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Emerging Pattern of Rabies Deaths and Increased Viral Infectivity

Sharon L. Messenger,* Jean S. Smith,* Lillian A. Orciari,* Pamela A. Yager,* and Charles E. Rupprecht*

Most human rabies deaths in the United States can be attributed to unrecognized exposures to rabies viruses associated with bats, particularly those associated with two infrequently encountered bat species (Lasioglossum noctivagans and Pipistrellus subflavus). These human rabies cases tend to cluster in the southeastern and northwestern United States. In these regions, most rabies deaths associated with bats in non-human terrestrial mammals are also associated with virus variants specific to these two bat species rather than more common bat species; outside of these regions, more common bat species contribute to most transmissions. The preponderance of rabies deaths connected with the two uncommon L. noctivagans and P. subflavus bat rabies viruses is best explained by their evolution of increased viral infectivity.

Bites by rabid dogs are the source of 35,000–50,000 human rabies deaths each year globally (1), yet most human rabies deaths in the United States are attributed to unrecognized exposures to rabid bats. Particular attention has focused upon two relatively rare bat species (Lasioglossum noctivagans and Pipistrellus subflavus) because rabies variants associated with these species account for approximately 70% of human cases and 75% of cryptic rabies deaths (2–6).

Molecular typing (i.e., phylogenetic analysis of DNA data) has shown that rabies viruses associated with insectivorous bats (L. noctivagans and P. subflavus variants in particular) are the culprits in what otherwise would have been unsolved cryptic human rabies deaths. However, phylogenetic analyses of human rabies cases have not provided insights into why an unexpectedly large proportion of human rabies deaths involve the uncommon L. noctivagans and P. subflavus variants. Passive surveillance systems used by state public health departments confirm that human encounters with Eastern Pipistrelle bats (P. subflavus) and Silver-haired Bats (L. noctivagans) are rare. Neither species exceeded 5% of all bats submitted for rabies testing in the southeastern United States, and Silver-haired Bats account for <12% of all bats submitted in the Northwest (7). Moreover, the prevalence of rabid individual bats within each species is also low and similar to the estimated prevalence in other, more common, bat species (8,9). While these surveillance studies are known to produce biased estimates of the true prevalence of rabies in natural bat populations, they should accurately reflect the prevalence of rabies in bat species encountered by the public. In addition, we have determined that although other bat species occasionally are infected with L. noctivagans and P. subflavus variants, the frequency of such spillover is low (6,10). A survey of rabid “house bats” (i.e., Eptesicus fuscus and Myotis lucifugus) in the United States revealed that only 2 of 117 E. fuscus and 4 of 15 M. lucifugus were infected with L. noctivagans and P. subflavus variants (6). The sample size of M. lucifugus is notably small because this species is rarely found rabid, despite submissions of thousands of individual bats each year (11). Thus, all available data suggest that L. noctivagans and P. subflavus variants are rare.

Given that L. noctivagans and P. subflavus variants appear to be infrequently encountered, two explanations have been proposed to explain their prevalence among cryptic human rabies deaths associated with bats. The small vector hypothesis suggests a failure to recognize that a bite has occurred when a small bat is involved (7,12). Absence of a bite history may result from inaccurate documentation when a patient could not be questioned directly or was not lucid. In 18 of 34 cases with no bite history, however, documented contact with a potentially rabid animal could have contributed to an unrecognized bite (i.e., physical contact with bats in 11 cases, bats in residence in 4 cases, and contact with a known sick domestic animal in 3 cases). Among these contacts, 11 involved L. noctivagans and P. subflavus variants. In addition, bites by smaller bat species, such as Eastern Pipistrelles, may be more likely to go unnoticed than bites by larger bats, which may explain why more cases are associated with this species. Wounds from the teeth of small bat species are not easily seen without careful examination (13,14), possibly leading to the impression that a bite has not occurred.

A second hypothesis was suggested by results from experimental data comparing rabies virus isolates from Silver-haired Bats with those from domestic dogs and a coyote (15,16). These data showed that, although both viruses replicated equally well in neuroblastoma cells, rabies viruses from Silver-haired Bats replicated to higher titers in fibroblast and epithelial cells, particularly at low temperatures of 34°C. Such growth characteristics might facilitate successful infection after a superficial bite. Thus, superficial contact may occur frequently between bats and terrestrial mammals (including humans), but may be unlikely to result in a productive infection unless L. noctivagans and P. subflavus variants are involved (the increased infectivity hypothesis). Although these experimental data suggest that L. noctivagans and P. subflavus variants have evolved increased infectivity, comparison between viruses associated with Silver-haired Bats and dogs

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(including one coyote) is not sufficient to pinpoint whether increased infectivity evolved in the common ancestor of *L. noctivagans* and *P. subflavus* variants and, thereby, is relevant to the noted prevalence of these variants among human rabies deaths.

We proposed a novel test of the increased infectivity hypothesis using a comparative phylogenetic approach in which we characterized transmission patterns from bats to nonhuman terrestrial mammals. Transmission of bat rabies to terrestrial mammals can be viewed as a natural experiment that removes the confounding effects of vector size and, therefore, can be used as a control in understanding transmission patterns between bats and humans. That is, such comparisons take advantage of a fundamental difference between rabies exposures in humans and rabies exposures in other terrestrial mammals (nonhuman terrestrial mammals cannot initiate postexposure prophylaxis even if they are aware of a bite). Given that size of the vector species is not a factor in terrestrial mammal deaths, we can control for the effect of bat vector size in our comparison. Therefore, a disproportionate number of *L. noctivagans* and *P. subflavus* cases among terrestrial mammals would be consistent with increased infectivity of the *L. noctivagans* and *P. subflavus* variants. If these variants are not over-represented in terrestrial mammal rabies cases, we can reject the increased infectivity hypothesis, leaving the small vector hypothesis as the most plausible alternative.

**Methods**

**Sequence Data**

Sequences were obtained from reverse transcriptase polymerase chain reaction (RT-PCR)-amplified portions of the nucleoprotein (positions 1,157–1,477), based on the reference strain M13215 (17). The taxa include 32 rabies virus samples obtained between 1958 and 2000 from frozen (n=27) and formalin-fixed (n=5) U.S. human brain tissue specimens, 17 of 41 U.S. bat species (n=54), and 98 nonhuman terrestrial mammals (24 cats, 3 dogs, 5 cattle, 9 horses, 1 sheep, 1 llama, 42 foxes [grey, red, and kit], 1 raccoon, 1 ringtail cat, and 11 striped skunks), determined to have been infected by a bat rabies virus by using monoclonal antibody screening. Reservoir species from the eight U.S. terrestrial rabies-endemic regions (n=24) were included as outgroups. Nucleotide sequences included in this study will be deposited in GenBank but are also available as an aligned matrix (batrabies.nex).

**Phylogenetic Analyses**

Phylogenetic analyses used PAUP* 4.0b2 (18), employing the neighbor-joining search algorithm (minimum evolution) using maximum likelihood to estimate Ti:Tv ratio and nucleotide base frequencies (settings correspond to Hasegawa-Kishino-Yano [HKY] 1985 model of nucleotide sequence evolution). We assessed tree support using the nonparametric bootstrap method (1,000 replicates). Phylogenetic analyses presented here included only frozen human brain samples (n=27). Additional analyses (not shown) that included incomplete taxa (formalin-fixed human case samples) did not alter tree topology but decreased nonparametric bootstrap proportions for nodes including formalin-fixed taxa.

**Results and Discussion**

Phylogenetic analysis (Figure 1) links 20 of 27 bat-associated human cases (24/32 cases, including formalin-fixed samples not shown in Figure 1) with *L. noctivagans* and *P. subflavus* variants. In contrast to the patterns seen in humans (Figures 1 and 2A), a wide variety of bat rabies variants are implicated in the terrestrial mammal cases across a broad geographic area and largely correspond to the most common bat rabies virus variants in that geographic region (Figures 1 and 2B). Two notable exceptions to this general geographic pattern occur in the northwestern and the southeastern United States (Figure 2B). In these two regions, *L. noctivagans* and *P. subflavus* variants account for a substantially larger percentage of transmission events to terrestrial mammals than expected, given the rarity of the host bat species and *L. noctivagans* and *P. subflavus* variants in those geographic areas (Figure 2C) (because spillover of these variants to other bat species is rare, we used the prevalence of rabid Silver-haired Bats and Eastern Pipistrelles as a surrogate for the prevalence of *L. noctivagans* and *P. subflavus* variants). These two geographic regions also match very closely the geographic distribution of human rabies deaths in the United States associated with these variants (Figure 2A). In the northwest region, delimited by the human cases associated with Silver-haired Bats (clade 1), the *L. noctivagans* rabies virus variant accounted for 57% of bat-associated cases in terrestrial mammals and 80% of bat-associated cases in humans, despite the fact that rabies-positive Silver-haired Bats account for only 5% of all bats submitted for testing. Similarly, in the southeast region, delimited by human cases associated with Eastern Pipistrelles (clade 2), where neither Eastern Pipistrelles nor Silver-haired Bats exceed an average of 2% of all rabies-positive bats submitted, the *P. subflavus* variant accounted for 63% of bat-associated cases in terrestrial mammals and 89% of bat-associated cases in humans. The high prevalence of *L. noctivagans* and *P. subflavus* variants among terrestrial mammals in the same regions where human cases have occurred suggests that a similar mechanism, such as increased infectivity of these rabies virus variants, is responsible for both epidemiologic patterns.

Our study included 13 terrestrial mammal species, both domesticated and wild, suggesting that exposure to bat species likely occurred in a variety of habitats, including more remote forest habitats. Our data set was comprised mostly of foxes and cats (two species likely to capture and feed upon bats), but we had no reason to suspect that their behavior would create a disproportionate opportunity for infection by *L. noctivagans* and *P. subflavus* variants compared with other bat variants.

While our data do not invalidate the small vector hypothesis, the terrestrial mammal data show that even when vector size does not play a role in determining whether bat bites are
recognized, *L. noctivagans* and *P. subflavus* variants are still the most prevalent rabies virus variants among bat-associated terrestrial mammal deaths in the northwestern and southeastern United States. For human rabies cases, small vector size may still play a role in the probability of detecting a bat bite, while the increased infectivity of *L. noctivagans* and *P. subflavus* variants enhances the likelihood of a successful infection following a superficial bite. Additional experimental data (e.g., site-directed mutagenesis) will be necessary to show definitively whether *L. noctivagans* and *P. subflavus* variants have evolved genetic changes associated with increased infectivity. Nonetheless, comparisons of phylogenetic patterns between independent datasets in which one data set can be used to control for one or more potentially relevant parameters offers a valid method of hypothesis testing. Additionally, through phylogenetic analyses such as these, we can target those taxa and genomic regions that warrant further investigation.

Figure 1. Phylogenetic tree of bat-associated rabies cases. Taxa represent 203 rabies virus variants from 27 human rabies cases (formalin-fixed taxa removed) and 98 terrestrial mammals infected with a bat rabies virus, 54 bat samples representing 17 species, and 24 terrestrial mammal outgroup taxa. Each circle represents a case (terrestrial mammal = closed circles, human = open circles) associated with the monophyletic clade in the phylogeny to the left. Numbers at tree nodes indicate nonparametric bootstrap proportions.
Figure 2. Geographic distribution of (A) human (including 5 formalin-fixed samples not shown in Figure 1) and (B) terrestrial mammal cases identified by rabies virus variant isolated. Minimum length polygons delimiting human cases associated with Silver-haired Bats (clade 1) and Eastern Pipistrelles (clade 2) shown in (A) and superimposed in (B). (C) Prevalence of Lasionycteris noctivagans (Ln) and Pipistrellus subflavus (Ps) variants in regions delimited by clades 1 and 2. Prevalence of variants in bats was estimated from unpublished state public health department reports that determined the percentage of rabies-positive Silver-haired or Eastern Pipistrelle bats from the total number of bats submitted (7, unpub. data). Prevalence of L. noctivagans and P. subflavus variants in terrestrial mammals and humans is estimated as the percentage of all spillover cases in each clade region infected with L. noctivagans or P. subflavus.

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Dr. Messenger has worked on bioterrorism preparedness programs at Louisiana State University and the Centers for Disease Control and Prevention. Her work includes training, development of rapid diagnostics laboratory procedures, and rapid diagnostic testing of clinical and environmental samples. She continues to employ the tools of molecular genetic and phylogenetic analyses to investigate the evolution and ecology of infectious disease pathogens and how that knowledge can be applied to improved detection, surveillance, and forensic technologies.

References

Araçatuba Virus: A Vaccinialike Virus Associated with Infection in Humans and Cattle

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We describe a vaccinialike virus, Araçatuba virus, associated with a cowpoxlike outbreak in a dairy herd and a related case of human infection. Diagnosis was based on virus growth characteristics, electron microscopy, and molecular biology techniques. Molecular characterization of the virus was done by using polymerase chain reaction amplification, cloning, and DNA sequencing of conserved orthopoxvirus genes such as the vaccinia growth factor (VGF), thymidine kinase (TK), and hemagglutinin. We used VGF-homologous and TK gene nucleotide sequences to construct a phylogenetic tree for comparison with other poxviruses. Gene sequences showed 99% homology with vaccinia virus genes and were clustered together with the isolated virus in the phylogenetic tree. Araçatuba virus is very similar to Cantagalo virus, showing the same signature deletion in the gene. Araçatuba virus could be a novel vaccinialike virus or could represent the spread of Cantagalo virus.

The poxviruses comprise a family of large DNA viruses capable of infecting vertebrate and invertebrate hosts (1). Viruses from this family have caused naturally occurring or introduced infections in all populated continents (2). In Brazil, as in other parts of South America, little is known about the occurrence and circulation of poxvirus in the wild (3–6). After the worldwide elimination of smallpox in the 1970s, a few reports of poxvirus isolation in South America have been published, including scattered reports of parapoxvirus outbreaks in sheep and goat herds and virus isolation from wild or captive animals (7,8). The existence of mouselopox outbreaks in animal facilities is also known, but most cases remain unpublished.

In recent years, however, many cases of unidentified diseases in dairy cattle with similar pathology have been reported in rural areas of Brazil, and some human infections have been associated with these illnesses in herds. Such diseases, characterized by the appearance of nodular and pustular lesions on bovine teats, are frequently related to viral infections such as bovine herpes mammillitis, pseudocowpox, and cowpox infections (9–12).

