce the marked decline in disease risk as age increases.

In 1979, Dickinson and Outram (5) conjectured that, in some experiments, scrapie responsiveness is the opposite of what one normally expects with an infection, "raising the possibility that, far from being inimical, some part of the host's immune system is essential and may even play the role of a Trojan Horse for these agents when infection occurs by a peripheral route." This theory appears well founded for transmissible spongiform encephalopathies in general. Disease-associated forms of resistant prion protein (PrP^{Res}) are likely transported from the gut to lymphoid tissue by cells such as migrating intestinal dendritic cells (6). Once in the lymphoid tissue PrP^{Res} appears to be amplified by follicular dendritic cells (6) and then enters the nervous system. Defects in either the complement pathway or follicular dendritic cells result in resistance to peripheral scrapie infection (7,8), and this resistance likely occurs for peripheral transmissible spongiform encephalopathy infections in general.

Both in vitro and in vivo animal and human studies demonstrate age-related declines in both humeral and cellular components of the immune system (9). In old (23 months) mice, the normal functioning of follicular dendritic cells appears to be strongly impaired when compared with young mice (10); according to researchers, "Antigen transport was defective and only a small fraction of antigen transport sites developed." (10). Furthermore, follicular dendritic cells were ultrastructurally atrophic, retained little antigen, and produced no iccosomes. By interfering with normal follicular dendritic cell function, age likely has the same effect on transmissible spongiform encephalopathies as has been observed due to dedifferentiation of follicular dendritic cells (8). Senescence of the immune system function could interfere with transmissible spongiform encephalopathy pathogenesis in other ways as well, such as impairing migrating intestinal dendritic cells or complement pathways involved in complexing PrP^{Res} to follicular dendritic cells.

This hypothesis could be readily tested by intracerebral versus peripheral PrP^{Res} challenge of young versus old animals. Because the intracerebral challenge bypasses the immune system portal, old, peripherally challenged animals should show a disproportionate reduction in disease risk if immune system senescence is important in regulating pathogenesis.

Dennis M. Heisey*
and Damien O. Joly†

*United States Geological Survey, Madison, Wisconsin, USA; and †University of Wisconsin, Madison, Wisconsin, USA

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Address for correspondence: Dennis M. Heisey, USGS-National Wildlife Health Center, 6006 Schroeder Road, Madison, WI 53711, USA; fax: 608-270-2415; email: dheisey@usgs.gov

SARS Epidemiology Modeling

To the Editor: To assess the effectiveness of intervention measures during the recent severe acute respiratory syndrome (SARS) pandemic, Zhou and Yan (1) used Richards model, a logistic-type model, to fit the cumulative number of SARS cases reported daily in Singapore, Hong Kong, and Beijing. The key to using mathematical models for SARS epidemiology is understanding the models (2). In the Richards model (1), the function *F(S)* in the model was described as measuring "the effectiveness of intervention measures." The parameters in *F(S)*, namely, the maximum cases load *K* and the exponent of deviation *a*, depict the actual progression of the epidemic as described by the reported data. In other words, the parameter estimates are used to quantify end results of the intervention measures implemented during the outbreaks. Simply put, the all-important question of "what if?" was not answered by their result. To gauge the effectiveness of intervention measures, one should consider a more complicated model with variable maximum case load and growth rate (*r*) that highlights the time-varying nature of an epidemic and its dependence on the intervention measures implemented during the epidemic.

Predicting the trend of an epidemic from limited data during early stages of the epidemic is often futile and sometimes misleading (3). Nevertheless, early prediction of the magnitude of an epidemic outbreak is immeasurably more important than retrospective studies. But how early is too early? Intuitively, the cumulative case curve will always be S-shaped and well-described by a logistic-type model. The essential factor is the time when the inflection of the cumulative case curve occurs, i.e., the moment when a rapid increase in case numbers is replaced by a slower increase. Since the inflection point, approximated by t_m (1), dictates the point in time when the rate of increase of cumulative case numbers reaches its maximum, the moment marks the key turning point when the spread of the disease starts to decline. As long as the data include this inflection point and a time interval shortly after, the curve fitting and predicting future case number will be reasonably accurate.

To illustrate this point more pre-

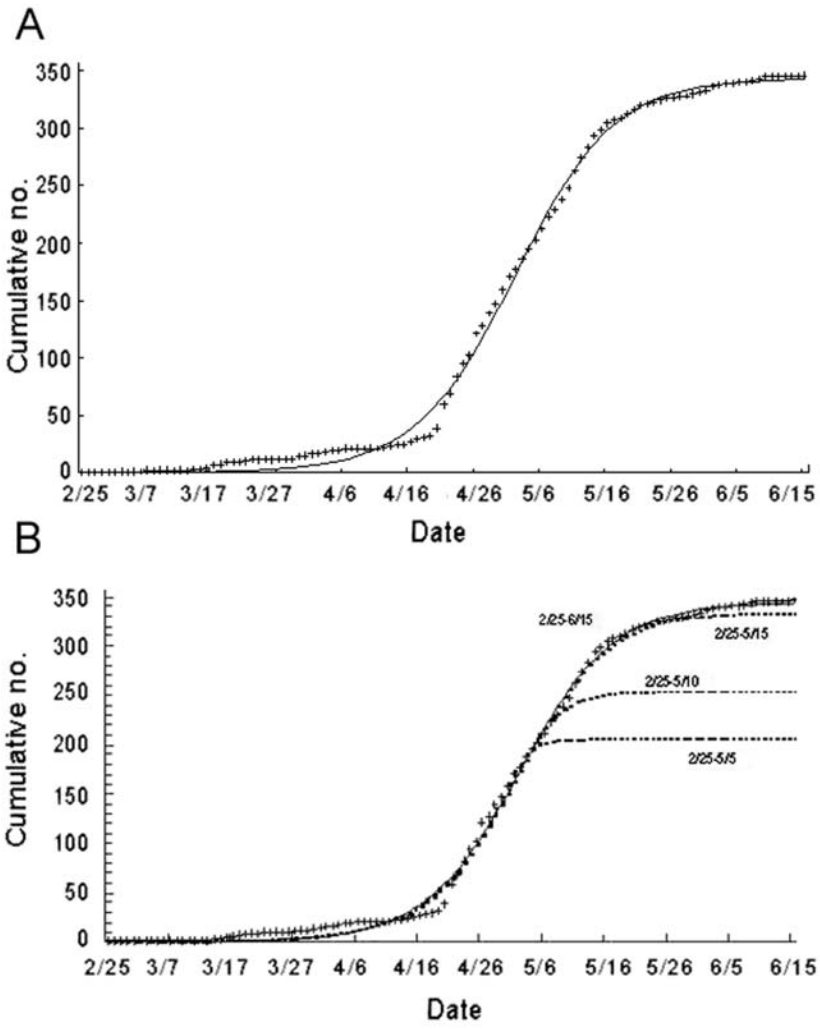

Figure. SARS cases, Taiwan 2003, Richards model; + = real data. A, confirmed cases; B, estimated cases using truncated data.

cisely, the cumulative SARS case data by onset date in Taiwan were obtained from the SARS databank of Taiwan Center for Disease Control. The data cover the time from February 25, 2003, the onset date of the first confirmed SARS case, to June 15, 2003, the onset date of the last confirmed case; a total of 346 SARS cases were confirmed during the 2003 outbreak in Taiwan (4). The cumulative case data are fitted to the cumulative case function *S(t)* in Richards model with the initial time $t_0 = 0$ being February 25 and the initial case number $S_0 = S(0) = 1$. Description of the model, as well as the result of the parameter estimation, is shown in the online Appendix (http://www.cdc.gov/ncidod/eid/vol10no6/03-1023_app.htm). The estimates for the parameters are $r = 0.136$ (95% confidence interval [CI] 0.121 to 0.150), $K = 343.4$ (95% CI 339.7 to 347.1), $a = 1.07$ (95% CI 0.80 to1.35), and the approximate inflection point at $t_m = 66.62$ (95% CI 63.9 to 69.3) with adjusted $r^2 > 0.998$, $p < 0.0001$ for the goodness-of-fit of the model (Figure). The result indicates that the infection occurred on May 3, and the estimate for the maximum case number $K = 343.3$ is 0.8% off the actual total case numbers.

Moreover, the case number data are sorted by onset date. Given a mean SARS incubation of 5 days (4–6 days) (5), the inflection point for SARS in Taiwan could be traced back to 5 days before May 3, namely April 28. On April 26, the first SARS patient in Taiwan died. Starting April 28, the government implemented a series of strict intervention measures, including household quarantine of all travelers from affected areas (6). In retrospect, April 28 was indeed the turning point of the SARS outbreak in Taiwan.

To address making projections during an ongoing epidemic, we used the same dataset but used various time intervals (all starting February 25) but truncated at various dates around the inflection point of May 3. The resulting parameter estimates are given in the Table of the online Appendix. For the truncated data ending on April 28 before the inflection, an unreasonable estimate of $K = 875.8$ was obtained. However, if we use the data ending on May 5, May 10, May 15, and May 20, we obtain estimates of $K = 204.9$, 253.1, 334.2, and 342.1, respectively. These estimates improve as we move further past the inflection time of May 3 (Figure). Moreover, the last estimate, using data from February 25–May 20 only, produces a 1.1% error from the eventual cumulative case number of 346, with 95% CI of 321.5 to 362.6. This retrospective exercise demonstrates that if the cumulative case data used for predictive purpose during an outbreak contain information on the inflection point and approximately 2 weeks afterwards, the estimate for the total case number can be obtained with accuracy, well before the date of the last reported case. This procedure may be immensely useful for deciding future public health policies although correctly determining the true inflection point during a real ongoing epidemic calls for scrutiny and judicious use of the model, as with all mathematical epidemic models.