After clinical and initial laboratory analysis, cowpox virus (CPXV) was considered to be the obvious etiologic agent causing this human and cattle infection. CPXV (genus Orthopoxvirus) is the causative agent of localized and painful vesicular lesions. The virus is believed to persist in wild host reservoirs (including mammals, birds, and rodents), cattle, zoo animals, and domestic animals, including cats in parts of Europe and Asia. Contact of these reservoirs with susceptible animals and people can trigger the onset of disease (13,14). When humans are affected, the lesions occur on the hands and sometimes on the arms, usually followed by axillary adenopathy (15). However, CPXV isolation has not been reported from cattle or humans in Brazil, which led investigators to consider the possibility that infections were caused by vaccinia virus (VACV), since VACV was used as a live smallpox vaccine throughout the country until the late 1970s.

The occurrence of VACV-infected animals (domestic or wild species) is believed to be a result of contact with people recently vaccinated against smallpox. In fact, during mass smallpox vaccination campaigns, VACV infections were occasionally transmitted from the vesicular lesion on the vaccinee to domestic animals, usually cattle. In turn, infected animals transmitted VACV to susceptible people (14,16,17). Such infections were shown to be reproducible in experimental conditions (18).

Vaccinialike viruses have been isolated from the wild in Brazil; at least one of these viruses, the Cantagalo virus, was specifically obtained from infected cattle and humans after an outbreak of a cowpoxlike disease (6,14,19). These facts indicate the long-term establishment and active circulation of different vaccinialike viruses in the wild in South America, similar to the well-documented establishment of buffalopox virus in India (19,20).

We describe the isolation and characterization of a vaccinialike strain linked to a cowpoxlike outbreak affecting a dairy herd and associated with human infection; a similar outbreak attributed to Cantagalo virus infection was recently described (14). The virus reported here, named Araçatuba virus, was readily identified as a poxvirus by conventional methods, including characterization of pock morphology on the chorio-
allantoic membrane of chick embryos and electronic microscopy, which allows a quick differentiation between CPXV, pseudocowpox virus, and herpesvirus. However, such techniques do not differentiate between closely related viruses such as CPXV and VACV. To obtain accurate phylogenetic information, we detected poxvirus-conserved genes, such as thymidine kinase (TK), vaccinia growth factor (VGF), and hemagglutinin (HA), in the genome of Araçatuba virus using polymerase chain reaction (PCR). These genes were sequenced and the data used to generate phylogenetic trees. We also analyzed the A-type gene (ATI) based on restriction length polymorphism, which is a phylogenetic tool used to differentiate and classify orthopoxviruses (13). Based on these techniques, Araçatuba virus was shown to be similar to VACV–Western Reserve (WR) strain, the prototype member of the poxvirus family and the Orthopoxvirus genus. In addition, in relation to the HA gene, Araçatuba virus was very similar to Cantagalo virus, showing the same signature deletion in the gene. Such findings specifically point to the ubiquity of VACV circulating in the wild in Brazil as well as to the public health problems that may arise from the presence of this virus.

Methods

Case Report

Five adult Girolanda cows from a herd of 40 animals were sent to the Veterinary Teaching Hospital at Unesp-Araçatuba, São Paulo State, Brazil; they had painful lesions on their teats, which interfered with milking. Lesions initially appeared on 2 cows and spread quickly to 35 animals, as well as the milker’s hands (Figure 1). Starting as a red focal area, the lesions developed quickly into a wound that healed with difficulty. No such episode had previously occurred on that farm. The cows had these symptoms for approximately 8 days before being taken to the veterinary surgeon. During the clinical examination, lesions in different stages were recognized; in most of the cows, nodular ulcerative wounds of 2–6 mm in diameter were predominant. Lesions were localized only on teats and udder, and many of them had dark, raw crusts. The teats had increased local temperature and were sensitive to touch. Because of the pain, cows avoided their suckling calves. At the farm, the only manual milker was also affected. The milker had approximately 10 lesions on both hands and arms, but he did not initially accept any medical help and did not consent to examination. Because aseptic measures were not carried out, contact between the cows’ teats and the milker’s hands during milking probably enhanced the rapid spread of virus within the herd. Oral vesicles were not observed on calves’ muzzles or on buccal mucosae. Sterile samples of the vesicles and crusts were collected and sent to the Laboratório de Viroses de Bovídeos, Instituto Biológico, São Paulo for analysis. The animals were isolated from the herd, and teat lesions were treated with glycerine and a topical antibiotic, while the milker received medication at a nearby hospital. Three months after onset of infection, the remaining lesions on the cows were in an advanced healing process; however, all affected cows produced substantially less milk.

Virus Isolation and Electron Microscopy

The material collected was prepared in 20% suspension of Eagle minimal essential medium (MEM) with 1% antibiotic to isolate the virus by inoculations in bovine fetal kidney cell monolayers at the Instituto Biológico, São Paulo. Samples that showed cytopathic effects were analyzed by transmission electronic microscopy. Material isolated from bovine fetal kidney cell monolayers was spread on the chorioallantoic membrane of embryonated chicken eggs and incubated at 37°C for 72 h (21).

Cells and Viruses

VACV, WR strain, was obtained from the National Institute for Medical Research (Mill Hill, London, U.K.) and CPXV, Brighton strain, was provided by Dr. C. Jungwirth, Würzburg, Germany. Viruses were propagated in Vero cells and purified in a sucrose gradient as described (22). Vero cells were propagated at 37°C in MEM, supplemented with 5% fetal calf serum. Vero cells were also used for viral titration (23).
Amplification and Cloning of Homologous VGF Gene and TK

The primers based on the TK and VGF nucleotide sequence of VACV–WR were produced as described by Fonseca et al. (6). The purified Araçatuba virus genome was used as a template, and temperatures of 45°C were used for annealing. Amplified fragments were cloned into the pGEMT vector (pGEM-T Easy Vector Systems, Promega Corp., Madison, WI). The portion of the HA coding sequence was amplified by using primers EACP1 and EACP2 as described by Roop et al. (24), and the approximately 900-bp fragment was produced and cloned into the pGEMT vector.

Amplification and Restriction Fragment Length Polymorphism (RFLP) of ATI Gene

A PCR-based method for rapid screening and taxonomic differentiation is currently used to explicate Orthopoxvirus taxonomy (25,26). The assay uses primers designed from the ATI gene sequence from CPXV. We performed PCR with the primer pair ATI-up 5′-AATACAAGGAGGATCCT3′ and ATI-low 5′-CTTAACCTTTTTCTTCT3′. After the amplification reactions were carried out, the amplicons were digested with XbaI at 37°C for 3 h, as described (26).

Nucleotide Sequencing

The PCR-amplified TK, VGF, and HA fragments of Araçatuba virus, cloned into the pGEMT plasmids, were sequenced in both orientations by the dideoxy-chain termination method (27) by using M13 universal primers (fmol DNA Sequencing System; Promega Corp.) and [α32P]dCTP for oligonucleotide labeling. Sequences were analyzed by using the BLASTN and BLASTX programs (28). The DNA sequences of the Araçatuba virus, TK, and VGF genes were deposited in GenBank (accession nos. AF503169 and AF503170). A phylogenetic tree was constructed by using the Treecon program with the Araçatuba virus–TK and Araçatuba virus–VGF nucleotide sequences (29).

Results

Virus Morphology

After Araçatuba virus was isolated in bovine fetal kidney cell monolayers, the samples were viewed by transmission electronic microscopy. Typical brick-shaped poxvirus forms were observed, measuring about 260 x 360 nm, with a superficial structure formed by tubules on long irregularly arranged filaments (data not shown). Samples were also added to embryonated chicken eggs so pock formations could be visualized on chorioallantoic membranes. White, nonhemorrhagic pocks were found (data not shown).

PCR of Conserved Genes in Orthopoxvirus Genus and Nucleotide Sequence Analysis

PCR amplification of TK, VGF, and HA genes generated 528-, 381-, and 960-bp fragments, respectively. Amplicons were cloned into pGEMT vector and sequenced in both orientations. When compared to nucleotide sequences available in the GenBank databases using the BLASTN program, the TK and VGF genes from Araçatuba virus were highly similar to homologous genes of VACV–WR. Optimal alignment showed similarity rates of up to 99.5% between Araçatuba virus and VACV–WR genes and minimal differences from nucleic acid substitutions. The coding region of HA gene was analyzed by alignment with similar sequences of VACV–WR and Cantagalo virus deposited in GenBank (accession nos. AF229247 and AF482758.1). The Araçatuba virus HA nucleotide sequence contained a signature deletion identical to a deletion detected in the sequence of Cantagalo virus (Figure 2A). This feature, absent in the sequence of most VACV strains, was used to correlate Araçatuba virus with VACV strain Instituto Ozwaldo Cruz (IOC), which was used as vaccine in some regions of Brazil during the smallpox eradication campaign (14). Using the nucleotide sequences from Araçatuba virus and other poxviruses, we constructed evolutionary trees with the Treecon program and placed Araçatuba virus isolate in the same cluster as other VACV strains (Figure 2B and 2C).

Analysis of the ATI Gene Amplicom

Although the formation of typical A-type inclusions is restricted to cells infected with cowpox virus, ectromelia virus, and raccoonpox virus (2), the sequence coding the N-terminus of the protein is highly conserved in many viruses, including CPXV, VACV, variola virus, camelpox virus, and ectromelia virus. These conserved sequences flank variable regions containing different size deletions, which may generate different size fragments after PCR amplification. The specificity of this assay is enhanced by the use of restriction enzymes, XbaI or BglII, allowing the detection of mutations at the restriction sites for these enzymes. We amplified the ATI gene from Araçatuba virus, VACV–WR, and CPXV for comparison. As described, the VACV–WR ATI amplicon generated 3 fragments after digestion with XbaI (26) (Figure 3). The larger fragment has approximately 900 bp, and the shorter fragments migrate closely, between the 300-bp and 400-bp markers. The profile obtained after digestion of Araçatuba virus ATI ampli con was similar to that of VACV–WR (Figure 3). The main difference, however, is that the larger fragment generated after XbaI digestion of the Araçatuba virus ATI amplicon is smaller than the VACV–WR fragments. These differences in size are also detected when nondigested ATI amplicons from Araçatuba virus and VACV are compared. Nevertheless, the pattern obtained for Araçatuba virus is completely different from the CPXV ATI pattern (Figure 3).

Discussion

In Brazil, few studies have been conducted on the existence and circulation of poxviruses in the wild. In recent years, however, a growing number of poxvirus isolates have been obtained from samples from wild and domestic animals as well as humans; some of these viruses have caused cowpox-
In this context, we isolated a novel virus, Araçatuba virus, from one of these cases of cowpox-like diseases. The infection affected a herd of milking cows as well as their milker, in a rural area of the state of São Paulo, Brazil. Overall, our results suggest that the isolated virus is a VACV variant. Sequencing of conserved and nonconserved genes from poxviruses, such as TK, VGF, and HA, respectively, has been used for the classification of unknown poxvirus isolates (6,14,19). In the case of Araçatuba virus, phylogenetic trees designed from the nucleotide sequences of these genes indicate clearly that the virus belongs to the VACV subgroup like other orthopoxviruses isolated in Brazil during the 1960s and 1970s, the BeAn 58058 and Cotia viruses (6,19,30). This proposition is strengthened by RFLP analysis of the Araçatuba virus ATI homologous gene. This strategy has also been widely used for poxvirus taxonomy studies (25,26). Although the Araçatuba virus ATI pattern is not identical to the VACV–WR pattern, the virus fits on the VACV subgroup, and the pattern differs decidedly from the CPXV ATI pattern. Such differentiation is important because CPXV was the most obvious candidate to be the agent of such diseases. The Cantagalo virus ATI gene was characterized only at protein level and showed the same pattern of bands as the VACV strains (14).

For now, the discussion about the probable origin of Araçatuba virus, as well as other VACV isolated from animals and people in the country, is purely speculative. Araçatuba virus could be another vaccinialike strain or could represent the spread of Cantagalo virus. A logical assumption is to associate these viruses with variola vaccine stocks that may have escaped to the wild when the vaccination program was taking place in the 1970s and early 1980s. However, identifying the origin of those isolated VACV is difficult since many different samples, such as VACV-Lister, VACV-WR (Brazilian Health Ministry, pers. comm.), VACV-IOC (14), and even mixtures of different samples were used during the smallpox elimination campaign in Brazil. Researchers have proposed that at least one of the isolates, the Cantagalo virus, may have been derived from VACV-IOC (14). However, this finding is based on the
nucleotide sequence of a single gene, and this issue is still a subject of some debate. Nevertheless, the Araçatuba virus HA nucleotide sequence revealed an interesting similarity with that of the same gene from Cantagalo virus, particularly at a signature sequence used to trace back the possible origin of this virus. Also of note, the Cantagalo virus was isolated in the city of Cantagalo (Rio de Janeiro state), about 850 km east of Araçatuba city. Moreover, a similar genetic feature of the HA gene was also detected in yet another cowpoxlike virus isolated from persons in the city of Muriaé (state of Minas Gerais), 800 km north of Araçatuba (data not published).

From the northern border at the Amazon region to the countryside of southeastern Brazil, an alarming number of genetically related vaccinialike viruses have been isolated from infected animals and humans. This fact clearly points to the existence and wide circulation of established, active VACV isolates in the vast wild and rural areas of Brazil. Whether the number of VACV infections has recently increased or whether only now they are being reported is difficult to determine. Nevertheless, the isolation of Araçatuba virus, together with other recently isolated viruses, was sufficient to trigger an alert by the Public Health Bureau in at least one of São Paulo’s neighboring states (Minas Gerais). How these viruses managed to persist in nature so long after the end of smallpox vaccination is a matter of speculation, but we think that they established circulation in some unknown wild hosts and were eventually transmitted to cattle and humans when they came in contact with populations of wild animals because of agricultural expansion.

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References


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To assess the role of horses as amplification hosts during the 1993 and 1996 Mexican Venezuelan equine encephalitis (VEE) epizootics, we subcutaneously infected 10 horses by using four different equine isolates. Most horses showed little or no disease and low or nonexistent viremia. Neurologic disease developed in only 1 horse, and brain histopathologic examination showed meningeal lymphocytic infiltration, perivascular cuffing, and focal encephalitis. Three animals showed mild meningoencephalitis without clinical disease. Viral RNA was detected in the brain of several animals 12-14 days after infection. These data suggest that the duration and scope of the recent Mexican epizootics were limited by lack of equine amplification characteristic of previous, more extensive VEE outbreaks. The Mexican epizootics may have resulted from the circulation of a more equine-neurotropic, subtype IE virus strain or from increased transmission to horses due to amplification by other vertebrate hosts or transmission by more competent mosquito vectors.

*Venezuelan equine encephalitis virus* (VEEV; *Togaviridae: Alphavirus*) is an emerging pathogen of humans and equines in many parts of the New World (1–3). Sporadic outbreaks date to the 1930s or earlier in South America and have affected hundreds of thousands of people, horses, donkeys, and mules, causing high mortality rates in equines and severe illness in humans. Most outbreaks have been confined to northern South America, but one that began in Guatemala and El Salvador in 1969 spread northward through Mexico and reached Texas in 1971 (2,4,5). The etiologic agents during all of the major VEE outbreaks were subtype IAB or IC VEEV. Other subtypes of VEEV, including subtype IE strains that circulate in sylvatic and swamp habitats of Central America and Mexico, are traditionally considered enzootic, equine-avirulent, and incapable of exploiting horses as amplification hosts to cause widespread disease (6–9).

Before 1993, the only VEE outbreak confirmed by virus isolation in Mexico was the 1969–1971 epizootic/epidemic (5). The etiologic agent was a subtype IAB VEEV strain that likely originated from an inadequately inactivated equine vaccine preparation (10,11). Another VEE outbreak in Tamaulipas, Mexico, was detected serologically in 1965–1966, but no virus strains were isolated (12,13).

During 1993 and 1996, small outbreaks of equine encephalitis occurred near the Pacific Coast in the Mexican States of Chiapas and Oaxaca, respectively. These outbreaks involved 125 and 32 equine cases, respectively, with case-fatality rates of 50% and 38%; human VEE was not confirmed, although human seroprevalence in the region is high (J.G.E.-F., S.C.W., unpub. data). Antigenic, sequencing and phylogenetic studies indicated that the VEEV strains isolated from horses belong to subtype IE and are closely related to enzootic strains isolated nearby in sylvatic habitats on the Pacific Coast of Guatemala from 1968–1980 (14–16). These Mexican outbreaks represented the first confirmed equine cases attributed to VEEV subtype IE infection. Although the 1966 Tamaulipas epizootic may have been caused by subtype IE viruses that circulate nearby on the Gulf Coast of Mexico (12), the IE subtype was not known to be equine virulent (8) and had never been shown to be capable of equine amplification or of producing equine epizootics. The 1993 and 1996 Mexican epizootics placed these assumptions into doubt.

Partial sequence analysis of the PE2 envelope glycoprotein precursor gene of four isolates made during the 1993 and 1996 Mexican outbreaks showed that they are strongly linked to enzootic subtype IE VEEV isolates made in 1968 and 1980 along the Guatemalan Pacific Coast (14,15). Complete structural gene nucleotide sequence comparison of a Guatemalan 1968 isolate with a 1993 Mexican isolate showed that they differ by only 3% at the nucleotide level (14), and more recent genomic sequencing of four strains has shown an overall nucleotide sequence divergence of <2%. Only eight amino acid changes are predicted to have accompanied the emergence of the epizootic strains (16). This genetic similarity suggests that small numbers of mutations in enzootic progenitors may have resulted in acquisition of the equine-virulent phenotype, as indicated by previous studies of subtype IC VEEV emergence in Venezuela (17).

To determine the virulence and viremia characteristics of VEEV strains isolated during these Mexican outbreaks, and to assess retrospectively the role of equines as amplification hosts during epizootic transmission, we conducted experimental infections of horses with four different Mexican strains iso-
lated from horses in 1993 and 1996. Although severe clinical neurologic disease occurred in one animal, low levels of viremia or none was detected after subcutaneous inoculation of any of the horses, which suggests that equines were not the principal amplification hosts in Mexico during the 1993 and 1996 epizootics.

**Methods**

**Virus Preparation**

The VEEV strains used for experimental infections are described in Table 1. These viruses (without further passage) were diluted in Eagle minimal essential medium (MEM) containing antibiotics and 10% antibody-negative normal horse serum before being used.

**Horse Infections**

Ten antibody-negative horses were identified, ranging in estimated age from 24 to 36 months. Only horses with no evidence of preexisting alphavirus immunity were used in this study of experimental infections. One week before the VEEV inoculations, all horses were treated with insecticide to eliminate external parasites, and their temperature and blood counts were recorded 3 days before the viral inoculations. The horses were selected at random for inclusion in two experimental groups of six and four animals, respectively.

Horses were inoculated with four different Mexican subtype IE VEE epizootic isolates (Table 1). Two strains were obtained from the 1993 Chiapas outbreak and two others from the 1996 Oaxaca outbreak. The first set of six horses was inoculated (three each) with strain CPA201, isolated from the brain of an encephalitic horse during the 1993 Chiapas outbreak, and strain OAX 131, isolated from the brain of a diseased horse from the 1996 Oaxaca outbreak. The last group of four horses (two each) was inoculated with OAX 142, isolated from the brain of a moribund horse from Oaxaca in 1996, and the I-290-93 strain, from the serum of a diseased horse from the 1993 Chiapas outbreak.

All horses were infected by subcutaneous inoculation in the shoulder region of 0.6–1.0 mL of MEM containing 10% antibody-negative normal horse serum and 10% antibody-negative normal horse serum before being used.

**Serum Viremia Assay**

Serum viremia levels were assayed by the inoculation of serial dilutions onto monolayers of Vero cell PFU of VEEV, a dose comparable to that inoculated by alphavirus-infected mosquitoes (18). Although infection by the bite of an infected mosquito may potentiate viremia levels generated by some bunyaviruses (19,20), we used needle inoculation for two reasons: 1) almost all past experimental equine infections with other epizootic VEEV have been conducted with needle infections, and we wanted to generate data that could be compared directly to the literature; and 2) lack of a high containment insectary near the large animal biocontainment facility precluded the use of infected mosquitoes.

Each pair or trio of horses inoculated with a given virus strain was housed together in an isolation room within a large animal biocontainment building. Each horse was placed in a single stall and fed daily with fresh hay and water, and the horse’s clinical signs were monitored at least twice daily. Rectal temperature was recorded twice daily until day 14 postinoculation, beginning on the day before inoculation. Blood was collected twice daily by venipuncture until day 9 and serum samples were stored at -70°C for virus titration. Additional blood was also collected into EDTA-containing tubes every morning for up to 9 days, beginning the day before inoculation, and analyzed for hematocrit, hemoglobin determination, and platelet and leukocyte counts (also counts of basophils, eosinophils, and monocytes).

The maintenance and care of animals complied with the guidelines of the Centro Nacional de Investigaciones en Microbiologia, Instituto Nacional de Investigaciones Forestales, Agricolas y Pecuarias.

**Histopathologic Studies**

All of the horses, except one (no. 4) that died on day 12, were killed 15 days after inoculation. Necropsies were carried out to extract tissues of interest. For histopathologic studies, samples were taken of white and gray matter from the anterior, median, and posterior brain. The medulla oblongata and a portion of the spinal chord were taken at random. Tissue samples <5 mm in thickness were fixed in 10% neutral buffered formalin and processed by routine methods for paraffin embedding. Sections were stained with hematoxylin and eosin.

**Virus and Viral RNA Assays**

Serum viremia levels were assayed by the inoculation of serial dilutions onto monolayers of Vero cells to assess plaque formation. Serial dilutions were also injected intracerebrally into 1- to 2-day-old mice, and titers were calculated as 50% lethal dose (LD50) values. Attempts to isolate VEEV from brain samples included the inoculation of Vero cell monolayers and inoculation of 1- to 2-day-old mice.

**Table 1. Mexican equine isolates of Venezuelan equine encephalitis virus used for experimental infections**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Date of isolation</th>
<th>Place of isolation</th>
<th>Tissue</th>
<th>Passage history a</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAX131</td>
<td>25 June 1996</td>
<td>Chahuites, Oaxaca State</td>
<td>Cerebrum</td>
<td>sm1, RK1</td>
</tr>
<tr>
<td>CPA201</td>
<td>29 June 1993</td>
<td>Rancho El Recuerdo, Mapastepec, Chiapas State; 15°, 25° N 93° 01” W</td>
<td>Brain</td>
<td>sm1, RK1</td>
</tr>
<tr>
<td>OAX142</td>
<td>5 July 1996</td>
<td>Tapanatepec, Oaxaca State</td>
<td>Cerebrum</td>
<td>sm1, RK1</td>
</tr>
<tr>
<td>I-290-93</td>
<td>12 July 1993</td>
<td>Rancho La Guadalupé, Mapastepec, Chiapas State 15°, 25° N 93° 06” W</td>
<td>Serum</td>
<td>sm1, RK1</td>
</tr>
</tbody>
</table>

aRK, rabbit kidney, sm, suckling mouse.
Reverse transcription–polymerase chain reactions (RT-PCR) were also carried out on RNA extracted from paraffin-embedded brain tissue from horses 1, 2, 3, 4, and 10 (insufficient brain tissue was left after histologic analyses on the remaining horses) to detect VEEV RNA. RNA was extracted from tissues by using the QIAGEN (Valencia, CA) RNase kit according to the manufacturer’s protocol. Approximately 20 mg of each sample was placed in a microfuge tube with 1,200 µL of 100% xylene and vortexed to dissolve the paraffin. The tissue was washed twice with an equal volume of 100% ethanol, then dried and resuspended in 350 µL of the QIAGEN RTL buffer before being ground with a pestle and 0.5 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK). The RNA was eluted in 30 µL of Rnase-free water and 3 µL of RNasin (Promega, Madison, WI) and used for RT-PCR.

RT-PCR was performed with the OneStep RT-PCR kit (QIAGEN). The primers were designed to amplify genome positions 8505–8882, yielding an expected 377-bp product: sense primer: 5′-CATAGACATCTCTGGTACGCGAG-3′, reverse primer: 5′-CAGCTTGCAAGCAGAAAGTATCC-3′. The 50-µL reaction mix was comprised of QIAGEN OneStep RT-PCR buffer, 400 µM of each dNTP, 600 nM of each primer, and 10 µL of RNA sample. The samples, along with negative control (water alone) and positive control (viral RNA) samples, were placed in a thermocycler for: 30 min at 50°C, 15 min at 95°C, followed by 40 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and a final extension of 10 min at 72°C. A seminested reaction followed with the same sense primer and a nested reverse primer 5′-GCACACCTGATGCACCTG-3′ to amplify genome positions 8505–8642, for an expected 137-bp product. For the seminested PCR, all initial PCR samples, including the controls, were diluted 1/50. The seminested PCR was performed by using Taq polymerase (Promega) for 30 cycles at 95°C for 30 sec, 56°C for 30 sec, 72°C for 2 min, and a final 72°C extension for 10 min.

PCR samples were analyzed on a 1% agarose gel, and visible DNA products were purified by using a QIAquick PCR purification kit (QIAGEN) and sequenced by using the sense primer and the ABI PRISM Big Dye Terminator v3.0 kit (Applied Biosystems, Foster City, CA). Results were compared to the genomic viral sequences for the Mexican viruses used (GenBank Accession nos. AF448536, AF448537, and AF448538).

Serologic Tests

Before the virus inoculation, serum samples from all horses were tested for preexisting antibodies to VEE, eastern (EEE), and western equine encephalitis (WEE) viruses by using an enzyme-linked immunosorbent assay (ELISA) with cell lysates prepared from BHK-21 cells infected with VEEV strain Trinidad donkey (21,22), EEE virus strain Fleming (24), and WEE virus strain Fleming (24). After the horses were infected, seroconversion was detected by using Vero cell plaque reduction (>80%) neutralization tests (PRNT) with the CPA201 strain.

Results

Clinical Signs and Symptoms

All animals became infected by VEEV as indicated by seroconversion detected by PRNT (titers ≥1:40) on day 12 or 15 after inoculation. Symptoms of VEEV infection were absent in the infected horses, and only clinical signs were observed. In 9 of the 10 horses, the only clinical sign consistent with VEE was intermittent fever. Horses nos. 1–5 and 10 showed a moderate temperature elevation of approximately 1°C–2°C, whereas horses 6–8 and 9 showed only a small elevation of approximately 0.5°C–1.0°C. Horse 6 did not display any febrile response, only normal variation in its temperature (Table 2). All animals had normal temperatures when they were killed on day 12 or 15. When it occurred, fever peaked 2–7 days after inoculation (Figure 1). Most horses displayed a slight anorexia, but none completely ceased eating during the study, except for horse no. 4 (described below). Diminished

### Table 2. Outcome for horses experimentally infected with Venezuelan equine encephalitis virus strains

<table>
<thead>
<tr>
<th>Horse no.</th>
<th>Gender</th>
<th>Virus strain</th>
<th>Maximum temperature (°C)</th>
<th>Maximum viremia levela</th>
<th>Clinical outcome</th>
<th>Histopathologic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>OAX131</td>
<td>40.3</td>
<td>2.8</td>
<td>No disease</td>
<td>Perivascular cuffing, minimal encephalitis</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>OAX131</td>
<td>40.2</td>
<td>1.4</td>
<td>No disease</td>
<td>Perivascular cuffing, minimal encephalitis</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>OAX131</td>
<td>39.1</td>
<td>1.4</td>
<td>No disease</td>
<td>Perivascular cuffing, minimal encephalitis</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>CPA201</td>
<td>40.8</td>
<td>2.4</td>
<td>Fatal encephalitis</td>
<td>Perivascular cuffing, lymphocytic menigitis, focal encephalitis</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>CPA201</td>
<td>38.4</td>
<td>1.4</td>
<td>No disease</td>
<td>Normal</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>CPA201</td>
<td>37.7</td>
<td>&lt;0.6</td>
<td>No disease</td>
<td>Normal</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>OAX142</td>
<td>38.2</td>
<td>&lt;0.6</td>
<td>No disease</td>
<td>Normal</td>
</tr>
<tr>
<td>8</td>
<td>Female</td>
<td>OAX142</td>
<td>38.9</td>
<td>&lt;0.6</td>
<td>No disease</td>
<td>Normal</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>1-290–93</td>
<td>38.6</td>
<td>&lt;0.6</td>
<td>No disease</td>
<td>Normal</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>1-290–93</td>
<td>39.8</td>
<td>&lt;0.6</td>
<td>No disease</td>
<td>Normal</td>
</tr>
</tbody>
</table>

aTiters expressed as log_{10} suckling mouse intracerebral lethal dose_{50}/mL serum.
eating tended to coincide with fever, and appetence coincided with a return to normal temperature.