Ying-Hen Hsieh,* Jen-Yu Lee,* and Hsiao-Ling Chang†

*National Chung Hsing University, Taichung, Taiwan; and †Center for Disease Control, Taipei, Taiwan

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Address for correspondence: Ying-Hen Hsieh, Department of Applied Mathematics, National Chung Hsing University, 250 Kuo-Kuang Rd., Taichung, Taiwan 402; fax: 886-4-22853949; email: hsieh@amath.nchu.edu.tw

In Reply: Our analysis of the dynamics of reported severe acute respiratory syndrome (SARS) clinical cases was conducted in May 2003 during the height of the public panic (1). Our primary goal in that study was to predict "when the epidemic might be brought under control if the current intervention measures were continued." (1). We used the Richards model and successfully predicted the epidemic cessation dates in Beijing, Hong Kong, and Singapore. Our predicted total number of SARS cases was close to the actual number of cases. In addition, we estimated the basic reproductive rate (R_0) of SARS infection, and our estimates based on the deterministic model were similar to those based on stochastic models (2,3). Therefore, our analysis provided useful information on the epidemiologic characteristic of SARS infections in three major Asian cities.

Hsieh et al. (4) commented that our article did not address the effect that specific intervention measures might have on the dynamics of SARS infection. Our study was not intended to measure this. As we stated in our article, "the transmission mechanism of the coronavirus that causes SARS and the epidemiological determinants of spread of the virus are poorly understood." Any models built on these unknowns are not suitable for assessing the effects of specific intervention measures. A method suggested by Hsieh et al. (4) to merely "consider a more complicated model with variable maximum case load and growth rate" will not answer the question to any extent.

The retrospective analysis of SARS case dynamics in Taiwan by Hsieh et al. (4) found that "as long as the data include this inflection point and time interval shortly after, the curve fitting and predicting future case number will be reasonably accurate." This notion holds only if the true inflection point is known before an epidemic ends. The main difficulty is how the true inflection point is correctly determined, as noted by Hsieh et al. (4). The time when inflection occurs varies tremendously if truncated data of cumulative SARS case numbers are used. To illustrate this point, we used the cumulative number of reported probable SARS cases in Hong Kong, starting March 17, 2003, but truncated at various dates, and calculated the date when inflection occurred (Table). For example, if the data period from the onset date (March 17, 2003) to the last case reported (June 12, 2003) was used, the date when inflection would occur was estimated as March 19, 2003. If the truncated data ending April 9, April 16, April 30, May 14, and May 28, 2003, were used, the dates when inflection would occur were estimated as April 2, February 7, March 3, March 23, and April 2, 2003, respectively (Table). Clearly, inflection point dates became a moving target as the epidemic progressed. When truncated data ending April 9, April 16, April 30, May 14, and May 28, 2003, were used, the corresponding estimated maximum numbers of cumulative cases (K) were 1,107, 1,907, 1,819, 1,749, and 1,733, respectively. Estimation of K improved when the data period used for prediction was at least one month past the March 19 inflection point obtained from the entire epidemic period. This analysis highlights the difficulty in identifying an optimal inflection point for prediction purposes during an ongoing epidemic when only a partial cumulative case number is available.

We fully agree with Hsieh et al. (4)

Table. Predicted inflection point and dates when inflection occurs based on truncated data of cumulative number of reported severe acute respiratory syndrome cases in Hong Kong

Data period (ending date)	t_m[a]	Date[b]	K[c]	r[d]	α[e]
April 9, 2003	16.62	April 2, 2003	1,107	0.20	0.74
April 16, 2003	–40.79	February 7, 2003	1,907	0.07	52.11
April 30, 2003	–13.52	March 3, 2003	1,819	0.07	10.21
May 14, 2003	6.80	March 23, 2003	1,749	0.09	2.84
May 28, 2003	17.31	April 2, 2003	1,733	0.10	1.38
June 12, 2003	2.63	March 19, 2003	1,751	0.09	3.77

[a] t_m is the inflection point of the model.
[b] Date refers to the date when inflection occurs.
[c] K is the predicted maximum number of cumulative cases.
[d] r is the intrinsic growth rate.
[e] α measures the extent of deviation of S-shaped dynamics from the classic logistic growth curve.

that the quantitative assessment of the effectiveness of public health intervention measures for SARS is a difficult task for modelers. To make models useful for assessing the effects of specific intervention measures and for predicting the future dynamics during an ongoing epidemic, we need improved knowledge on the transmission mechanisms, pathogenesis, and the epidemiologic determinants of the spread of the virus. Any retrospective analysis of the 2003 SARS epidemic that improves our knowledge of SARS epidemiology is welcome.

Guofa Zhou* and Guiyan Yan*

*State University of New York, Buffalo, New York, USA

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Address for correspondence: Guofa Zhou, Department of Biological Sciences, State University of New York, Buffalo, NY 14260, USA; fax: 716-645-2975; email: gzhou2@buffalo.edu

Diagnostic Criteria during SARS Outbreak in Hong Kong

To the Editor: A novel coronavirus caused more than 8,000 probable cases of severe acute respiratory syndrome (SARS) worldwide (1,2) during the 2003 outbreak. Before the etiologic agent was identified, the diagnosis of SARS was made according to a set of clinical-epidemiologic criteria as suggested by the Centers for Disease Control and Prevention (CDC) (1–3). These criteria remained important in the initial diagnosis and prompt isolation of patients because the overall sensitivity of initial reverse transcriptase-polymerase chain reaction (RT–PCR) testing for SARS-associated coronavirus (SARS CoV) RNA on upper respiratory specimens ranged from approximately 60% to 70% (though sensitivity improved with a second test) (4,5). In a SARS screening clinic at the Prince of Wales emergency department, the positive predictive value (PPV) of these criteria was estimated to be 54% (95% CI 39% to 69%) (6). The relative importance of the clinical versus epidemiologic criteria had not been evaluated. By using paired serologic testing to determine SARS-CoV infection (3), we evaluated the relative importance of the clinical-epidemiologic diagnostic criteria during an outbreak.

Patients with a diagnosis of SARS, and who were admitted to one of five regional hospitals in Hong Kong for isolation and treatment from March 4 to June 6, 2003, were included in this retrospective analysis. Probable SARS case-patients were those who met the CDC clinical criteria for severe respiratory illness of unknown etiology (3), and met the epidemiologic criterion for exposure in either a close or a possible contact. Close contact was defined as caring for, living with, or having direct contact with body fluids of a probable SARS patient (e.g., working in the same medical ward or staying in the same household) within 10 days of initial symptoms. Because Hong Kong was the documented SARS transmission site from February 1 to July 11, 2003, a modified epidemiologic criterion of possible contact was adopted. Possible contact was defined as staying or working in the same hospital compound, or residing in the same building where case clusters of SARS had been reported, within 10 days of symptoms onset.

Laboratory testing of paired immunoglobulin (Ig) G antibody to SARS-CoV was used to determine infection (7). Positive serologic evidence of infection was defined as a four-fold rise in antibody titer or detection of antibody in convalescent-phase serum. Seronegativity was defined as absence of antibody in convalescent-phase serum obtained ≥21 days after symptom onset (3). Seronegativity in this defined time frame (≥21 days – serum collected before July 11, 2003, and beyond 28 days) excluded the diagnosis of SARS (3). Samples from patients showing nonspecific fluorescent signals were considered negative for SARS-CoV infection. RT-PCR was performed on clinical specimens (respiratory, fecal) from all patients (1,3–5).

Demographic and laboratory parameters and history of close contact were compared between the seropositive and seronegative groups. Student *t* test was used to analyze continuous variables. A p value of <0.05 was considered statistically significant. Odds ratio (OR) and 95% confidence interval (CI) were calculated for categorical variables.

During the study period, 475 patients were hospitalized with probable SARS. One hundred patients were excluded because their serologic results were either missing (n = 37) or they died before day 21 of illness (no convalescent-phase serum, n = 63). Three hundred seventy-five patients were included in the analyses; 353 (94.1%) patients were serology-positive for SARS-CoV. Two hundred sixty-three of the 353 patients (74.5%) had a 4-fold increase in antibody titers, and 90 of the 353 patients

Table. SARS contact history and demographic and initial laboratory parameters in seropositive and seronegative patients

	Seropositive patients, n = 353 (%)	Seronegative patients, n = 22 (%)	p value or OR (95% CI)[a]
Demographic data			
Age	40.9 ± 17.2	51.2 ± 24.3	0.008
Healthcare workers (HCW)	193 (54.7)	2 (9.1)	12.1 (2.8 to 52.4)
Non-HCW	160 (45.3)	20 (90.9)	
Laboratory parameters on admission			
Total leukocyte count (x 10^9/L)	6.2 ± 3.2	9.4 ± 7.4	< 0.001
Lymphocyte count (x 10^9/L)	1.0 ± 0.4	1.2 ± 0.8	0.027
Level of contact			
Definite close contact	322 (91.2)	7 (31.8)	22.3 (8.4 to 58.7)
Possible contact	31 (8.8)	15 (68.2)	
Possible contact plus lymphopenia			
Lymphocyte < 0.8 x 10^9/L	13 (76.5)	4 (23.5)	
Lymphocyte < 1.0 x 10^9/L	21 (72.4)	8 (27.6)	
Venue of contact			
Hospital	290 (82.2)	8 (36.4)	8.1 (3.2 to 20.0)
Community	63 (17.8)	14 (63.6)	

[a]OR, odds ratio; CI, confidence interval.