Platelet counts increased slightly after day 5 in horses infected with strain CPA201 (Figure 2). A modest leukopenia occurred from day 2 to day 6 in two of three animals infected with strain CPA201. Horse no. 4, infected with strain CPA201, developed severe neurologic disease and showed the greatest reduction in leukocyte counts (Figure 3). A slight leukocytosis was seen in animals infected with OAX131 from day 3 to day 5. Otherwise, most animals showed little evidence of changes in leukocyte counts. Hematocrit values did not drop (data not shown) as in most previous studies of equines infected with epizootic VEEV (6,9). Strains I-290–93 and OAX142 produced no apparent reduction in platelet counts (data not shown).

Horse no. 4 was the only animal that exhibited clinical signs of encephalitis. On day 6 postinoculation, this male appeared weak, and on day 8 it exhibited nervousness, anorexia, pendular head movements, marked incoordination of the extremities, penile relaxation, teeth grinding, muscular tremors, restlessness, dyspnea, head shaking, excessive sweating, movement of the ears in all directions, circular walking, and blindness. On day 9 this animal was more tranquil, with less sweating. It assumed an abnormal posture with its head resting on the wall, and blindness was evident because the horse began walking into various objects. On day 10, horse no. 4 showed incoordination, nervous ticks of the head, and complete blindness. By day 11 a weight loss was evident; on day 12 the horse was prostrate and was therefore killed.

Viremia Levels

Of 10 horses infected with VEEV strains from the two Mexican epizootics, none showed detectable viremia levels when serum specimens were assayed for plaques on Vero cells. When inoculation of newborn mice with serial dilutions was used, two of three horses infected with strain CPA201 and three horses infected with strain OAX131 demonstrated viremia with low titers on days 1–3 and 2–4, respectively (Figure 4). The other two VEEV strains (I-290–93 and OAX142) did not produce detectable viremia levels.

Virus Isolation and Viral RNA Detection

Virus could not be isolated from brain tissues of any of the horses by inoculation of either Vero cells or baby mice. This was not unexpected because the animals were killed 12–15 days after infection, when the virus was presumably cleared by the immune response (neutralizing antibodies detected in all animals). Of the brain samples from horses 1–4 and 10 which were available for viral RNA detection by RT-PCR, all were positive (Figure 5). Sequences of the PCR amplicons were all identical to the parent strains, except for horse no. 4 infected with strain CPA201, which had a thymine to cytosine synonymous transition at nucleotide position 8562. Strain I-290–93 used to infect horse 10 had not been sequenced previously, but the sequence of the amplicon derived from horse 10 was identical to that of strain OAX131.

Necropsy and Histopathologic Findings

Gross pathologic lesions attributable to VEEV infection were not observed upon necropsy in any of the horses. Sec-
tions of brain from all 10 horses were examined. The fatally infected horse, no. 4, showed multifocal perivascular cuffing, lymphocytic meningitis, and focal encephalitis, characterized by focal neuronal necrosis and neuronophagia with associated microglial nodules (Figure 6B). Lesions were seen in gray and white matter of the cerebrum and cerebellum, but were not present in available sections of hippocampus or spinal cord. Samples of skeletal muscle and kidney from horse no. 10 showed no pathologic changes. Lymphoid tissue was not examined for any of the animals. All three horses infected with isolate OAX131 also showed evidence of meningitis with perivascular cuffing, and minimal encephalitis (Figure 6A). Animal no. 2 displayed more intense lesions, with numerous admixed eosinophils within leptomeningeal and perivascular infiltrates (Figures 6C and 6D).

Discussion

Equine Amplification and VEE Emergence

Previously described experimental infections of equines with epizootic subtype IAB and IC VEEV generally resulted in high rates of overt disease with high-titered viremia levels, often reaching $10^{5-8}$ suckling mouse intracerebral lethal dose 50% units (SMICLD$_{50}$/mL serum) (4,6,7,9,25–28). This high-titered viremia, combined with the attractiveness of equines to many potential mosquito vectors and poor anti-mosquito defensive behavior, results in their high efficiency as amplification hosts for epizootic virus transmission. Equine case-fatality rates in these previous studies were generally approximately 50%, similar to those measured during natural epizootics. In contrast, enzootic VEEV belonging to subtypes ID (6,9), IE (8,9), II (Everglades virus) (25), III (Mucambo virus), and IV (Pixuna virus) (29) generally produces little or no clinical illness and viremia less than $10^5$ SMICLD$_{50}$/mL serum after experimental equine infection. These enzootic viruses, including a subtype IE virus strain isolated near Veracruz near the Gulf Coast of Mexico, also have not generally been associated with equine disease in nature (3,8).

During 1969–1971, a VEE epizootic involving a subtype IAB strain spread northward from Guatemala along the Pacific Coast of Chiapas and Oaxaca States and then crossed into Veracruz State on the Gulf Coast before moving north to Texas (5). The reasons why the 1993 and 1996 Mexican outbreaks did not spread northward along the same route are unknown, but our studies suggest that the recent Mexican outbreaks were fundamentally different than those caused by subtype IAB and IC VEEV. The maximum viremia levels we measured in experimentally infected horses were approximately 1,000-fold lower than titers measured in previous studies using subtype IAB and IC VEEV strains from more extensive outbreaks (4,6,7,9,25–28,30). The titers we measured with the Mexican equine strains were also much lower than the infection thresholds for mosquitoes, as predicted by most experimental infections with VEEV (31–34). This finding indicates that equines probably did not serve as amplification hosts to support epizootic transmission cycles in Mexico during 1993 and 1996. This lack of equine amplification, which is believed to be a critical factor in VEE spread, probably limited the duration and scope of the 1993 and 1996 Mexican epizootics. The low-titered viremia level, even in the horse in which fatal encephalitis developed, also indicates that the Mexican subtype IE epizootic viruses may be more neurotropic than other VEEV strains that apparently reach the central nervous system only after exceeding higher viremia titer thresholds (4,6,9).

Other possible explanations for sudden appearance of equine disease during the 1993 and 1996 outbreaks include increased transmission to horses by efficient amplification in other vertebrate hosts, or enhanced transmission by adaptation of local VEEV strains to equiphilic mosquito vectors. Although robust vector surveillance was not conducted during the 1993 and 1996 Mexican epizootics, rainfall patterns and

Figure 3. Mean leukocyte counts in horses infected with virus strains CPA201 and OAX131. Bars indicate standard deviations. Data for horse no. 4, in whom severe neurologic disease developed after infection with strain CPA201, are shown individually. Bars indicate standard deviations; shaded box indicates approximate normal values. Normal temperature ranges from The Merck Veterinary Manual, SE Ariello and A. Mays (eds.), 1998, Merck & Company, Inc., Whitehouse Station, New Jersey.

Figure 4. Mean viremia titers in horses infected with four different Mexican strains of subtype IE Venezuelan equine encephalitis virus. A log$_{10}$ titer of 0.6 suckling mouse intracerebral lethal dose$_{50}$ represents the maximum sensitivity of the assay. Bars indicate standard deviations.
anecdotal reports do not indicate unusually large mosquito populations. Vector incrimination and susceptibility testing are needed to assess the hypothesis that the Mexican epizootic strains are more infectious for mosquitoes and more readily transmitted than putative enzootic IE progenitors identified in phylogenetic studies (15,16).

Equine Virulence of the Mexican VEEV Strains

In some respects, our results agreed with those of previous experimental studies of VEEV in equines. Infection with the Mexican VEEV strains resulted in mild leukopenia as has been reported (9). However, the mild hematologic abnormalities were more like those caused by enzootic VEEV strains than epizootic, subtype IAB and IC variants (9). In contrast to many VEE outbreaks attributed to subtype IAB and IC strains (3), the four Mexican epizootic virus strains we tested in horses produced an overall mortality rate of 10% but a case-fatality rate of 100%. These results also contrast with the reported case-fatality rates during the 1993 and 1996 Mexican outbreaks of 50% and 37%, respectively, and with the 1993 attack rate of 30% (14). The case-fatality rate differences probably reflect sampling error because our experimental infections produced disease in only one horse. Possible explanations for the discrepancy in apparent:inapparent ratios in our study versus data collected during the outbreaks include an underreporting of true inapparent infections during the 1993 epizootic, or phenotypic differences between the virus populations we inoculated into horses versus those circulating naturally during the epizootics. The latter possibility is discussed below.

Detection of VEEV RNA in the brain of five horses killed 12–14 days after infection, despite the absence of detectable infectious virus, was somewhat surprising. This result indicates that VEEV replicated in the brain of all horses, consistent with some pathologic lesions in the asymptomatic animals infected with strain OAX131 (Table 1). Persistence of viral RNA in the brain after the disappearance of infectious virus has also been reported for other alphaviruses (35).

Previous sequencing, rodent infection, and in vitro studies with these Mexican VEEV strains suggested that mixed populations of viruses may circulate in nature, represented by differences in amino acid composition and charge on the surface of the E2 envelope spike glycoprotein (16). Genomic sequencing studies of the four Mexican VEEV strains we studied revealed one amino acid difference: strain OAX142 has a Glu residue at position 117 of the E2 envelope glycoprotein, whereas the other three strains have Lys (16). The possibility that the Lys residue does not represent the wild-type sequence was considered previously because alphaviruses including VEEV are known to accumulate artificial, positive charge amino acid changes in the E2 protein as the result of selection for binding to heparan sulfate on the surface of cells in culture (36). Because these changes reduce the virulence of alphaviruses in laboratory rodents, they can place into question the wild-type phenotype of VEEV strains.

However, our results further support the previous conclusion that the E2-117 Lys residue was present in natural VEEV isolates during the Mexican outbreaks as the consensus amino acid, but possibly in a mixed quasispecies population (16). The only strain that produced neurologic disease in our study was CPA201, which has a consensus Lys at E2-117. Strain OAX142, which has Glu-117, produced no detectable disease, viremia, or hematologic alterations. These results are not consistent with the hypothesis that the Lys residue results from heparan sulfate adaptation during RK cell passage, which would be expected to result in artificial attenuation (36).

Figure 5. Results on 1.5% agarose gel of reverse tranferase–polymerase chain reaction (RT-PCR) products stained with ethidium bromide and imaged under UV light. M: 100-bp marker; H1–4, H10: horses 1–4 and 10; (+): positive control viral RNA, 1.8 x 10^2 PFU amplified; (N-): negative control for nested PCR (-); negative control from single round RT-PCR. All horse samples and the positive control show a band at the expected size (134 bp), and negative controls show only the primers (below 100 bp).

Figure 6. Representative histopathologic changes of brains of infected equines. A. Cerebrum, horse no. 3 (strain OAX131), showing perivascular cuff of lymphocytes (original magnification x200, hematoxylin and eosin [H&E] stain). B. Cerebrum, horse no. 4 (strain CPA201), showing perivascular cuff of lymphocytes (original magnification x200, H&E stain). C. Cerebral gray matter, horse no. 2 (strain OAX131), showing infiltration of mononuclear cells into brain parenchyma (original magnification x200, H&E stain). D. Cerebral gray matter, horse no. 2, showing numerous eosinophils within perivascular infiltrate (arrow) (original magnification x200, H&E stain).
Because the Lys-117 mutation produces the small plaque phenotype characteristic of most epizootic, equine-virulent VEEV strains, it remains a potential virulence determinant worthy of further characterization by using cDNA clones and reverse genetics.

Acknowledgments

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Elimination of Epidemic Methicillin-Resistant Staphylococcus aureus from a University Hospital and District Institutions, Finland

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From August 1991 to October 1992, two successive outbreaks of methicillin-resistant Staphylococcus aureus (MRSA) occurred at a hospital in Finland. During and after these outbreaks, MRSA was diagnosed in 202 persons in our medical district; >100 cases involved epidemic MRSA. When control policies failed to stop the epidemic, more aggressive measures were taken, including continuous staff education, contact isolation for MRSA-positive patients, systematic screening for persons exposed to MRSA, cohort nursing of MRSA-positive and MRSA-exposed patients in epidemic situations, and perception of the 30 medical institutions in that district as one epidemiologic entity brought under surveillance and control of the infection control team of Turku University Hospital. Two major epidemic strains, as well as eight additional strains, were eliminated; we were also able to prevent nosocomial spread of other MRSA strains. Our data show that controlling MRSA is possible if strict measures are taken before the organism becomes endemic. Similar control policies may be successful for dealing with new strains of multiresistant bacteria, such as vancomycin-resistant strains of S. aureus.

Methods

Background

The Turku University Hospital is a teaching facility that serves as a tertiary referral center for southwestern Finland. Approximately 500,000 inhabitants live in the Southwest Finland Medical District; the density of the population varies from 20–100 inhabitants per square kilometer. The institutions include 1 university hospital, 1 central hospital, 7 regional hospitals, and 22 health-care centers.

From August 1991 to October 1992, two successive outbreaks caused by different MRSA strains occurred in the departments of surgery and medicine at University Hospital. During and after these outbreaks, these two MRSA strains were isolated from patients and staff members in five additional institutions in our district.

Screening Policy

Our policy of screening contact patients of the MRSA-positive patients and the hospital staff for MRSA varied during the different phases of the outbreaks. Unless otherwise indicated, the term contact patient refers to a patient hospitalized on the same ward at the same time with an MRSA-positive patient.

Surgical Unit Outbreak

During the outbreak in the surgical unit, in most cases MRSA was isolated from a clinical specimen. Initially, a policy decision was made not to screen either the contact patients...
of MRSA-positive patients or the staff on outbreak wards for MRSA. When the number of MRSA cases increased, we performed one cross-sectional study to screen all patients cared for in the department of surgery during that particular day for nasal and wound colonization by MRSA.

Medical Unit Outbreak

After the first two cases were diagnosed in the medical unit, we began screening other patients treated in the medical intensive-care unit (ICU). During weeks 6–8, all contact patients connected with MRSA-positive patients (treated in the ICU after admission of the index case) were screened once by using nasal swabs if they were still hospitalized. If a new case was identified on a ward, the roommates of the patient were screened. If transmission of MRSA was observed on a ward, all patients were screened. Initially, screening involved only nasal swabbing, but from the first week of June 1992 on, cultures were taken also from the perineum, groin, and axillae, as well as from all open wounds, skin lesions, and, later, the throat. After 10 identified cases of MRSA, we began to label case records of the colonized patients and contact patients with tags showing MRSA information. The contact patients were screened on the next visit; previous roommates of MRSA-positive patients were isolated while waiting for culture results. After providing two sets of negative MRSA cultures, contact patients were no longer screened on subsequent admissions. However, patients once found to be MRSA positive were screened on subsequent admissions and placed in single rooms to be cared for in contact isolation. All patients previously treated at hospitals abroad or with a known MRSA problem were screened at the time of admission and nursed in contact isolation until results from colonization cultures were negative. We screened the staffs of the medical ICU and the hematology and infectious diseases units by nasal swab at varying intervals during the medical outbreak. Screening cultures were done, as described (7,8).

Identification of MRSA

The isolates grown on culture plates were identified as MRSA following the National Committee for Clinical Laboratory Standards guidelines (9). Genetic resistance to methicillin was verified by the presence of the mecA gene (10). All MRSA isolates were submitted to the Staphylococcal Reference Laboratory at the National Public Health Institute, where they were typed with the international phage set and ribotyping and pulsed-field gel electrophoresis (PFGE), as described (6). Two isolates were defined as different strains if they had different phage types and/or PFGE and ribotypes. We considered phage types different if two or more strong phage reaction differences occurred. Ribotypes were considered different if any band difference occurred. Before 1995, PFGE types were considered different if any band difference occurred. After 1995, PFGE types were considered different if four or more band differences occurred.

Elimination Treatment

Elimination treatment with topical or combined topical and systemic antimicrobial therapy was given to selected patients (e.g., long-term care patients of health-center wards and nursing homes) and those with severe underlying diseases who were frequently admitted to any hospital in the district (7,8,11). Long-term carriers among the staff were also given elimination treatment. Detailed data on drug regimens will be reported separately.

Results

MRSA Strains

From 1991 through 2000, a total of 202 persons in the Southwest Finland Medical District were infected or colonized by MRSA (Table). On the basis of phage typing and molecular typing, we identified 15 different MRSA strains isolated from two or more persons. These strains included 10 isolated from hospitalized patients (outbreak strains) and 5 causing intrafamilial clusters in the community (familial strains). The strain causing the surgical outbreak (referred to as the surgical strain) belonged to phage type 75,77,84,85III and had a characteristic ribotype and PFGE pattern. The strain causing the medical outbreak (referred to as the medical strain) was nontypable with phages (NT), but the strain relatedness between different isolates could be ascertained by ribotyping and PFGE. A third MRSA strain typed 54,84,85III/96V/95 caused the Mynamaki Health Center outbreak described previously (7). A detailed typing analysis, including a picture of the PFGE profiles of these major epidemic strains, has been published (6) and describes the corresponding strain identification code as E6 for the surgical strain, E7 for the medical strain, and O9 for the Mynamaki strain. The cases involved in these three outbreaks, as well as the clusters caused by 12 additional MRSA strains, are summarized in the Table. The remaining 63 strains were isolated from one person each.

Three (30%) of 10 outbreak strains and 22 (35%) of 63 unique strains were designated as of foreign origin. None of the five familial strains were of foreign origin.

MRSA Outbreaks at the University Hospital

Surgical Unit Outbreak

The hospitalization periods of the patients during the surgical outbreak and the times when MRSA was first isolated in each case are shown in Figure 1. In August 1991, the surgical strain was isolated from a bone sample of patient 1 who was cared for on an orthopedic ward for posttraumatic osteomyelitis. The patient was referred to the infectious diseases unit to be cared for in contact isolation, but she was readmitted to the orthopedic ward three times during the following 4 months for treatment of osteomyelitis. Each time, the isolation precautions followed by hospital personnel did not comply with the standard adopted later.
MRSA was next isolated from head wound of a colonized male patient on the same ward. He was placed in a single room to be cared for in contact isolation, but when the wound healed, the patient was transferred to a three-bed room. Subsequently, three of his roommates (patients 3, 4, and 5) acquired MRSA. By the 3rd week of December 1991, the combined number of patients colonized by epidemic MRSA had increased to eight cases on two wards and in the surgical ICU. A shortage of single rooms and the threat of an expanding outbreak led to implementation of the following control measures: 1) intensive education of the staff on hospital hygiene, 2) nursing of all MRSA-positive patients in single rooms in contact isolation, preferably in the infectious diseases unit, 3) strict adherence to contact isolation precautions and minimal duration of hospitalization whenever an MRSA-positive patient was treated at the department of surgery (e.g., operative treatment required), and 4) cross-sectional screening of all patients nursed on surgical wards and in the surgical ICU on December 19, 1991, for nasal and wound colonization. The screening uncovered three new cases of MRSA on epidemic wards. By year end, all patients identified as MRSA positive had been either discharged or transferred to the infectious diseases unit. Thereafter, no new transmission of MRSA was observed after these patients were transferred to the infectious diseases unit. The total number of University Hospital patients infected or colonized by the surgical strain was 24.

In November 1995, the surgical strain was unexpectedly isolated from an endotracheal aspirate of a patient in the surgical ICU. This patient had also been cared for on the orthopedic ward during the 1991 outbreak. Subsequent screening of contact patients in the ICU showed MRSA colonization in three other patients who had ventilatory support at the same time. No new transmission of MRSA was observed after these patients were transferred to the infectious diseases unit. The total number of University Hospital patients infected or colonized by the surgical strain was 24.

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### Table. Yearly number of new cases caused by different methicillin-resistant *Staphylococcus aureus* (MRSA) strains, Southwest Finland Medical District, 1991–2000

<table>
<thead>
<tr>
<th>Year</th>
<th>Surgical outbreaka</th>
<th>Medical outbreakb</th>
<th>Mynamaki outbreakc</th>
<th>MRSA outbreak IVd</th>
<th>MRSA outbreak IVe</th>
<th>Other outbreaks</th>
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*aStrain was recovered from 24 patients at the university hospital, 10 patients at a regional hospital, and 3 staff members in these hospitals.

*bStrain was recovered from 30 patients and 18 staff members at the university hospital and from 9 patients in other district institutions.

*cStrain was recovered from 12 patients and 1 staff member in a health-center ward and associated nursing home (7).

*dStrain caused a cluster of four cases in an intensive-care unit of a central hospital.

*eStrain caused a cluster of four infected patients and one infected staff member in a health-center ward at the beginning of 2000, but was subsequently eliminated.

*fIntrafamilial clusters of two to four MRSA cases in the community.

*gMRSA strain was transmitted from one patient to another at the university hospital.

*hMRSA strain was transmitted from one patient to another at a regional hospital.

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![Figure 1. Spread of surgical outbreak strain. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated August 1991–October 1992 in 17 patients cared for on two surgical wards and the surgical intensive-care unit. Hospitalization periods of these patients are shown as horizontal lines. Symbol • indicates the time point when the first culture positive for MRSA was taken.](image-url)
**Medical Unit Outbreak**

The index patient was treated for cerebral hemorrhage in an ICU in Rome, Italy. After his referral to the department of neurology of University Hospital in December 1991, the medical strain was isolated from his endotracheal aspirate. For the next 3 months, the patient was cared for in contact isolation in a single room on a neurologic ward; we found no evidence of MRSA transmission to other patients on that ward.

**Medical ICU**

In March 1992, the index patient became ill with septic shock caused by MRSA and was admitted to the medical ICU for respiratory support. For the first 24 hours, he was not isolated because of a misunderstanding but treated in the same room with three other patients who had ventilatory support. His contact patients were neither screened nor isolated.

Two weeks later (Figure 2), the medical strain was cultured from an endotracheal aspirate of a patient who had died in the ICU a few days earlier. The devised screening program was delayed, and subsequent screening on weeks 6–8 found six new patient carriers and two staff carriers of MRSA. The medical ICU was closed to new admissions, and an auxiliary ICU was established for those patients who had not been exposed to MRSA.

In the auxiliary ICU, a new staff carrier of MRSA was identified on week 12 and a new patient carrier and a staff carrier on week 13. The MRSA-positive patient was immediately referred to the infectious diseases unit. The six other patients who had shared the ICU room with him were simultaneously transferred to that same unit. Screening cultures later revealed MRSA colonization in five of them, indicating that early cohorting of these contact patients may have been critical in preventing further spread.

**Hematology Unit**

In May 1992, we identified MRSA colonization in four patients cared for on the hematology ward. Two of them became colonized while being treated in the medical ICU in April and transmitted MRSA to their two roommates on the ward before carriage became manifest. Using nasal swabs, we screened a number of patients treated at that time on the same ward. Many other contact patients already discharged were not screened when they were readmitted, rendering further spread of MRSA possible. At the beginning of July 1992, MRSA was isolated from an endotracheal aspirate of a bone marrow transplant patient cared for on the hematology ward (Figure 2). Subsequent screening showed colonization in 11 additional hematologic patients and 12 staff members.

We prevented nosocomial transmission by immediately closing the hematology ward. For the next 3 months, hematologic patients were cared for in three separate cohorts: 1) those not exposed to MRSA were admitted to the hematology unit when it was reopened, 2) those potentially exposed to MRSA during the previous 4 months were cared for in a separate cohort in the infectious diseases unit until three sets of coloni-
hospital ICU outbreak was controlled after four cases (outbreak strain IV). In 2000, MRSA outbreak strain V was eliminated from a long-term care facility after five cases occurred. Of the five additional epidemic MRSA strains, one was eliminated after two cases in a regional hospital, and the other four strains were eliminated after causing two cases each at University Hospital (Table).

Long-Term Follow-Up of Patients

Of the 37 patients who acquired the surgical strain, 6 died within 1 month after MRSA was identified, 20 died during the following years, and 3 were not part of follow-up. Eight patients remain residents in our district, two of them still carrying the surgical strain. The majority of the 39 patients who acquired the medical strain had severe underlying diseases. Of all 39 patients, 21 died within 3 months of colonization, 12 died during the following years, and 1 was not part of follow-up. Five patients who still live in Turku, three of whom were treated to eliminate carriage, no longer carry MRSA. Thus, the medical strain has been eliminated from our district.

Discussion

During past few years, news on MRSA has usually been discouraging. Clinicians and infection control practitioners appear to have lost confidence in their capability to control the nosocomial spread of this pathogen. The number of papers focusing on the overwhelming spread of MRSA is increasing (1,12–17), whereas those addressing successful efforts of control or stating that nosocomial spread of MRSA can and should be controlled are few (18–22). A number of researchers debating the control of MRSA have questioned whether controlling this microorganism is reasonable, feasible, or justified (23–25) and whether the tracing of colonized people is justified (26). We describe the elimination of MRSA from a university hospital and a medical-district–wide control policy for MRSA after the outbreak. Our results show that controlling or even eliminating MRSA is possible, if strict measures are systematically taken before the organism becomes endemic. Our experience should encourage other countries with a low incidence of MRSA to continue efforts to prevent the spread of this microorganism in hospitals and long-term care facilities.

According to 46 published reports on outbreaks, 10% of the hospitals with ≥40 cases have achieved definite or probable elimination of MRSA (27). Although >100 patients and staff members in our district initially became colonized by epidemic MRSA, this microbe is being controlled almost 10 years after these first outbreaks. We eliminated the medical strain from the whole district, and only a few outpatients presently carry MRSA in the community. Moreover, we were able to prevent nosocomial spread of the almost 100 additional MRSA strains encountered in our area. Even the 22 MRSA strains introduced by patients transferred from hospitals abroad have remained solitary cases, despite their epidemic potential. In fact, after the small university hospital ICU outbreak in 1995, nosocomial transmission of MRSA has been detected in our district hospitals only three times; on each occasion, MRSA colonization of the index case was not known or suspected on admission.

Containment of the Turku outbreaks in 1991–1992 was greatly impeded by the fact that we had no national guidelines on how to control MRSA in Finland at that time and very little previous experience with these microorganisms. Detailed guidelines published by authorities from abroad advised an active control policy (28), but stringent measures were perceived by our colleagues as too disruptive for the patient care in our institution. One major argument against adopting an aggressive line of control was the lack of severe MRSA infections because many of our patients were colonized without clinical infection. During the early phase of the medical outbreak, the infection control team adjusted to a lenient control policy because of our previous experience with the surgical strain, which was easily contained. The behavior of the medical strain, however, was quite different from the surgical strain, and the inadequacy of the control measures at the beginning of the medical outbreak is now evident. We may have been able to restrict this outbreak to only a few cases if all ICU patients had been screened for MRSA and MRSA-positive patients had been isolated as soon as we discerned that appropriate control measures had not been taken when caring for the index patient or if the medical ICU had been closed to new admissions after the second or third MRSA case. Similarly, screening of only some of the MRSA contact patients in the hematology unit in May 1992 was clearly insufficient. Had our efforts initially been more aggressive and the outbreaks quickly controlled, we may have saved many persons from becoming colonized with MRSA and considerably reduced the costs of infection control measures required.

The most important lesson from these first epidemics was that an ambivalent and permissive control policy for MRSA easily fails. We have subsequently made every effort to avoid making the same mistake. Whenever MRSA has been introduced into our hospitals, rapid steps have been taken to adopt appropriate control measures. The mainstays of our present policy involve continuous staff education, caring for MRSA-positive patients in single rooms in contact isolation, systematic screening of patients exposed to MRSA, including all patients transferred from hospitals abroad or with a known MRSA problem, and cohort nursing of MRSA-positive and exposed patients, at least in epidemic situations.

National guidelines have proved most beneficial in preventing the spread of MRSA in a few low-incidence countries (2,6). Medical-district–wide guidelines may be equally important when an individual hospital is struggling with MRSA and needs practical or moral support. The Turku MRSA policy involves perceiving our medical district with its approximately 30 institutions as one epidemiologic entity; the infection control team of University Hospital is responsible for the control of MRSA (and also of other multiresistant bacteria) in the whole entity. This overall responsibility ensures that the same control policy for MRSA is followed in all district institutions.
If MRSA is encountered in any local hospital or health-center ward, consultation is given; if nosocomial transmission of the microbe is observed, the infection control team visits the institute. We continuously strive to prevent the development of MRSA reservoirs in our extended-care facilities. In so doing, treatment to eliminate MRSA carriage in long-term patients has been favored, while in contrast, efforts to eliminate MRSA in outpatients have not had the same focus.

Many of our experiences were taken into account when the National Guidelines for the Control of MRSA in Finland were prepared in 1995 (6). To a great extent, the MRSA control policy finally adopted is in line with that currently followed in the Netherlands (2) and initially recommended by the British authorities in 1990 (28). Because of the increasing prevalence of MRSA, those guidelines were replaced in the U.K. by more lenient instructions in 1998 because the situation in many parts of the country was such that a more flexible approach was considered appropriate (29,30). With the dramatic increase of MRSA, other countries (including the United States) where these microbes are already endemic in hospitals have adopted more flexible control policies (31,32). However, now that vancomycin-resistant S. aureus (VRSA) exists (33), controlling MRSA is even more imperative. A lenient or ambivalent policy is especially inappropriate in those countries where MRSA remains uncommon, since they may still have a fair chance of eliminating this pathogen. In southwestern Finland, the factors possibly contributing to our success include active education and excellent compliance of health-care personnel, a uniform health-care system, and low population density.