(25.5%) had detectable antibody in either acute- or convalescent-phase serum samples (titer 80–5,120). Twenty-two patients (5.9%) had antibody titer <40 in their convalescent-phase serum samples (median = 31 days; range = 21–61 days). No clinical specimens were positive for SARS-CoV by RT-PCR. Thus, the PPV of the clinical-epidemiologic criteria for SARS in our cohort was 0.94 (95% CI 0.91–0.96).

The contact history and demographic and laboratory parameters for both seropositive and seronegative groups are depicted in the Table. The proportion of patients with a history of close contact was significantly higher in the seropositive group than in the seronegative group (91.2% vs 31.8%, OR 22.3; 95% CI 8.4–58.7). Only 8.8% of the patients with serologically confirmed results had no close contact history; 68.2% of the seronegative patients were in this category. The PPV of close contact was 0.98 (95% CI 0.96–0.99), and the PPV of possible contact was 0.67 (95% CI 0.54–0.81). Seropositive patients had a significantly lower lymphocyte count on admission compared to the seronegative patients (1.0 ± 0.4 vs 1.2 ± 0.8 x 10^9/L) (p = 0.027). The PPVs for possible contact plus lymphopenia <0.8 x 10^9/L and <1.0 x 10^9/L were 0.76 (95% CI 0.56–0.97) and 0.72 (95% CI 0.56–0.89), respectively. Seronegative patients were older (51.2 ± 24.3 vs. 40.9 ± 17.2 years), were less likely to be healthcare workers (90.9% vs. 45.3%), had their venue of contact in the community (63.6% vs. 17.8%), and had a higher total leukocyte count on admission (9.4 ± 7.4 vs. 6.2 ± 3.2 x 10^9/L). No differences were found in the lactate dehydrogenase, activated partial thromboplastin time, creatinine phosphokinase, and alanine-aminotransferase levels between the two groups.

Fifteen of the 22 seronegative patients responded to antibiotics (8); five died of comorbid illnesses (one of carcinoma of lung, one of metastatic carcinoma of prostate, two of chronic pulmonary diseases, and one of congestive heart failure), and two died of bacterial pneumonia. In four patients, bacterial pathogens were identified (one methicillin-resistant *Staphylococcus aureus*, two *Stenotrophomonas maltophilia*, and one *Pseudomonas aeruginosa*). Also, 15 (68.2%) of the patients had coexisting medical conditions: three had congestive heart failure, four had chronic pulmonary diseases, two had chronic renal failures, two had advanced malignancies, two had diabetes mellitus, and two had Parkinson's disease.

Our findings showed that 5.9% of cases defined as probable SARS on the basis of clinical-epidemiologic criteria had no serologic evidence of coronavirus infection. This set of criteria was associated with a PPV as high as 0.94 in a local outbreak. The PPV of the CDC epidemiologic criterion of close contact was higher (0.98). The PPV of possible contact was 0.67, but when applied with lymphopenia, the PPV became higher. Our analysis illustrated that a history of close contact with patients with SARS-CoV infection is of major importance when diagnosing such infection. This finding supports the hypothesis that SARS-CoV is transmitted through respiratory droplets and physical contact with a patient's body fluids. Although not specific, lymphopenia and its subsequent progress was highly prevalent among SARS patients (8–10). Clinicians are now advised by the World Health Organization that hematologic deviations (e.g., lymphopenia) should be considered in SARS evaluations (1).

Our study was limited by sample size and its retrospective status. Nonetheless, we demonstrated the accuracy of diagnostic criteria in an outbreak and the importance of epidemiologic criteria. Further studies are needed to evaluate the diagnostic accuracy of these criteria in a nonoutbreak situation when the case prevalence is low.

Louis Y. Chan,* Nelson Lee,† Paul K.S. Chan,† Alan Wu,† Timothy H. Rainer,† Philip K.T. Li,* Hong Fung,* and Joseph JY Sung†

*Prince of Wales Hospital, Hong Kong, Special Administrative Region (SAR); and †The Chinese University of Hong Kong, Hong Kong, SAR

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Address for correspondence: Nelson Lee, The Chinese University of Hong Kong, Department of Medicine and Therapeutics, Prince of Wales Hospital, Shatin, Hong Kong; fax: 852-26375396; email: leelsn@yahoo.com

Malaria Control and Public Health

To the Editor: Malaria continues to cause disease and death in millions of persons living in areas of the world where it is endemic, despite 4 decades of research on vaccines, new drugs, and alternative methods of control. Still, by far the most effective method for reducing and controlling the impact of this disease is indoor residual spraying (IRS) of insecticides. The most cost-effective and safe insecticide has been, and in many instances still is, dichlorodiphenyltrichloroethane (DDT). This intervention is continually under scrutiny, and we address these issues in this letter.

Chen and Rogan (1) claim that DDT causes reduced duration of lactation and increased incidence of preterm births, and they posit that DDT used for malaria control would do as much harm as good. The validity of their arguments requires substantial evidence of a causal relationship between DDT and adverse consequences of DDT IRS for malaria control.

Chen and Rogan dismiss a field study on births and duration of lactation in South African mothers, some of whom occupied houses sprayed with DDT for malaria control (2). However, if claims of large numbers of adverse health effects of DDT IRS are correct, then the study should have detected large differences between DDT-exposed and unexposed populations. According to Chen and Rogan, the median duration of breastfeeding could be as low as 3–4 months when mothers are exposed to high levels of DDT. Thus, a cross-section of breastfeeding infants in the DDT-exposed population should, on average, have been considerably younger than in the unexposed population. In fact, the average age of breastfeeding infants was slightly greater in the DDT-exposed population (8.3 months versus 7.7 months). For both populations, only an insignificant fraction of mothers could not donate milk. Furthermore, twice the level of dichlordiphenylethylene (DDE, metabolic breakdown product of DDT) that is claimed to cause reduced duration of lactation in humans has no adverse affect on lactation in rats (3). The authors of the South African study (2) report no difference in rates of stillbirths between the sprayed and unsprayed areas.

The National Institute of Environmental Health Sciences study (4) reported a causal association between DDT and preterm and small-for-gestational-age births but this has not been replicated for African births. The study was not based on a random population of births, and no explanation is offered for including diverse categories of births in the study population.

An earlier study in Sri Lanka presented data on deaths attributed to malaria and to premature births years before DDT was used and years when DDT IRS was used in 21 districts (5). Districts varied greatly in levels of malaria endemicity. After DDT was introduced in 1946, levels of IRS in 21 districts were commensurate with levels of endemic malaria. After 1946, malaria deaths declined greatly and the reduction was greatest where DDT usage was highest. During the same period, deaths attributable to premature births increased slightly. Inves-tigators attributed this to "improvements in reporting and diagnosis rather than any declines in the health of expectant mothers, which on all other criteria showed improvement." (5). Spearman's correlation analysis for 21 districts shows that the increase in premature birth deaths was slightly greater in areas with less malaria and DDT use. Thus, the evidence does not support the idea that the reported increase in premature births was a side effect of DDT use. In any case, the increase in deaths attributable to premature births was orders

of magnitude less than the reduction in deaths directly caused by malaria and other conditions indirectly related to malaria (5).

Similar major benefits of DDT use were seen in Guyana, where in 2 to 3 years, near elimination of malaria halved maternal deaths and reduced infant deaths by 39% (6). Anemia-associated deaths in pregnant females were reduced from 10 to 2.3 per 1,000 adult deaths (7). There was no offset of infant deaths attributable to adverse effects of DDT. Data from Guyana are particularly relevant to the present issue because malaria control was entirely due to DDT, i.e., drug treatments were not included (7). Health improvements related to DDT use accounted for 21% to 56% of increased population growth in Guyana during the postwar years (5).

In summary, these data from South Africa, Sri Lanka, and Guyana are clearly contrary to the claims of Chen and Rogan (1) that ill effects of DDT on maternal health and infant survival would counterbalance the beneficial effect of malaria control. Their claim that alternative chemicals are cheaper than DDT is incorrect (8). Recent data on pyrethroid-treated bed nets are encouraging for situations in which sustained provision of spray pumps and trained spray teams are not feasible. However, even the best results with these nets do not match those obtained in the past with IRS, e.g., the suppression of malaria infection in Zanzibar from holoendemic levels to <5% (9).

In recent years, programs in South Africa and Madagascar (10) that again started IRS with DDT have greatly reduced malaria and malaria-related deaths. DDT is still needed and research is required to improve its use. The Stockholm Convention on Persistent Organic Pollutants specifically allows continued public health use of DDT.

Donald Roberts,*
Christopher Curtis,†
Richard Tren,‡ Brian Sharp,§
Clive Shiff,¶ and Roger Bate#

*Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA; †London School of Hygiene & Tropical Medicine, London, United Kingdom; ‡Africa Fighting Malaria, Sandton, South Africa; §Medical Research Council, Durban, South Africa; ¶Johns Hopkins University, Baltimore, Maryland, USA; and #Africa Fighting Malaria, Washington, D.C., USA

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Address for correspondence: Donald R. Roberts, Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA; fax: 301-295-3731; email: droberts@usuhs.mil

In Reply: We do not believe that causality has been demonstrated for the relationship between dichlordiphenylethylene (DDE) and shorter period of lactation or preterm birth. However, we think the evidence is sufficiently strong that the possibility of causality cannot be dismissed and testing this hypothesis will require data from appropriately designed studies in areas where dichlorodiphenyltrichloroethane (DDT) is used.

We think that the cross-sectional study (1) referred to by Roberts et al. (2) cannot determine whether DDE shortens lactation. Women with higher levels of DDE and shorter lactation periods would be less likely than women with lower levels of DDE and longer lactation periods to appear in such a study, which would mask any associations.

As noted in the Longnecker report on the association between DDE and preterm birth (3), several previous studies have shown such an effect, but they were relatively small. That the perinatal collaborative study was not a random sample of U.S. births does not seem relevant. Women could not choose whether to participate on the basis of their DDT level because they did not know it and could not choose whether to participate on the basis of a preterm birth because they were enrolled during pregnancy.

Roberts et al. reference success stories of DDT use from the 1930s to

the 1950s in Sri Lanka and Guyana. The reports of the Sri Lanka study are not in the peer-reviewed literature. Data from the Guyana sugar plantations must include factors other than DDT and malaria because profound differences existed in all-cause mortality in adults and children over the span of the reports. Whether DDT was effective in those areas at that time cannot determine whether it would be a two-edged sword now.

Although the p,p′ isomer of DDE is in human tissue at the highest concentration, technical DDT contains approximately 10% of the o,p isomer, and o,p-DDE can be detected if sought (4). We and others measure p,p′-DDE as an index of total DDE, but our hypothesis for the estrogenic mechanism by which lactation time might be shortened involves the estrogenic isomer p,p'-DDE. The Kornbrust study of DDE and lactation in rodents used pure p, p-DDE, the most prevalent congener but also the least estrogenic one (5). Since the hypothesis concerned o,p-DDE, the estrogenic congener, the work was unfortunately noncontributory.

Malaria is a major public health problem, and vigorous efforts to prevent and treat it are necessary. We fear, though, that DDT is not entirely benign and have some evidence to show this. Proceeding as if the safety of DDT had been demonstrated absolutely does not seem a prudent course. DDT is inexpensive; however, cost is irrelevant if DDT use causes as many infant deaths as it prevents.

Aimin Chen* and Walter J. Rogan*

*National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

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Address for correspondence: Walter J. Rogan, Epidemiology Branch, National Institute of Environmental Health Sciences (NIEHS), P.O. Box 12233, Mail Drop A3-05, Research Triangle Park, NC 27709, USA; fax: 919-541-2511; email: rogan@niehs.nih.gov

CTX-M and Plasmid-mediated AmpC-Producing *Enterobacteriaceae*, Singapore

To the Editor: In gram-negative bacteria, β-lactamases are an important cause of antimicrobial resistance. In the 1990s, several new β-lactamases, including CTX-M type and plasmid-mediated AmpC β-lactamases, emerged.

CTX-M extended spectrum β-lactamases (ESBLs) differ from those derived from TEM and SHV enzymes by their preferential hydrolysis of cefotaxime and ceftriaxone compared with ceftazidime. They also differ from an evolutionary standpoint and are more closely related to the chromosomal enzymes of *Kluyvera* species (1). These enzymes are increasingly described worldwide, particularly in South America, Europe, and East Asia.

AmpC enzymes confer resistance to oxyimino- and 7-α-methoxycephalosporins. They occur naturally in the chromosomes of bacteria such as *Enterobacter*, *Citrobacter*, *Serratia*, and *Pseudomonas* species. In the last decade, genes coding for AmpC β-lactamases have made their way into plasmids and are increasingly detected in other species, notably *Klebsiella* and *Escherichia coli* (2).

We describe five strains of *Enterobacteriaceae* with unusual antimicrobial susceptibility patterns, which were isolated from patients in an 800-bed hospital in Singapore. *K. pneumoniae* EU17113, EU2673, and *E. coli* EU2657 were noted to be more susceptible to ceftazidime than ceftriaxone, whereas *E. coli* EU4855 and EB9505 were resistant to both cephalosporins. The National Committee for Clinical Laboratory Standards ESBL confirmatory test (3) was positive for strains EU17113, EU2673, and EU2657 but negative for strains EU4855 and EB9505. All strains were isolated from urine cultures except EB9505, which was isolated from blood culture. The isolates were identified by VITEK 2 (bioMérieux, Marcy l'Etoile, France) or Microbact 12E/A (Medvet Diagnostics, Thebarton, Australia).

The MICs by Etest (AB Biodisk, Solna, Sweden) and isoelectric points of β-lactamases in crude extracts are shown in the Table. Polymerase chain reaction (PCR) for CTX-M genes was performed on strains EU17113, EU2673, and EU2657 by using primers CTX-1 and CTX-2 as described by Pai et al. (4). This yielded an ≈780-bp product with DNA extracts from strain EU17113 but not the others. The sequence of this product was identical to $bla_{\text{CTX-M-9}}$ as found in the GenBank database (accession no. AJ416345.1). PCR was repeated for strains EU2673 and EU2657 with a different primer set as described by Gniadkowski et al. (5). This produced

Table. Characteristics of isolates containing CTX-M and plasmid-mediated AmpC β-lactamases

Isolate	Species	MIC (μg/mL)[a]				pI[b]	Sequenced β-lactamase
		CAZ	FEP	CRO	AZT		
EU17113	*K. pneumoniae*	4	4	128	2	7.2, 7.5, 8.0	CTX-M-9 type
EU2673	*K. pneumoniae*	128	128	>256	>256	5.8, 7.2, 7.5, 9.0	CTX-M-15 type
EU2657	*E. coli*	16	128	>256	64	5.1, 6.3, 7.2, 7.5, 8.0	CTX-M-2 type
EU4855	*E. coli*	>256	4	>256	64	8.8	CMY-2 type
EB9505	*E. coli*	128	1	256	8	5.1, 8.8	CMY-2 type

[a]*K. pneumoniae*, *Klebsiella pneumoniae*; *E. coli*, *Escherichia coli*; CAZ, ceftazidime; FEP, cefepime; CRO, ceftriaxone; AZT, aztreonam.
[b]Isoelectric points of β-lactamases.

≈600-bp products which were identical to $bla_{\text{CTX-M-11}}$ and $bla_{\text{CTX-M-15}}$ (accession no. AJ310929.1 and AY463958.1) for EU2673, and $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-20}}$ and $bla_{\text{Toho-1}}$ (accession no. X92507.1, AJ416344.1, and D37830.1) for EU2657.

CTX-M ESBLs can be grouped into four clusters based on the similarity of their amino acid sequences: CTX-M-1 type (CTX-M-1, -3, -10, -11, -12, -15, -23, -28), CTX-M-2 type (CTX-M-2, -4, -5, -6, -7, -20, Toho-1), CTX-M-9 type (CTX-M-9, -13, -14, -16, -17, -18, -19, -21, Toho-2), and CTX-M-8. Three of the four clusters of CTX-M-type enzymes are represented in our small sample of isolates. The only other report of CTX-M enzymes in Southeast Asia of which we are aware describes CTX-M-14 and -17 in Vietnam (6).

Diagnostic laboratories may fail to identify CTX-M–positive isolates as ESBL producers if ceftazidime resistance is used as the sole screening criterion, which is unlikely in Singapore because ceftriaxone is usually in the first-line panel for antimicrobial susceptibility testing of *Enterobacteriaceae*. In addition, ESBL screening is conducted in most laboratories by the Jarlier double disk diffusion method (7), with at least two different β-lactam disks placed on either side of an amoxicillin-clavulanate disk. However, CTX-M producers may not be distinguished from other ESBL producers because once the ESBL test is positive, the organism is reported as resistant to all third-generation cephalosporins. The importance of the characteristic antimicrobial susceptibility pattern (cefotaxime- or ceftriaxone-resistant, ceftazidime-susceptible) may not be appreciated and may be lost in the edited report. We paid special attention to these isolates because we were evaluating a VITEK 2 at the time and noted the original susceptibility pattern while reviewing the machine reports.

Multiplex PCR for plasmid-mediated AmpC genes was performed on strains EU4855 and EB9505 by using the method described by Perez-Perez and Hanson (8). Amplified products of ≈460 bp were produced which, when sequenced, were identical to the gene sequences encoding C-1 molecular subgroup plasmid-mediated AmpC enzymes CMY-2 (accession no. X91840.1), and LAT-3 (accession no. U77414.1) (9). This family of enzymes is thought to be derived from the chromosomal β-lactamase of *C. freundii* and includes some of the most widely distributed plasmid-mediated AmpC β-lactamases. CMY-2 has been recently found in *E. coli* strains from Malaysia, which shares a common border with Singapore (10).