Anticipating the emergence of new and even more serious strains of multiresistant staphylococci poses a demanding challenge to clinicians and infection-control practitioners worldwide to seek novel methods, which could effectively prevent the spread of these microorganisms. Despite an inability to control MRSA in many countries, we believe that confining these newly emerging multiresistant strains may be possible, provided that vigorous efforts are taken early while the microbe still remains rare. If rapidly begun, aggressive measures may not be needed for long and thereby be cost-effective. To meet future challenges successfully, a stringent and consistent international control policy should be issued and universally obeyed.

We have shown that controlling or even eliminating MRSA is possible, if strict measures are taken before the organism becomes endemic. A similar policy may be successful when combating new and even more serious strains of multiresistant bacteria (e.g., VRSA). The recent emergence of VRSA emphasizes the need for unremitting and vigorous control of MRSA. National guidelines for MRSA control policy have proven beneficial in a few low-incidence countries. Our results suggest that firm international guidelines will aid countries in preventing the global spread of any newly emerging multiresistant bacterial pathogen. An ultimate prerequisite for success is the commitment of the health-care personnel worldwide to struggle for that important goal.

Dr. Kotilainen is professor of infectious diseases at the Medical School of the University of Turku, Finland. During the outbreaks, she was the infection control physician of Turku University Hospital with responsibility for the overall district. Her recent research interests focus on the epidemiology and control of multidrug-resistant Staphylococcus aureus and S. epidermidis; the application of molecular methods in establishing etiologic diagnoses of bacterial diseases in a clinical setting; and the increasing antimicrobial resistance problems of Salmonella enterica and Campylobacter jejuni.

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25. Pittet D, Waldvogel FA. To control or not to control colonization with MRSA...that’s the question. QJM 1997;90:243–6.
Several recent studies have used proportions of tuberculosis cases sharing identical DNA fingerprint patterns (i.e., isolate clustering) to estimate the extent of disease attributable to recent transmission. Using a model of introduction and transmission of strains with different DNA fingerprint patterns, we show that the properties and interpretation of clustering statistics may differ substantially between settings. For some unindustrialized countries, where the annual risk for infection has changed little over time, 70% to 80% of all age groups may be clustered during a 3-year period, which underestimates the proportion of disease attributable to recent transmission. In contrast, for a typical industrialized setting (the Netherlands), clustering declines with increasing age (from 75% to 15% among young and old patients, respectively) and underestimates the extent of recent transmission only for young patients.

We conclude that, in some settings, clustering is an unreliable indicator of the extent of recent transmission. Annual infection risk and interpretation of clustering statistics are provided in the Appendix.

Studies are increasingly using levels of clustering of isolates from tuberculosis cases (proportion sharing identical DNA fingerprint patterns) to estimate the extent of disease attributable to recent transmission. To date, few studies have been conducted in unindustrialized countries, where the impact of tuberculosis and the proportion of disease attributable to recent transmission are greatest. Whether or not the properties and interpretation of clustering statistics in such settings are similar to those in industrialized populations is unclear.

Studies in industrialized countries have found relatively low overall levels of clustering (e.g., 30% to 40% during a 3-year period [1–3]) but much higher levels among younger versus older patients. This age differential probably reflects past trends in the annual risk for infection, which was high in the early 20th century (e.g., >2% per year before 1940 in the Netherlands [4]) and is currently very low. Thus, a large proportion of disease in older patients is attributable to reactivation of infections acquired many years ago, and, given the short half-life of DNA fingerprint patterns (5), only a small proportion of old patients share identical isolates with other patients. In some unindustrialized countries, on the other hand, where the annual risk for infection may not have changed much over time, the age differential in clustering might be small, given that a large proportion of disease even among older persons may be attributable to recent (re)infection. Understanding the effect of the magnitude of the annual risk for tuberculosis infection on clustering frequency helps determine how molecular epidemiologic data can be best applied to estimate the extent of ongoing transmission of *Mycobacterium tuberculosis*, and hence to identify optimal control strategies.

We explored how the magnitude and trend in the annual risk for infection influence the age-specific proportion of clustered cases and its relationship to the extent of disease attributable to recent transmission. We use a model of the transmission dynamics of *M. tuberculosis* previously calibrated to data from the Netherlands (6), where isolates from all tuberculosis cases with onset since 1993 have been routinely DNA fingerprinted (1). We describe the general epidemiologic assumptions in the model and how it distinguishes between cases according to the DNA fingerprint pattern of the strain causing the disease episode, which is needed to calculate clustering statistics.

**Methods**

Our analysis is based on a model developed recently to interpret data on clustering of DNA fingerprint patterns in the Netherlands (6). Equations describing the model’s formulation are provided in the Appendix.

**Epidemiologic Assumptions in the Model**

The model’s structure, parameters, and assumptions have been published (6). Persons are assumed to be born uninfected. Infected persons are divided into those in whom primary disease has not yet developed (defined by convention as disease within 5 years of initial infection [7]), and those in the “latent” class, who are at risk for endogenous reactivation or for reinfection, which can be followed by exogenous disease. Exogenous disease is here defined as the first disease episode within 5 years of the most recent reinfection; endogenous disease includes disease occurring >5 years after the most recent (re)infection event, and second or subsequent disease episodes occurring <5 years after the most recent (re)infection event. (These definitions differ slightly from those of Sutherland et al. (8) to include the assumption that once persons have recovered from disease during the first 5 years after initial infection...
or reinfection, their risk of developing disease becomes the same as that of developing disease through reactivation, until they are newly reinfected.

The infection and reinfection risks are assumed to be identical, but reinfection is less likely to lead to disease than is initial infection, due to some immunity induced by the prior infection (9). We explored the implications of four assumptions for the magnitude (and trend) in the annual risk for infection, namely, that the risk for infection 1) declined over time, as estimated for the Netherlands (from approximately 2% in 1940 to approximately 5/10,000 by 1979 [4,10]); 2) remained unchanged over time at a very low level (0.1%); 3) remained unchanged at 1%; or 4) remained unchanged at 3%. Infection risks of 1% have been found in several populations (e.g., Malawi [11]). Infection risks of 3% are uncommon today but have been reported in parts of South Africa (12). For simplicity, we assumed that persons cannot be reinfected during the period between initial infection (or reinfection) and onset of the first primary episode (or exogenous disease).

The risks of developing disease depend on age and sex (Figure 1A; [6]); they are based on previous analyses, in which we fitted predictions of disease incidence to observed notifications in the U.K. (9). The risks of developing either a first primary episode or disease following exogenous reinfection also depend on the time since infection and reinfection, respectively (Figure 1B). The probability that a disease episode is infectious (sputum smear/culture-positive) is age dependent (Figure 1C) (9). The demography of the population described in the model is assumed to be that for the Netherlands. Analyses are restricted to respiratory (pulmonary) forms of tuberculosis, since these are far more likely than extrapulmonary forms to lead to transmission. Although additional factors such as immigration and HIV can influence the extent of clustering in complicated ways (14), these factors are not considered here, where the focus is upon the effect of the magnitude and trend in the annual risk for infection on clustering.

**Derivation of Clustering Statistics**

Recent studies suggest that the half-life of DNA fingerprint patterns based on IS6110 restriction fragment length polymorphism (RFLP, which has been used for the DNA fingerprinting conducted to date in most studies) is 2–5 years (5,15). If the molecular clock speed for IS6110 RFLP patterns of strains involved in latent infection (currently unknown) were to be similar, this relatively short half-life implies that most of the fingerprint patterns of the strains causing disease today differ from those that caused disease many years ago. Similarly, this short half-life implies that the M. tuberculosis fingerprint types and cluster distributions in tuberculosis cases today depend only loosely upon those that existed 50 years ago. Based on this assumption, to derive clustering estimates for a given population for recent years, we designed the model to simulate the introduction and subsequent transmission of strains with new DNA fingerprint patterns from a sufficiently distant time in the past (taken to be 1950), so that a) all cases with onset in recent years involved a strain whose DNA fingerprint pattern had first appeared since then and b) no assumptions would be required about the distributions of strains that existed before 1950. The general steps in the calculations are outlined briefly below.

The numbers of persons of each age in each of the epidemiologic categories for 1950 were calculated by using the model, based on described equations (9). From 1950, each of these age-sex classes was stratified to distinguish between those who had, versus those who had not, been (re)infected since 1950. Those who had been (re)infected since 1950 were subdivided further according to the time of infection or reinfection. The transmission dynamics were tracked simultaneously for all persons with the equations described in the Appendix and elsewhere (6), by using time steps of 6 months and 1 year for calendar year and age, respectively.

In each interval, disease was assumed to develop in a proportion of infected persons, and a proportion of these disease episodes was attributed to a strain for which the DNA finger-
print pattern differed from that of the strain with which the persons were originally infected. This latter proportion depended on the time since infection (see below), and each of the new DNA fingerprint patterns was assigned a unique identity number. Each infectious patient with onset at a given time was assumed to contact a different number of persons (see Appendix and Figure 2). The frequency distributions of the number of persons contacted by each patient were used to derive the total number of persons who were newly (re)infected at this time. The corresponding equations were then applied to this number to determine the total number of persons in whom disease developed at a later time, \( T \), among those who had been infected at time \( t \). The DNA fingerprint patterns of the strains in these diseased persons were then determined by using the frequency distribution of the number of persons contacted by each case-patient at time \( t \). These calculations are described further in the Appendix.

**Estimating the Effect of the Annual Risk for Infection on Clustering as an Indicator of Recent Transmission**

Our model was used to calculate the age-specific proportion of disease attributable to primary and exogenous disease from 1993 to 1997 for the Netherlands and for settings in which the annual risk for infection is assumed to have remained unchanged over time at 0.1%, 1%, and 3%. Primary and exogenous disease involve disease occurring during the first 5 years after the most recent (re)infection event, although the majority of persons in whom primary or exogenous (reinfection) disease develops acquire the disease within 2–3 years (Figure 1B). The clustering by sex and age for cases with onset in different periods between 1993 and 1997 for the Netherlands, and for settings in which the annual risk for infection is assumed to have remained unchanged over time at 0.1%, 1%, and 3%, was also calculated by using the age and sex distribution of the cases with onset in that period (see equations in [6]). For simplicity, we present age-specific levels of clustering for male patients only. Model predictions for male patients generally compared better against the observed data in the Netherlands than did those for female patients (6).

The predictive values of clustering for the identification of recent transmission were calculated as follows. The positive predictive value of clustering for identifying recent transmission in different age groups in different periods was calculated as the proportion of case-patients who were in a cluster in a given period who had been infected or reinfected \(<5\) years before disease onset. The negative predictive value of clustering for identifying recent transmission in different age groups was calculated as the proportion of case-patients who were not in a cluster in a given period who had been infected or last reinfected \(>5\) years before disease onset.

**Results**

**Model Predictions of the Extent of Clustering and Disease Attributable to Recent Transmission**

As shown in Figure 3, very different age patterns in the proportion of disease attributable to recent transmission were predicted for the Netherlands and for settings in which the annual risk for infection has remained unchanged over time. In the Netherlands, the proportion of disease attributed to recent infection decreased dramatically with age, e.g., from 100% in the young to approximately 50% and 10% for 45- to 54-year-old patients and persons \(>65\) years of age, respectively. The proportion of disease attributed to recent reinfection was very low for all age groups \(<3\%\). For constant infection risk settings, the predicted proportion of disease attributable to recent
transmission (i.e., recent infection or reinfection) was very similar, falling from 100% in the young to 85%, 88%, and 90% in the oldest age groups for the 0.1%, 1%, and 3% infection risk scenarios, respectively. On the other hand, large differences between settings were predicted in the proportion of disease attributed to initial infection or to reinfection. In all instances, the proportion attributed to reinfection was zero in the youngest age groups, but this proportion increased with age to 3%, 35%, and 80% for the 0.1%, 1%, and 3% annual infection risk assumptions, respectively. The proportion attributed to recent initial infection in these settings decreased from 100% in the young to 80%, 50%, and 15%, respectively, in old patients.

As shown in Figure 4A, for each setting, the overall clustering (i.e., that seen among all age groups) was predicted to increase with study duration, e.g., from 15% for the Netherlands for a 1-year period to approximately 25% for a 5-year period. The clustering predicted for all the constant infection risk scenarios was similar in magnitude for each study period and increased from 60% to 70% for a 1-year period to 75% to 85% for a 5-year period. Since the overall clustering was not predicted to increase much for study periods of more than 3 years, clustering is defined using a 3-year period in the remainder of these analyses (represented by 1993–1995). As shown in Figure 4B, the clustering predicted for each age group was similar for each of the settings in which the annual risk for infection remained unchanged over time, and declined only slightly with age, e.g., from 83% for the youngest age group to approximately 75% for the oldest age category. In contrast, for the Netherlands, the clustering was predicted to decrease dramatically with age, from approximately 75% among young case-patients to approximately 15% in very old patients. This prediction is consistent with observed data (Figure 4B).

Reliability of Clustering as a Measure of the Extent of Recent Transmission

For settings in which the annual risk for infection remained unchanged over time at 0.1%, 1%, and 3%, the predicted clustering in each age group underestimated the proportion who had been recently infected or reinfected (Figure 5). In settings with an annual risk for infection of 0.1%, at least 90% of cases in each age group were predicted to have been recently (re)infected, whereas the proportion clustered decreased from about 85% in the youngest age group to approximately 70% for the oldest persons. For the Netherlands (described elsewhere [6]), clustering underestimated the proportion of disease attributable to recent transmission in the young (by up to 43%) and overestimated that for older patients (by up to 50%). The positive and negative predictive values of being in a cluster, as an indicator of recent transmission, depended both on age and the study setting (Figure 6). For settings with a high annual risk for infection that had remained unchanged over time, model predictions suggested that most patients clustered in each age group were likely to have been recently (re)infected, corresponding to a positive predictive value of clustering for recent transmission of almost 100% in each age group.
group (Figure 6A). The positive predictive value was estimated to decrease with age in the Netherlands from 100% in the very young to about 20% for the oldest patients.