Although identifying these enzymes has little impact on managing a patient, recognizing CTX-M and plasmid-mediated AmpC enzymes affects antimicrobial drug-resistant surveillance because important new mechanisms of extended-spectrum cephalosporin resistance are represented.

The following sequences have been submitted to GenBank: EU17113 (accession no. AY517474), EU2673 (accession no. AY517476), EU2657 (accession no. AY517475), EU4855 (accession no. AY517473), and EB9505 (accession no. AY514304).

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Tse Hsien Koh,*
Grace Chee Yeng Wang,*
Li-Hwei Sng,* Zhao Yi,*
and Tse Yuen Koh†

*Singapore General Hospital, Singapore; and †Ngee Ann Polytechnic, Singapore

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Address for correspondence: Tse Hsien Koh, Department of Pathology, Singapore General Hospital, 1 Hospital Drive, 169608, Singapore; fax: +65-6222-6826; email: gptthk@sgh.com.sg

ANTIMICROBIAL DRUGS

Novel Antimicrobial Class

Bacterial adaptation makes antibacterial drug resistance inevitable. As old medicines lose their effectiveness, scientists must find new drugs that can safely treat a broad spectrum of bacterial infections. For more than 30 years, the problem has been addressed by improving old classes of drugs. However, with this approach, we stay just ahead of the evolving bacteria; and the strategy becomes more difficult with each iteration. No truly new classes of orally active, broad-spectrum antimicrobial agents have been discovered since quinolones. A class of broad-spectrum novel ribosome inhibitors (NRI) has been found that shut down bacterial protein synthesis. Although many existing antimicrobial agents act against the ribosome, the NRIs exploit a new mechanism of action. Because bacterial populations are not familiar with NRIs, no preexisting resistance mechanisms exist in bacteria, and NRIs have consistent antimicrobial activity even against multiple drug-resistant strains.

The team conducted a comprehensive series of biologic and biochemical experiments to discover and characterize the new class, as recently reported in the journal Antimicrobial Agents and Chemotherapy. NRIs inhibit ribosomes of both gram-positive and -negative pathogenic bacteria but will not disturb eukaryotic protein synthesis. Furthermore, the new compounds inhibit bacterial growth without toxicity to human cells, consistent with developing a new drug that kills bacteria without disturbing the human host. As further evidence of the ribosomal mechanism, the group showed that bacteria treated with NRI compounds respond by trying to overproduce ribosomal proteins. As with other ribosome inhibitors, the bacteria seem to realize that their ribosomes are failing, and they desperately try to make more to survive. In the laboratory, bacteria could be made less susceptible to NRIs by certain mutations in their ribosomes. Although, fortunately, these mutations would be difficult to generate outside the laboratory, they were useful in supporting the novel mechanism of action, since these genetic alterations did not affect the action of other ribosomal drugs. The recent data hold hope for new antimicrobial agents that can combat the rising tide of microbial resistance.

Dandliker PJ, Pratt SD, Nilius AM, Black-Schaefer C, Ruan X, Towne DL, et al. Novel antibacterial class. Antimicrob Agents Chemother. 2003;47:3831–9.

INFLUENZA

Influenza Viruses with Genes from the 1918 Pandemic Virus

Eighty-six years ago, an influenza A H1N1 virus swept the globe and killed an estimated 20–40 million people. The exceptionally high death rate, especially among young adults, was not observed during later influenza pandemics of 1957 and 1968. Genetic sequence analysis of the 1918 "Spanish" influenza virus genes has not shown any features that could account for its high virulence. Therefore, we generated recombinant influenza (A/WSN/33) viruses possessing 1918 influenza gene segments to provide insights into the pathogenicity and to identify possible vaccine strategies against potentially reemergent 1918 or 1918-like viruses. Analysis of the recombinant influenza viruses points to a critical role of the 1918 hemagglutinin (HA) and neuraminidase (NA) influenza genes in virulence in the mouse model. The antigenic analysis demonstrated that the 1918 recombinant viruses most closely resembled a common influenza laboratory strain, A/Swine/Iowa/30. In fact, human survivors of the 1918 influenza pandemic had antibodies that neutralized both the 1918 HA/NA (1918 HA/NA:WSN) recombinant virus and the A/Swine/Iowa/30 virus. In studies, the protection provided by A/Swine/Iowa/30 vaccine was similar to that observed in mice that received inactivated 1918 HA/NA:WSN virus vaccine. Mice that were immune to A/Swine/Iowa/30 were protected against death and major weight loss and had undetectable virus in respiratory tissues on day 5 after virus challenge with the lethal 1918 HA/NA:WSN virus. The protection induced by A/Swine/Iowa/30 virus vaccine correlated with detectable virus–neutralizing antibodies measured in the mouse. These vaccine strategies, with data demonstrating that existing anti-influenza drugs would be effective against 1918 virus genes, provide the basis for prophylactic measures against the reemergence of new 1918-like viruses.

Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, Basler CF. Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. Proc Natl Acad Sci U S A. 2004;101:3166–71. Epub 2004 Feb 12.

Influenza Virus Tropisms

Human and avian influenza viruses target different cell types in human airway epithelium. Recent outbreaks of avian influenza infections in humans highlighted the threat of pathogenic influenza viruses emerging from a huge natural reservoir in birds. To initiate the infection, avian influenza viruses bind to cell-surface receptors containing terminal sialyl-galactosyl residues linked by 2-3-linkage, whereas human viruses, including the earliest available

pandemic isolates, bind to receptors that contain terminal 2-6–linked sialyl-galactosyl residues. It is believed that a nonoptimal receptor specificity of avian viruses limits their replication in human respiratory tract and pandemic spread, but the mechanism of this restriction is not clear. Ciliated epithelium of conducting airways consists of several distinct cell types with different functions, but the roles of specific cell types in virus replication have not been defined. To investigate cellular tropism of influenza viruses, the authors employed cultures of differentiated human airway epithelial cells which closely mimic airway epithelium in vivo. The authors found that human viruses preferentially infected nonciliated cells, whereas avian viruses mainly infected ciliated cells; this pattern correlated with cell type–specific distribution of sialic acid receptors recognized by the viruses. This study suggests that two widely held concepts concerning influenza viruses (uniform susceptibility of airway epithelial cells to human viruses and a lack of receptors for avian viruses) are incorrect. These data provide insight on the emergence of pandemic viruses and open avenues for cellular studies on influenza virus replication and pathogenicity in humans.

Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci U S A. 2004;101:4620–4. Epub 2004 Mar 15.

INFECTIOUS DISEASE ARCHAEOLOGY

Chagas Disease

In this study, investigators reconstructed the behavior of Chagas disease (American trypanosomiasis) in the Atacama Desert over the past 9,000 years. The researchers analyzed ancient DNA to identify kinetoplast DNA of *Trypanosoma cruzi*, the disease's infectious agent transmitted by the insect vector, a triatomid bug. Specimens analyzed were from muscle and visceral tissues in excavated, naturally mummified human bodies buried in that hyperarid desert during the past 9 millennia.

Results indicated that 41% of these bodies were infected by *T. cruzi* at the time of death. Among the 11 represented populations, no statistically significant differences in prevalence rates could be demonstrated when studied by the time period, sex, or age, except for lower rates (28%) for infants. Such prevalence rates are similar to those of modern *T. cruzi*–endemic areas. These results demonstrated the well-established presence of Chagas disease in this region among wild forest animals when the first humans (the Chinchorro) arrived. By settling this region, the new arrivals initially and inadvertently exposed themselves to the triatomid bug transmitting this disease, and joined the wild animals as part of the disease's reservoir. At some undetermined time during this 9,000-year interval, a few of the vector species became adapted to the thatched roof and other features of the region's human dwellings and initiated the independent domestic cycle involving only humans and their domesticated animals. The study also suggests that, given available specimens, the history of other infectious diseases can be similarly reconstructed.

Aufderheide AC, Salo W, Madden M, Streitz J, Buikstra J, Guhl F, et al. A 9,000-year record of Chagas' disease. Proc Natl Acad Sci U S A. 2004;101:2034–9. Epub 2004 Feb 06.

MICROBIAL VIRULENCE

Polysaccharide Intercellular Adhesin

Coagulase-negative staphylococci, with *Staphylococcus epidermidis* as the most frequently isolated species, have become the leading cause of hospital-acquired infections and infections of indwelling medical devices. In the course of these infections, biofilm formation and the ability to escape from host immune defense are regarded as the main virulence determinants. However, the factors protecting *S. epidermidis* from the immune system have remained elusive. Scientists have discovered the first specific molecule involved in immune evasion in *S. epidermidis*. The exopolysaccharide polysaccharide intercellular adhesin (PIA) was located at the cell surface of *S. epidermidis;* it protected organisms against phagocytosis by neutrophils, antibacterial peptides from human skin, and neutrophil granula. PIA was also indispensable for the formation of cellular aggregates. The positively charged PIA likely functions both as a mechanical barrier against peptides and phagocytes, and by electrostatic repulsion of the predominantly cationic antibacterial peptides. Thus, by inhibiting major mechanisms of the human innate immune defense, PIA may significantly contribute to the success of *S. epidermidis* in chronic infections. Interestingly, the genetic basis for PIA production is present in an increasing number of microorganisms, including such pathogenic species as *Yersinia pestis*, the causative agent of plague. Targeting PIA as a crucial component of both cell-cell aggregation and immune evasion processes might therefore constitute a promising way to interfere with the virulence of a series of important bacterial pathogens.

Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, et al. Polysaccharide intercellular adhesion (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol. 2004;6:269–75.

Quinolone Antimicrobial Agents, 3rd Edition

David C. Hooper and Ethan Rubinstein

American Society of Microbiology Press, Washington, DC 20036, USA
ISBN: 1-55581-231-7
Pages: 485, Price: $125.95

The quinolone class is one of the more important classes of antimicrobial agents discovered in recent years and one of the most widely used classes of antimicrobial drugs in clinical medicine. Their broad spectrum of activity and pharmacokinetic properties make the quinolone agents ideal for treating a variety of infections. Their clinical importance is further demonstrated by their activity against a wide range of diseases of public health importance such as anthrax, tuberculosis, bacterial pneumonia, and sexually transmitted diseases.

Recent research has provided new data on the agent's structure-function relationships, modes of action, resistance, pharmacokinetics, and drug interactions. The third edition of Quinolone Antimicrobial Agents nicely addresses these advances. The book is organized into four sections, each containing chapters written by leading experts.

Mechanisms and Spectrum of Activity and Resistance, the first major section, explores the basic biology of the quinolone class. The interrelationships between structure, antimicrobial activity, and side effects associated with various side chain positions of the quinolones are discussed here. Pertinent information on the bacterial topoisomerases and DNA gyrases, quinolone binding, DNA/RNA synthesis inhibition, cell death in the absence of protein synthesis, specific mutations within the quinolone resistance–determining region, and mutations that lead to altered access to target enzymes (efflux systems) is also highlighted.

In the next section, Pharmacology, the intricate field of quinolone pharmacokinetics (PK) and pharmacodynamics (PD) is evaluated. Information regarding absorption, distribution, metabolism, and excretion in a range of patient types is provided, followed by data gathered from pharmacokinetic/pharmacodynamic studies in a variety of models. This section also provides a review of the role of pharmacologic evaluation in optimizing therapy.

The last two sections of the book, Clinical Applications and Adverse and Other Effects, will be of particular interest to clinicians. The chapters in this section include a comprehensive reference on general considerations, antimicrobial aspects, treatment models, clinical and comparative studies, and suggested treatment regimens for a host of infections. The chapter on adverse effects has been greatly expanded from the second edition because a substantial body of new information has since been gathered. Topics covered here include allergic reactions, effects on connective tissue structures and pregnancy, phototoxicity, and central nervous system and immune system toxicity.

The organization and content of this text make it a superlative reference. The discussions on treatments and adverse effects contain some of the most current data available. The field of antimicrobial resistance, however, is one of the most rapidly evolving areas with constant discoveries of new cases and data. Users should understand that while this text serves as a valuable reference, other sources should be consulted to ensure that the most comprehensive data are obtained. The references provided throughout, however, give the reader starting points to other literature.

Gregory J. Anderson*
*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Address for correspondence: Gregory J. Anderson, Centers for Disease Control and Prevention, Mailstop C12, 1600 Clifton Road, Atlanta, GA 30333, USA; fax: 404-639-4197; email: gca5@cdc.gov

Risk Analysis of Prion Diseases in Animals

David Adams and Corrinne Ida Lasmézas, editors

OIE Scientific and Technical Review 22 (2003)
ISBN 92-9044-573-4
Pages: 346, Price: 45 Euros

Although scrapie, a prion disease of sheep, has been recognized since the 18th century, it was the dramatic emergence of bovine spongiform encephalopathy (BSE) in British cattle in the late 1980s that brought the dangers of prion diseases into prominence. The subsequent spread of this disease into humans as new variant Creutzfeldt-Jakob disease (CJD) remains one of the unsolved emerging infectious disease mysteries of the 20th century. This issue (OIE Scientific and Technical Review 22 [2003]) of the Scientific and Technical Review of the Office International des Epizooties comes 11 years after a previous issue about BSE. Much has happened since then; this multi-author volume provides an excellent account of what is known about prion diseases in animals, including BSE, which has now spread from the United Kingdom to 14 other countries, and remains an important risk to human health. As a consequence of BSE emergence, research

has been expanded considerably into transmissible spongiform encephalopathies (TSE). This research has resulted in improved diagnostic tests, which have contributed to risk management, even though our understanding of the underlying molecular pathogenesis of TSE remains limited.

The 17 chapters in this book are written by experts from many countries and illustrate the various approaches to risk management in specific regions of the world. In addition to BSE, the book contains chapters about TSE of North America such as scrapie, transmissible mink encephalopathy, and chronic wasting disease of deer and elk; the last disease has recently spread from its endemic Colorado-Wyoming area to six other states in the United States, as well as Alberta and Saskatchewan in Canada.

With the recognition that BSE had spread in the United Kingdom and many other countries through feeding contaminated mammalian meat-and-bone meal to ruminants, a ban on this practice was instigated in the European Union in 1994. This ban has since been adopted by many other regions of the world. In 2000, the European Union strengthened the ban to prohibit feeding processed animal proteins to farmed animals kept, fattened, or bred to produce food. This measure has undoubtedly helped to prevent or reduce numbers of cases of BSE in ruminants but incidentally has led to a new science-based industry for feed analysis. This industry uses sophisticated molecular techniques such as near infrared spectroscopy and microscopy, polymerase chain reaction, and immunoassays to check feed for animal by-products, and to prevent intraspecies recycling (cannibalism), which undoubtedly caused the BSE epidemic that resulted in the death of 186,000 cattle from 1985 to 2002.

I recommend this book as an excellent source of information on all aspects of prion diseases in animals.

Brian W.J. Mahy*

*Centers for Disease Control and Prevention, Atlanta, GA, USA

Address for correspondence: Brian W.J. Mahy, Centers for Disease Control and Prevention, Mailstop C12, 1600 Clifton Road, Atlanta, GA 30333, USA; fax: 404-639-4197; email: bxm1@cdc.gov

On February 25, 2003, more than 30 representatives from United Nations agencies, international nongovernmental organizations, research institutions, professional associations, and private companies met in Geneva, Switzerland, to establish the International Network to Promote Household Water Treatment and Safe Storage, sponsored by the World Health Organization (WHO). The group has since convened at the Kyoto World Water Forum, Kyoto, Japan, in Washington, D.C., and in Cape Town, South Africa. At the next plenary meeting, in Nairobi, Kenya, on June 14–15, 2004, participants will consider a 5-year strategic plan to reduce waterborne disease through specific actions in research, advocacy, communication, and implementation.

This first phase has seen progress in the organization and expansion of the network's participant base and real work in the field, building on the evidence that household water management can contribute to meeting the Millennium Development Goals for child survival and water security. This fieldwork has reaffirmed the conclusion of a WHO-sponsored review: simple, low-cost interventions for home water treatment and storage lead to dramatic improvements in drinking water quality and reductions in diarrheal disease (1).

The Safe Water System, developed by the Centers for Disease Control and Prevention (CDC), the Pan American Health Organization (PAHO), and WHO, combines point-of-use water disinfection with locally produced sodium hypochlorite, safe storage in narrow-mouth containers, and community education and has consistently been effective in preventing diarrhea (2,3). In recently published trials, the Safe Water System reduced diarrhea by 24% in Bangladesh (4) and 25% in Guatemala (5). In a 2003 study, the Safe Water System reduced diarrhea by 30% among persons with HIV infection in rural Uganda (6).

In 2003, accounts of field trials of a household-based flocculant-disinfectant for water treatment were published for the first time. Developed by the Procter & Gamble Company and CDC, the intervention combines a chemical flocculant with a timed-release hypochlorite disinfectant. Through precipitation, coagulation, and flocculation, the combined product physically removes a broad range of microbial pathogens and chemicals, including arsenic, and concurrently inactivates remaining microbes with free chlorine (7,8). In a randomized, controlled trial in Guatemala, use of the product reduced the incidence of diarrhea among intervention households by 24%, or 29% when the treated water was stored in a vessel designed specifically for safe storage (5).

In 2003, considerable progress was made in evaluating the impact of household-based filtration. In a large field trial, Rita Colwell and colleagues showed that simple filters made from sari cloth or nylon, combined with appropriate education, reduced cholera by 48% compared to controls (9). Locally produced slow sand and ceramic filters were evaluated by Massachusetts Institute of Technology postgraduate students (10–12). In a trial in Bolivia, locally fabricated filters that used imported ceramic candles eliminated all detectable fecal coliform bacteria in household drinking water and reduced levels of diarrhea by 64% (13).

In 2004, a systematic review of 57 studies assessed the extent and causes of microbiological contamination of household drinking water between the source and the consumer (14). The reviewers concluded that water quality declines substantially after collection and recommended household treatment and safe storage of water. A systematic review of the health impact of improved water quality is under way, driven in part by the burgeoning evidence indicating that substantial health gains result when water is treated in households and protected against recontamination (15,16).