When unclustered cases were considered, the proportion of clinical case-patients who were estimated to have been infected >5 years previously was low (<5%) for young patients and increased with age for all settings, approaching 100% for patients >55 years of age in the Netherlands (Figure 6B). Almost all case-patients of ages >55 years who were not in a cluster in the Netherlands were therefore estimated to have been infected >5 years previously and thus owed their disease to reactivation of latent foci. Of the adult case-patients who were not in a cluster in the other settings, the proportion who had been infected >5 years previously was <45%, 35%, and 20% if the annual risk for infection was 0.1%, 1%, and 3%, respectively.

Discussion

The availability of DNA fingerprinting techniques has led to a large number of studies that measure clustering of isolates from tuberculosis cases (1–3,16). Most of these studies have been conducted in industrialized settings and have found relatively low levels of clustering (30% to 40%) and decreases in clustering with age. Our analyses indicate that those findings have been influenced strongly by the large secular decline in the annual risk for infection that occurred in industrialized settings during the 20th century and that very different findings are expected in settings where the annual risk for infection has changed little over time. The clustering predicted is high (>60% for 2-year periods) in such settings, similar for all age groups, and may nevertheless still underestimate the extent of disease that is due to recent transmission.

Our conclusions are based on a model of the transmission dynamics of *M. tuberculosis* that includes several simplifications. The most obvious is our assumption that the risks for disease, given infection in settings in which the infection risk is high, are the same as those estimated for industrialized populations. HIV influences these risks (17,18), although its effect on clustering is not yet understood (14). Another simplification is our assumption that the half-life of DNA fingerprint patterns is identical for strains involved in active disease and in latent infection. If latent infections are associated with a slow rate of genetic change of the bacilli, our assumption would have led to an underestimate of clustering but would not have affected our conclusions for settings in which the annual risk for infection has remained unchanged over time, where only a small proportion of disease is attributed to reactivation of a latent infection (Figure 3). The effect of this assumption on clustering estimates for the Netherlands is discussed elsewhere (6).

Our finding that the overall amount of clustering in populations with a low (constant) annual infection risk should be similar to that observed in populations with a high (constant) infection risk may appear paradoxical. Our finding follows from the fact that in such populations any decline in the pro-

![Figure 5](image)

Figure 5. Comparison between model predictions of the clustering in different age groups and the proportion of disease attributable to recent infection or reinfection in the Netherlands and in settings in which the annual risk for infection has remained unchanged over time at 0.1%, 1%, and 3%.

![Figure 6](image)

Figure 6. Summary of model predictions of the A) positive predictive values of clustering (proportion of cases who are in a cluster who have been infected or reinfected <5 years before onset) and B) negative predictive values of clustering (proportion of cases who are not in a cluster who are experiencing disease as a result of infection or reinfection acquired >5 years before onset) in different age groups in the Netherlands and in settings in which the annual risk for infection has remained unchanged over time at 0.1%, 1%, and 3%.
portion of disease attributable to recent primary infection with age is compensated by increases in the proportion attributable to recent reinfection with age (Figure 3). As a result, both the overall and age-specific predicted proportions of disease attributable to recent transmission in these populations are very similar; this finding leads to predictions that the overall and age-specific levels of clustering in these settings would also be similar.

Previous model-based analyses (6) have indicated that in industrialized settings such as the Netherlands clustering among young case-patients will underestimate the extent of disease attributable to recent transmission (because some sources of infection have onset outside the study period and because DNA fingerprint patterns can change between infection and disease onset), and clustering among old case-patients may overestimate recent transmission (because clustering among older case-patients is more likely to be attributable to their being sources of infection rather than their being recently reinfected). These analyses extend those findings and indicate that in settings in which the annual risk for infection has not changed much over time, the overall level of clustering in any given age group is likely to underestimate the extent of recent transmission (Figure 5). This underestimate follows from the fact that in these settings, most disease in all age groups is attributable to recent transmission, and some patients will have been infected or reinfected immediately before the study started and thus may not be in a cluster.

These analyses provide the first estimates of the positive and negative predictive values of clustering. Overall, these analyses highlight the fact that in settings in which the annual risk for infection has not changed greatly over time, most clustered case-patients are likely to have been recently infected or reinfected (i.e., the positive predictive value of clustering is high) (Figure 6). This finding suggests that in such settings, application of the “n-1” rule (2), which assumes that each cluster comprises an index case attributable to reactivation and the other cases result (in)directly from that case, will lead to even more unreliable estimates of the extent of recent transmission than those based on the “n” rule. Similarly, estimates of the proportion of disease attributable to reactivation will be unreliable if they are based on the proportion of patients who fail to be in a cluster in a given period.

Our analyses demonstrate that the properties and interpretation of clustering statistics depend strongly on the trend and magnitude in the annual risk for infection and thus will vary between settings. For example, in settings in which the annual risk for infection has remained unchanged at either a high or a low level, the age differential in clustering is likely to be small, in contrast with that in industrialized settings, and clustering is likely to underestimate the extent of recent transmission in all age groups. Given the growing importance of clustering studies, which, to date have been conducted in populations in which the annual risk for infection declined dramatically over time and is currently very low, these insights are important for an improved understanding of the natural history of tuberculosis.

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RESEARCH

Simulating Contact between Persons

For simplicity, we assumed that all effective contacts (defined as those sufficient to lead to infection by an infectious case-patient if the contacted person has never been infected) occurred immediately after onset of (infectious pulmonary) disease in the source case. This assumption is reasonable for industrialized countries in recent years (see, for example, 19) but is less realistic for some unindustrialized countries because of longer diagnostic delays in such populations. The number of persons effectively contacted by each case-patient during the infectious period (the effective contact number) was assumed to follow a negative binomial distribution, defined by a time-dependent mean and variance (Figure 2).

The infection risk at time \( t \) is given by the total number of persons experiencing their first primary episode, i.e.,

\[
i(t) = \sum T_aT \int_0^t d_aT(t, s)ds - (m_t, s)\Delta_t(a)\]

(0 < s ≤ 5)

For notational convenience, we denote \( 1 - d_s(a) \) by \( d(a) \). The infection risk at time \( t \) is given by

\[
i(t) = \sum T_aT \int_0^t d_aT(t, s)ds - (m_t, s)\Delta_t(a)\]

(0 < s ≤ 5)

Boundary conditions:

\[
U(0,t) = B(t)
\]

\[
I_r(a,T,0) = i(T)U(a,T)
\]

\[
I_r(a,T,0) = i(T)\sum L_a(a,T)
\]

For notational convenience, we denote \( 1 - d_s(a) \) by \( d(a) \). The infection risk at time \( t \) is given by

\[
i(t) = \sum T_aT \int_0^t d_aT(t, s)ds - (m_t, s)\Delta_t(a)\]

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Though assumptions about contact patterns between persons influence the predicted cluster distributions, they do not affect the overall levels of clustering (6). The data used for calibrating the model’s assumptions have been described (6).

Appendix

Summary of Equations Used in Model

We use the notation summarized in the Table and reference 6 to describe the transmission dynamics of Mycobacterium tuberculosis. Note that all of the variables are stratified by sex; for reporting convenience, we have omitted this stratification in the following description. The equations are as follows:

\[
\frac{\partial U(a,t)}{\partial a} + \frac{\partial U(a,t)}{\partial t} = -i(t) + m_g(a,t)U(a,t)
\]

(1)

\[
\frac{\partial P_a(a,t,s)}{\partial a} + \frac{\partial P_a(a,t,s)}{\partial t} + \frac{\partial P_a(a,t,s)}{\partial s} = \int d_s(a-s,s)P_a(t,s)ds - (m_t, s)\Delta_a(a)
\]

(0 < s ≤ 5)

\[
\frac{\partial L_a(a,t)}{\partial a} + \frac{\partial L_a(a,t)}{\partial t} = (i(a,t,5) + l_s(a, t,5))k(5) + r(a, t,2)P_a(a, t, 2) + E_{st}(a, t, 2) + E_{st}(a, t, 2) - (i(t) + d_s(a) + m(a, t))L_a(a, t)
\]

(4)

\[
\frac{\partial E_{sa}(a,t,s)}{\partial a} + \frac{\partial E_{sa}(a,t,s)}{\partial t} + \frac{\partial E_{sa}(a,t,s)}{\partial s} = \int d_s(a-s,s)E_{sa}(a,t,s)ds - (m_t, s)\Delta_{sa}(a)
\]

(0 < s ≤ 5)

\[
\frac{\partial E_{sa}(a,t,s)}{\partial a} + \frac{\partial E_{sa}(a,t,s)}{\partial t} + \frac{\partial E_{sa}(a,t,s)}{\partial s} = d_s(a)\Delta_{sa}(a) - r(a,t, s)E_{sa}(a,t, s) - (m_t, s)\Delta_{sa}(a)
\]

+ m_g(a,t)d(a) + r(a,t,s)E_{sa}(a,t,s)

(7)

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Appendix table. Definitions of state variables used in the model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B(t)$</td>
<td>No. of live births at time $t$. Obtained from the Dutch Central Bureau for Statistics (data available from 1892 to present).</td>
</tr>
<tr>
<td>$U(a,t)$</td>
<td>No. of uninfected persons of age $a$ at time $t$.</td>
</tr>
<tr>
<td>$I_T(a,t,s)$</td>
<td>No. of persons of age $a$ at time $t$ who were infected at time $T$ and have been infected for time $s$ ($\leq 5$ years) without having yet developed disease.</td>
</tr>
<tr>
<td>$P_T(a,t,s)$</td>
<td>No. of persons of age $a$ first infected at time $T$ who are experiencing their first primary episode at time $t$, who have been diseased for time $s$.</td>
</tr>
<tr>
<td>$L_T(a,t)$</td>
<td>No. of persons of age $a$ at time $t$ in the “latent” class (comprising those who have either just recovered from their first primary episode, or who have been infected for $&gt;5$ years) whose most recent (re)infection event occurred at time $T$.</td>
</tr>
<tr>
<td>$I_r(a,t,s)$</td>
<td>No. of persons of age $a$ at time $t$, whose most recent reinfection occurred at time $T$, who have been reinfected for time $s$ ($\leq 5$ years) and in whom exogenous disease has not yet developed.</td>
</tr>
<tr>
<td>$E_{x0}(a,t,s)$</td>
<td>No. of persons of age $a$ with exogenous disease at time $t$, who have been diseased for time $s$ and whose most recent reinfection occurred at time $T$.</td>
</tr>
<tr>
<td>$E_{x+}(a,t,s)$</td>
<td>No. of persons of age $a$ with endogenous disease at time $t$, who have been diseased for time $s$ and whose most recent reinfection occurred at time $T$.</td>
</tr>
</tbody>
</table>

Calculating the Distribution of Strains among Cases at a Given Time

We assumed that all reactivations (which generally involve persons infected for $>5$ years) of infections acquired before 1950 were with unique strains and that the strain isolated from persons who had been reinfected was from the most recent (re)infection event. The DNA fingerprint pattern of the strain causing disease among each of the case-patients with onset at time $T$ and whose most recent (re)infection had occurred at time $t$ since 1950 was assumed to be identical to that with which the source of infection of that person (identified by using the algorithm described in [6]) had been infected, unless the DNA fingerprint pattern had since changed through random mutations. The proportion of case-patients who had been infected at time $t$ for whom the DNA fingerprint pattern was assumed to have changed was given by the expression $1-e^{-0.21661(T-t)}$ which describes a half-life of 3.2 years for DNA fingerprint patterns, as found in a recent study [5]. These analyses assume implicitly that clustered cases were involved, at some level, in the same chain of transmission and that clustering was not attributable, e.g., to preferential insertion of IS6110 into any particular location in the genome.
Endemic Babesiosis in Another Eastern State: New Jersey

Barbara L. Herwaldt,* Paul C. McGovern,† Michal P. Gerwel,‡ Rachael M. Easton,† and Rob Roy MacGregor†

In the United States, most reported cases of babesiosis have been caused by *Babesia microti* and acquired in the northeast. Although three cases of babesiosis acquired in New Jersey were recently described by others, babesiosis has not been widely known to be endemic in New Jersey. We describe a case of babesiosis acquired in New Jersey in 1999 in an otherwise healthy 53-year-old woman who developed life-threatening disease. We also provide composite data on 40 cases of babesiosis acquired from 1993 through 2001 in New Jersey. The 40 cases include the one we describe, the three cases previously described, and 36 other cases reported to public health agencies. The 40 cases were acquired in eight (38.1%) of the 21 counties in the state. Babesiosis, a potentially serious zoonosis, is endemic in New Jersey and should be considered in the differential diagnosis of patients with fever and hemolytic anemia, particularly in the spring, summer, and early fall.

In the United States, most of the hundreds of reported cases of babesiosis have been caused by *Babesia microti*, a parasite of small mammals transmitted by *Ixodes scapularis* (deer ticks); these ticks also transmit *Borrelia burgdorferi* and *Anaplasma (Ehrlichia) phagocytophila*. Most reported cases of babesiosis have been acquired in the northeast, specifically in New York, Massachusetts, Connecticut, and Rhode Island. Another focus of *B. microti* infection is in Wisconsin and Minnesota (1). Although three cases of babesiosis acquired in New Jersey in 1998 were described by Eskow et al. (2), babesiosis has not been widely known to be endemic in New Jersey. Of interest, the index case–patient who acquired *B. microti* infection in the northeast (on Nantucket Island in 1969) actually was hospitalized in New Jersey (3). We describe a case of babesiosis acquired in New Jersey in 1999 and provide composite data that include this case, the three cases previously reported by Eskow et al. (2), and 36 other cases acquired in New Jersey from 1993 through 2001. Our data strengthen the conclusion that babesiosis is endemic in New Jersey.

Case Report

A previously healthy 53-year-old woman was admitted to a community hospital on June 24, 1999, because she had had 1 week of fever (38.9°C–39.4°C), rigors, a nonproductive cough, an occipital headache, and increasing malaise. Three days before her hospitalization, she started therapy with cefuroxime axetil for presumed bronchitis but did not improve. She had a >50 pack-year history of smoking and drank two to three beers per day. She lived in Burlington County (Figure 1) in southcentral New Jersey and had not traveled outside the county recently. Although she did not recall recent exposure to deer ticks, she occasionally had seen deer in her backyard and she gardened frequently.

On admission to the hospital, she had a temperature of 39.2°C, a blood pressure level in the 80/60 mm Hg range, and otherwise unremarkable results on physical examination. She was anemic and thrombocytopenic, with elevated total bilirubin and lactate dehydrogenase values (Table). On the basis of a blood smear from June 24, which showed intraerythrocytic ring forms in approximately 5% of the erythrocytes on her
Peripheral blood smear, treatment for babesiosis was begun on June 25. The treatment included intravenous clindamycin, 900 mg three times a day, and oral quinine, 650 mg three times a day; and she was transfused with two units of packed erythrocytes. Also on June 25, hypoxic respiratory failure developed, and she was intubated. A chest radiograph showed diffuse alveolar infiltrates, which were attributed to the adult respiratory distress syndrome (ARDS).