In Nairobi, network members will review recent progress and plan their next steps for advancing household-based water management. Stakeholders from all organizations are urged to participate in, contribute to, and take full advantage of this important new movement in the battle against waterborne disease. For more information on the network and to register for the Nairobi meeting, readers are referred to: http://www.cdc.gov/safewater/network.htm

Thomas F. Clasen* and Eric D. Mintz†

*London School of Hygiene and Tropical Medicine, London, United Kingdom; and †Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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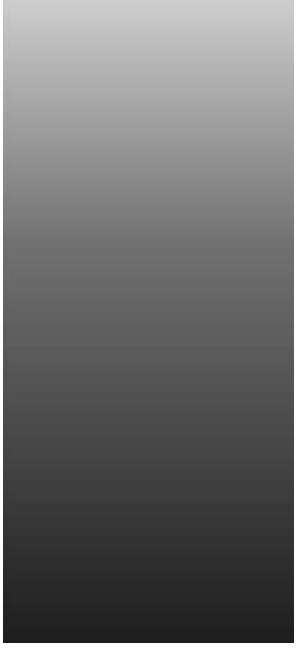
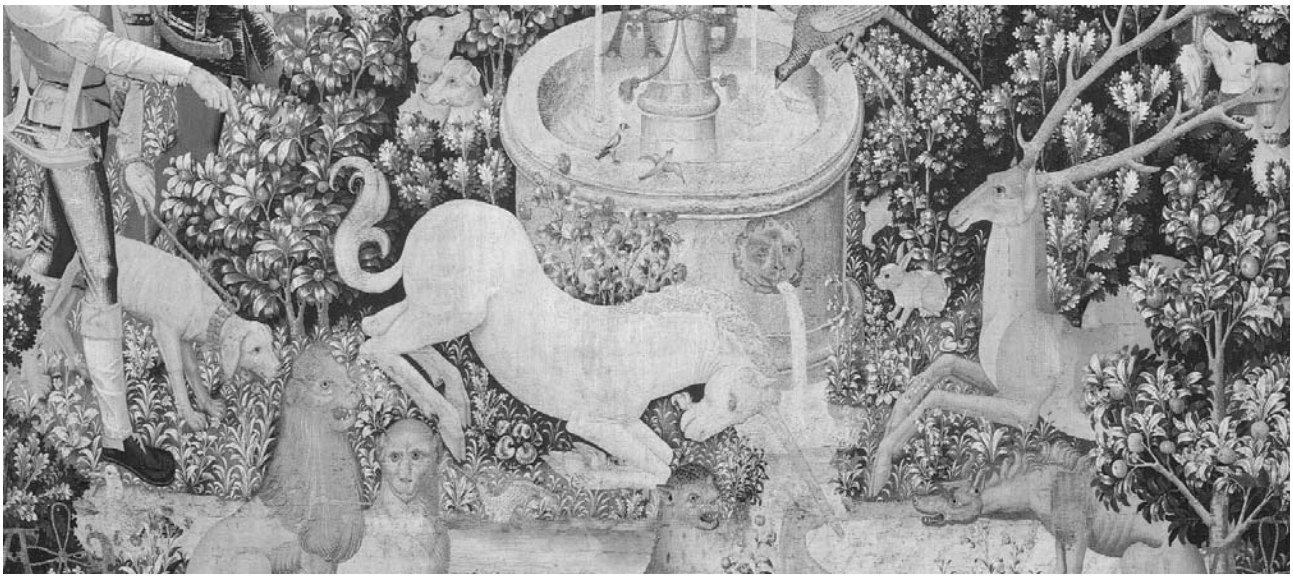
Address for correspondence: Eric D. Mintz, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop A38, Atlanta, GA 30333, USA; fax: 404-639-2205; email: emintz@cdc.gov





South Netherlandish, The Unicorn Is Found, from Hunt of the Unicorn (1495–1505)
Wool warp, wool, silk, silver and gilt wefts, 368 cm x 379 cm. The Metropolitan Museum of Art, Gift of John D. Rockefeller Jr., 1937

Unicorn Tapestries, Horned Animals, and Prion Disease

Polyxeni Potter

"The written text is a recent form of textile, ancillary to those primary texts 'told' or 'tooled' in cloth" (1). Thousands of years before humans could write, they could weave, turning cloth into a commodity, symbol of wealth, and decorative art. In many cultures, weaving has become a common linguistic metaphor as "weave" is used broadly to mean "create." A weaver "not only fashions textiles but can, with the same verb, contrive texts" (2).

Textiles, which date back to the Neolithic and Bronze Ages, have strong egalitarian roots and an ancient connection with personal expression (3). The primary purpose of weaving was storytelling. In Greek mythology, skilled Arachne challenged Athena, the goddess of wisdom and patron of the loom, to a contest. Arachne wove an elaborate account of the scandalous lives of gods and won respect from the judges but wrath from Athena, who turned her into a spider. In 11th-century France, unknown weavers stitched the conquest of England on 70 meters of linen in the Bayeux Tapestry.

Countless techniques and styles of weaving evolved throughout the ages, producing imaginative designs through a tactile process (4). In the Middle Ages, representational and other scenes commissioned by wealthy patrons and composed in tapestry workshops all over Europe decorated and insulated large structures (5). Luxurious wool, silk, and other threads were used in massive tapestries depicting love of nature and the outdoors; a penchant for luxury; and a desire to communicate, shown in banners, initials, and other messages encrypted on the scenes.

The Unicorn Is Found, on this month's cover of Emerging Infectious Diseases, is one of seven famed tapestries depicting the Hunt of the Unicorn. The works are housed at The Cloisters, a part of the Metropolitan Museum of Art, New York, designed specifically to evoke the context in which these and other medieval artifacts were created (6). The brilliant descriptions in these tapestries of exotic plants and animals, local customs, and human endeavor document a thriving arts scene in an era often labeled the "Dark Ages."

The tapestries, whose origins and history are as mysterious and enigmatic as their designs, could have belonged to Anne of Brittany, who married King Louis XII in 1499 (6). All tapestries prominently display the cipher AE and have a unifying theme: the struggle between humans and the unicorn. This creature, whose mythologic origins go back to ancient China, dominates the scenes and not only for its unique phenotypic demeanor. Strong and purposeful, it claims center stage, interceding on behalf of animals and working for the common good—purifying the stream. Although the motives and intentions of the medieval weavers and their patrons are unknown, the tapestries are likely allegorical. Why else would a beautiful, light-hearted, and benign creature on a positive mission be hunted like a common stag?

The Unicorn Is Found is thick with foliage, animals, and hunters crowding around a fountain to observe the outlandish creature. Woven in minute detail, the gothic landscape captures a universal fascination with myth and magic. As the horn is dipped into the stream, the "hunters," decked out in finery inappropriate for the hunt, form a conspiratorial circle, plotting the kill. Hunting-dogs afoot, reason aside, they close in. No one knows where the madness started or how it spread. Tension mounts as animals, beneficiaries of the unicorn's gesture, gather sheepishly in the foreground, anticipating its capture and fall. The color is intense, the crowd fickle, the unexplained madness infectious.

As travel to remote parts of the globe put myths and legends first under scrutiny and then to rest, unicorn sightings have ceased. Less exotic horned creatures, among them deer, elk, mule deer, greater kudu, have persevered in spite of at times heavy hunt and an array of infections, including, recently, prion diseases. Chronic wasting disease, which spreads by unknown routes among susceptible deer and elk, is on the increase (7). Like the madness in Hunt of the Unicorn and bovine spongiform encephalopathy (BSE) in other mammals, this disease could break the species barrier through foodborne or other transmission and extend its devastating neurologic effects to humans. Of all species naturally exposed to BSE, the greater kudu, perhaps the exotic unicorn's closest relative on the tapestry scene, appears most susceptible (8).

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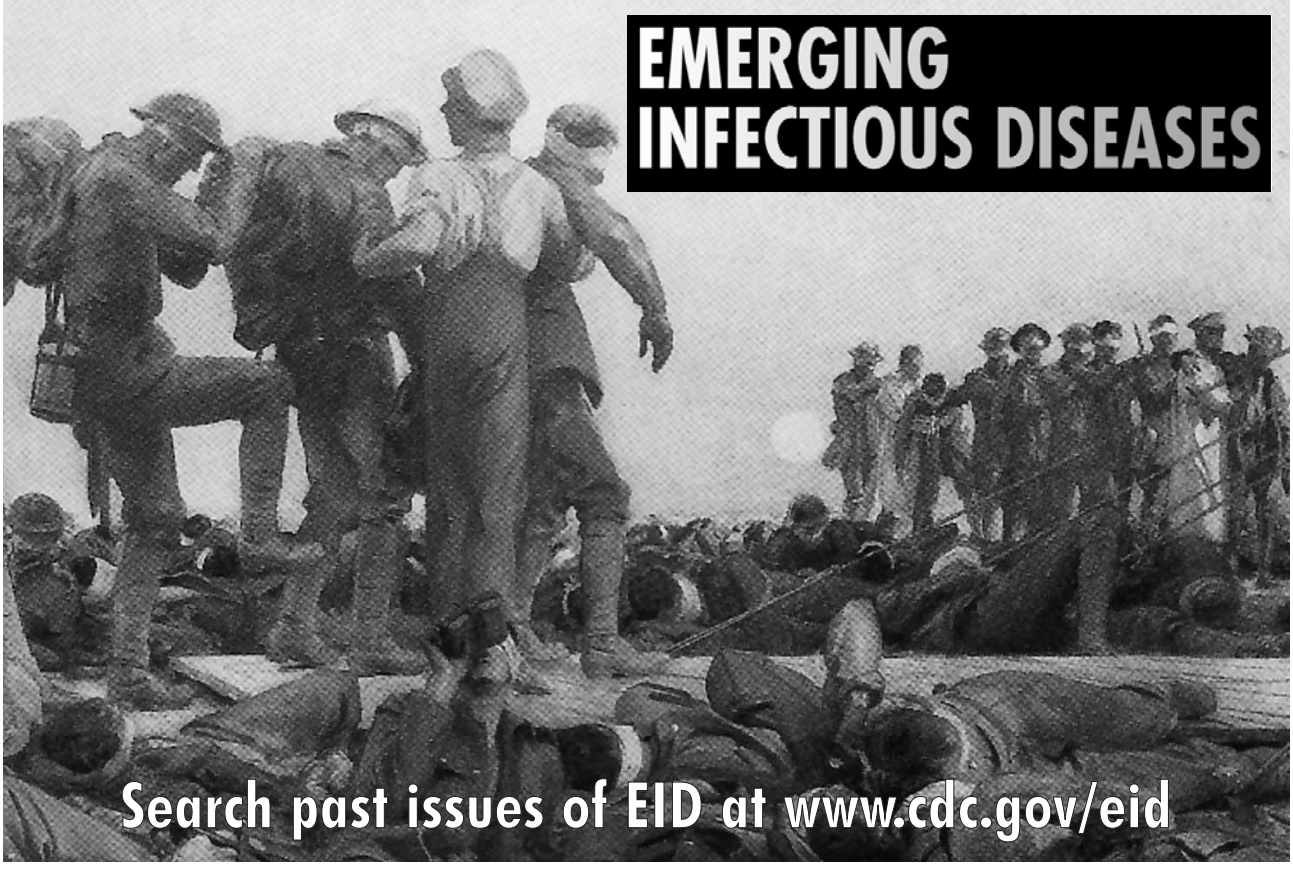
Address for correspondence: Polyxeni Potter, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., Mailstop D61, Atlanta, GA 30333; fax: 404-371-5449; email: pmp1@cdc.gov



Upcoming Infectious Disease Activities

June 3–5, 2004
13th International Symposium on HIV & Emerging Infectious Diseases
Toulon, France
http://www.avps.org/2003/hiv.htm

June 14–15, 2004
Plenary Metting,
International Network to Promote Household Water Treatment and Safe Storage
Nairobi, Kenya
Contact: 301-656-0003
resistance@nfid.org
http://c3.org/partnerships/wrn/int-water-treat

June 28–30, 2004
2004 Annual Conference on Antimicrobial Resistance
Bethesda, MD
Contact: 301-656-0003
resistance@nfid.org
http://www.nfid.org/conferences/resistance04

August 6–8, 2004
FACES 2004 Encephalitis Conference
Enfield, CT
http://www.encephalitisglobal.com

September 19–23, 2004
Extremophiles 2004
American Society for Microbiology
Cambridge, MD
http://www.asm.org/Meetings/index.asp?bid=19177

September 23-26, 2004
JPGM Gold Con
International conference on journal writing and publishing
Parel, Mumbai, India
Contact: Atul Goel, Editor, Journal of Postgraduate Medicine,
91-22-24129884
goldcon@jpgmonline.com
http://www.jpgmonline.com/goldcon.asp

EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol. 10, No. 7, July, 2004

Upcoming Issue

Look in the July issue for the following topics:

Response to SARS, Taiwan

Passive Surveillance and Imported Malaria in Canada

Situational Awareness: Estimating Time and Size of Bioterror Attack

SARS Control and Psychological Effects of Quarantine, Toronto, Canada

SARS-associated Coronavirus in Oral Specimens and Early Diagnosis

Sporadic Cryptosporidiosis Case-Control Study with Genotyping

Fluoroquinolone and Other Antimicrobial Resistance in Pneumococci, Hong Kong, 1995–2001

Model Parameters and Outbreak Control for SARS

Alert Threshold Algorithms and Malaria Epidemic Detection

Measuring Haemophilus influenzae type b Disease in Developing Countries

Recombinant Viruses and Early Global HIV-1 Epidemic

Nosocomial Infection with Vancomycin-dependent Enterococci

Q Fever Outbreak in Industrial Setting

Complete list of articles in the July issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

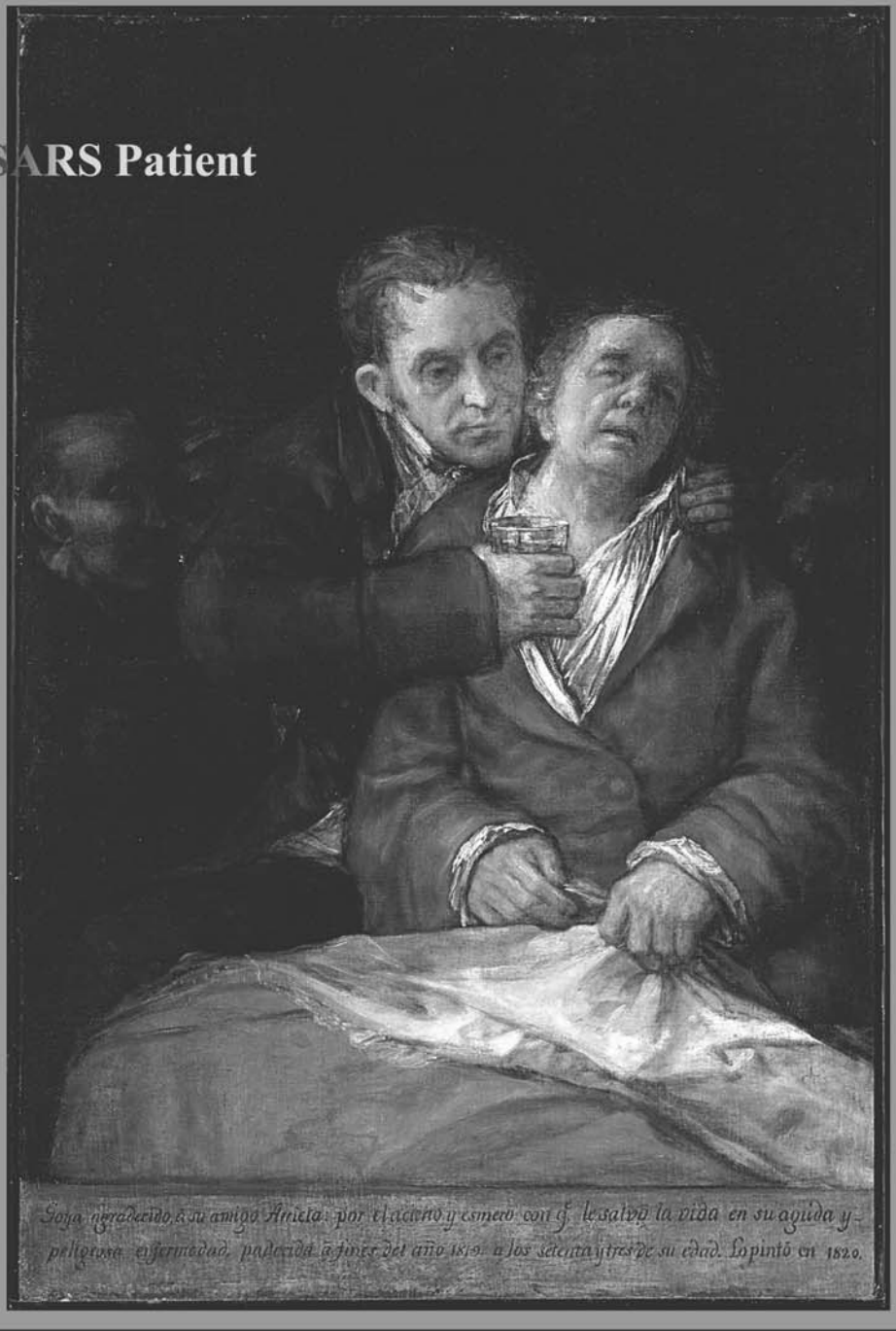
EMERGING INFECTIOUS DISEASES

EID Online
www.cdc.gov/eid

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Vol.10, No.5, May 2004

The SARS Patient



CDC
SAFER·HEALTHIER·PEOPLE

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (email).

Emerging Infectious Diseases is published in English Chinese, French, and Spanish translations of some articles can be accessed at http://www.cdc.gov/eid/ncidod/EID/trans.htm. The journal features the following types of articles: Perspectives, Synopses, Research Studies, Policy and Historical Reviews, Dispatches, Commentaries, Another Dimension, Letters, Book Reviews, and News and Notes. The requirements of each type of article are described in detail below and at http://www.cdc.gov/eid/ncidod/EID/instruct.htm. To expedite publication, we post journal articles on the Internet as soon as they are edited.

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases website (www.cdc.gov/eid).

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, 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.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. This section includes letters that present preliminary data or comment on published articles. Letters (500–1,000 words) should not be divided into sections should contain no more than one figure or table. References (no more than 10) may be included.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. (500–1,000 words) of emerging infectious disease conferences may provide references to a full report of conference activities and should focus on the meeting's content rather than on individual conference participants.