On June 28, she was transferred to the Hospital of the University of Pennsylvania. When admitted, her blood pressure was 84/52 mm Hg, despite therapy with dopamine. She continued therapy for babesiosis for a total of 15 days (the dose of clindamycin was decreased to 600 mg three times a day on June 28). Although the level of parasitemia had decreased to 0.3% by June 29, she had ongoing hemolysis and received six more units of packed erythrocytes during her hospital stay (Table). No parasites were noted on a blood smear on July 9, the last day of antibabesial therapy. She had been successfully weaned from inotropic blood-pressure support on July 2 and underwent extubation on July 4.

Additional laboratory testing at CDC provided further evidence that she was infected with \( B. \) microti. Serum specimens assayed in parallel, in serial fourfold dilutions, by indirect fluorescent antibody testing for antibody to \( B. \) microti (4), had titers of 1:1,024 (June 30, 1999) and 1:16 (July 16, 2000). In addition, PCR analysis of whole blood from June 30, 1999, by using \( B. \) microti-specific primers (5), confirmed she was infected with \( B. \) microti. Serologic testing performed at the Hospital of the University of Pennsylvania by enzyme immunoassay for antibody to \( B. \) burgdorferi was negative.

Complications during her hospitalization unrelated to babesiosis included nosocomial pneumonia, acute tubular necrosis from hypoperfusion, bilateral deep venous thromboses, pulmonary embolism, and thrombocytopenia temporarily associated with the initiation of heparin therapy (Table). On July 23, after 30 days in the hospital, she was sent home. She was continuing to do well as of October 2002.

**Composite Data**

The 40 cases in our analyses include the case described above, the three cases previously described by Eskow et al. (2), and 36 other cases. We did not include six other reported tick-borne cases that occurred in New Jersey residents, because the laboratory data did not meet our criteria or information about the probable state in which infection was acquired was not known or provided.

The number of reported cases of babesiosis increased over time (Figure 2); 28 (70.0%) of the 40 cases occurred in 2000 or 2001. The 40 cases were acquired in eight (38.1%) of the state’s 21 counties (Figure 1). Burlington County, on the inner coastal plain, and Ocean County, on the outer coastal plain, which are neighboring counties in southcentral New Jersey, accounted for 25 (62.5%) of the 40 cases; these two counties are the 7th (Ocean) and 10th (Burlington) most populous counties in the state. None of the cases were acquired in the northernmost or southernmost counties of New Jersey.

Most of the cases were in elderly persons (median age, 67 years; range, 11–87 years). Over half of the cases (22 [55.0%]) were in male patients. The median date of diagnosis was July 20 (range, June 10–September 9; n=36). Two patients (5.0%) died: an 86-year-old man with multisystem organ failure and an 80-year-old man with ARDS. The patient whose case we described here also developed ARDS.

The following information about the patients was not collected systematically. However, three patients were reported to be asplenic, 18 to have recalled tick bites, 34 to have been hospitalized, and three to have had Lyme disease (no details available). Underlying conditions included HIV infection in one patient, who had a CD4 count of 50; diabetes in five patients; a history of breast or prostate cancer in three patients (no details available); and a condition that led to chemotherapy in one person (no details available).

Various types of laboratory tests were used to diagnose the 40 babesiosis cases. Not all patients were tested with the same methods. However, 34 patients had positive blood smears; for 27 of these patients, the positive smear was the only laboratory result that met our diagnostic criteria. All three cases reported by Eskow et al. were in patients who had negative blood
One of the two patients who had whole blood inoculated into hamsters had positive results (i.e., the hamsters became parasitemic). Four patients had positive PCR results from a reference laboratory; for one of these patients, these results were the only ones that met our diagnostic criteria. Twelve patients had serologic data that met our criteria; for four patients, the serologic results were the only data that met our diagnostic criteria. These cases were considered probable rather than confirmed. CDC confirmed the diagnosis of Babesia infection in 11 (27.5%) of the 40 cases; specimens from the other 29 case-patients were not sent to CDC.

Discussion

Our report strengthens the evidence that New Jersey is one of the eastern states in which babesiosis is endemic. In addition, the risk for acquisition of infection is widely distributed in the state. Whether the fact that most of the reported cases occurred in southcentral and northcentral counties reflects the degree of endemicity of babesiosis in various areas of New Jersey is unknown.

The fact that babesiosis is endemic in New Jersey is not surprising, given that Lyme disease, the etiologic agent of which also is transmitted by I. scapularis, is highly endemic in New Jersey (6,7) and given the geographic proximity of New Jersey to areas in the northeast where babesiosis is highly endemic. In a 1996 study, of 100 I. scapularis ticks collected in Hunterdon County, New Jersey, 43 were infected with Borrelia burgdorferi, 5 were infected with B. microti, and 2 were infected with both organisms (8).

The increase in reported cases of babesiosis, which began in 1998 (Figure 2) and was even more notable in 2000 and 2001, could indicate an increased risk for B. microti infection and illness. If true, possible reasons for the increased risk could include a growing abundance of local I. scapularis populations or the introduction of a more virulent strain of B. microti (9). However, the increased numbers of reported cases could simply represent an increased awareness of the disease and increased reporting. Even so, the 40 cases of babesiosis that we tallied probably represent only a fraction of the clinical cases of B. microti infection acquired in New Jersey from 1993

Table. Clinical data on selected dates for a patient who acquired babesiosis in New Jersey

<table>
<thead>
<tr>
<th>Date</th>
<th>Temperature (°C)</th>
<th>Hematocrit (%)</th>
<th>Leukocyte count (10⁹/L)</th>
<th>Platelet count (10⁹/L)</th>
<th>Parasitemia level (%)</th>
<th>Creatinine level (mg/dL)ᵃ</th>
<th>Total bilirubin level (mg/dL)ᵇ</th>
<th>Lactate dehydrogenase level (U/L)ᵇ</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>June 24</td>
<td>39.2</td>
<td>25</td>
<td>4.6ᵇ</td>
<td>92</td>
<td>5.0</td>
<td>1.1</td>
<td>2.2ᵈ</td>
<td>646</td>
<td>Hospitalized</td>
</tr>
<tr>
<td>June 25</td>
<td>38.2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Antibabesial therapy started; intubated; 2 units packed erythrocytes transfused</td>
</tr>
<tr>
<td>June 26</td>
<td>40.0</td>
<td>31</td>
<td>6.0</td>
<td>55</td>
<td>—</td>
<td>1.1</td>
<td>2.9</td>
<td>1,019</td>
<td>—</td>
</tr>
<tr>
<td>June 28</td>
<td>—</td>
<td>28</td>
<td>8.6</td>
<td>93</td>
<td>—</td>
<td>0.9</td>
<td>2.7ᵈ</td>
<td>—</td>
<td>Transferred to the Hospital of the University of Pennsylvania</td>
</tr>
<tr>
<td>June 29</td>
<td>39.2</td>
<td>27</td>
<td>9.0</td>
<td>99</td>
<td>0.3</td>
<td>1.9</td>
<td>—</td>
<td>5,147</td>
<td>—</td>
</tr>
<tr>
<td>June 30</td>
<td>38.3</td>
<td>22</td>
<td>7.9</td>
<td>109</td>
<td>—</td>
<td>2.8</td>
<td>1.5</td>
<td>4,047</td>
<td>2 units packed erythrocytes transfused</td>
</tr>
<tr>
<td>July 1</td>
<td>38.7</td>
<td>26</td>
<td>9.7</td>
<td>138</td>
<td>—</td>
<td>3.5</td>
<td>1.9</td>
<td>4,140</td>
<td>—</td>
</tr>
<tr>
<td>July 2</td>
<td>38.0</td>
<td>26</td>
<td>9.0</td>
<td>139</td>
<td>—</td>
<td>2.9</td>
<td>1.6</td>
<td>3,669</td>
<td>2 units packed erythrocytes transfused</td>
</tr>
<tr>
<td>July 4</td>
<td>38.7</td>
<td>29</td>
<td>14.7</td>
<td>144</td>
<td>—</td>
<td>2.2</td>
<td>2.2</td>
<td>2,864</td>
<td>Extubated</td>
</tr>
<tr>
<td>July 7</td>
<td>38.2</td>
<td>28</td>
<td>19.3</td>
<td>277</td>
<td>—</td>
<td>1.5</td>
<td>—</td>
<td>—</td>
<td>Developed nosocomial pneumonia</td>
</tr>
<tr>
<td>July 8</td>
<td>38.1</td>
<td>23</td>
<td>16.3</td>
<td>308</td>
<td>—</td>
<td>1.7</td>
<td>2.1</td>
<td>2,008</td>
<td>2 units packed erythrocytes transfused</td>
</tr>
<tr>
<td>July 9</td>
<td>38.3</td>
<td>31</td>
<td>11.7</td>
<td>300</td>
<td>0.0</td>
<td>1.8</td>
<td>—</td>
<td>—</td>
<td>Antibabesial therapy stopped</td>
</tr>
<tr>
<td>July 11</td>
<td>Afebrile</td>
<td>28</td>
<td>15.3</td>
<td>310</td>
<td>—</td>
<td>1.8</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>July 16</td>
<td>Afebrile</td>
<td>29</td>
<td>15.8</td>
<td>396</td>
<td>—</td>
<td>1.5</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>July 19</td>
<td>Afebrile</td>
<td>30</td>
<td>15.8</td>
<td>322</td>
<td>—</td>
<td>1.6</td>
<td>—</td>
<td>1,081</td>
<td>—</td>
</tr>
<tr>
<td>July 21</td>
<td>Afebrile</td>
<td>26</td>
<td>12.3</td>
<td>71</td>
<td>—</td>
<td>1.9</td>
<td>—</td>
<td>—</td>
<td>Developed heparin-associated thrombocytopenia</td>
</tr>
<tr>
<td>July 23</td>
<td>Afebrile</td>
<td>28</td>
<td>12.4</td>
<td>50</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Sent home</td>
</tr>
</tbody>
</table>

ᵃNormal ranges for community hospital (June 24–June 27): creatinine level, 0.6–1.3 mg/dL; total bilirubin level, 0.2–1.0 mg/dL; indirect bilirubin level, 0.2–1.2 mg/dL; lactate dehydrogenase level, 91–180 U/L. Normal ranges for the Hospital of the University of Pennsylvania: creatinine level, 0.6–1.0 mg/dL; total bilirubin level, 0.0–1.2 mg/dL; indirect bilirubin level, 0.0–1.2 mg/dL; lactate dehydrogenase level, 313–618 U/L.
ᵇ25% segmented neutrophils, 33% band forms, 22% lymphocytes, 2% atypical lymphocytes, 8% monocytes.
ᶜIndirect bilirubin level, 1.4 mg/dL.
ᵈIndirect bilirubin level, 0.2 mg/dL; serum haptoglobin level, <38 mg/dL (normal range, 60–160 mg/dL); results of direct and indirect Coombs test, negative.
through 2001. Presumably, other symptomatic cases (as well as many more subclinical cases) occurred but were not diagnosed or reported. In fact, several other possible symptomatic cases were reported that we did not count because we received insufficient information about them. Also, as is commonly true for surveillance data, the amount and quality of the information provided to the health department and CDC about the cases varied widely; some of the information might have been inaccurate, and not all of the cases were confirmed by reference laboratories (e.g., not all of the blood smears that were reported as positive were reexamined by a reference laboratory).

The laboratory tests CDC offers for babesiosis, when indicated, include examination of blood smears, hamster inoculation, and PCR (5) for parasitologic diagnosis and an indirect fluorescent antibody assay for total immunoglobulin for serologic diagnosis (4). Using PCR for detection of DNA from Babesia spp. has not yet become a routine diagnostic method, and the analysis should be conducted by experienced reference laboratories.

Immunoblot testing for IgG and IgM is investigational. However, an immunoblot test for IgG performed well in a recent evaluation, with a sensitivity of 96% and a specificity of 99% (10). A positive serologic result for IgM (11) is insufficient for diagnosis without a positive result for IgG. If the IgM result is positive but the IgG result is negative, a follow-up specimen should be tested. If IgG seroconversion is not noted, the IgM result likely was a false positive. Future serologic testing might involve recombinant and synthetic antigens (12) rather than whole parasites or soluble antigens.

The case we described in detail demonstrates that babesiosis can be life threatening (1,13,14). In fact, two (5.0%) of the 40 case-patients died. In the patient we described, the following conditions developed: severe anemia, for which she was transfused with eight units of packed erythrocytes; hypotension that required inotropic support; ARDS, which has previously been reported (13–17); and various nosocomial complications. The fact that she was ill for approximately 1 week before therapy for babesiosis was initiated might have contributed to the severity of her illness. Fortunately, treatment for babesiosis was begun soon after she was hospitalized. Although she was treated successfully with clindamycin and quinine, a recent clinical trial indicated that the combination of azithromycin and atovaquone is also effective (18). However, patients with life-threatening babesiosis were excluded from the study. Severely ill patients, particularly those with high levels of parasitemia (e.g., >10%), may benefit from exchange transfusion (1,19).

In summary, babesiosis, a potentially serious zoonosis, is endemic in New Jersey and should be considered in the differential diagnosis of patients with fever and hemolytic anemia, particularly in the spring, summer, and early fall.

Acknowledgments

We thank the laboratory staff of the Division of Parasitic Diseases, CDC, and the other laboratorians and physicians who provided data about the cases of babesiosis.

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References


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Molecular Typing of IberoAmerican Cryptococcus neoformans Isolates

Wieland Meyer,* Alexandra Castañeda,† Stuart Jackson,*‡ Matthew Huynh,* Elizabeth Castañeda,† and the IberoAmerican Cryptococcal Study Group†

A network was established to acquire basic knowledge of Cryptococcus neoformans in IberoAmerican countries. To this effect, 340 clinical, veterinary, and environmental isolates from Argentina, Brazil, Chile, Colombia, Mexico, Peru, Venezuela, Guatemala, and Spain were typed by using M13 polymerase chain reaction-fingerprinting and orotidine monophosphate pyrophosphorylase (URA5) gene restriction fragment length polymorphism analysis with HhaI and Sau96I in a double digest. Both techniques grouped all isolates into eight previously established molecular types. The majority of the isolates, 68.2% (n=232), were VNI (var. grubii, serotype A), which accords with the fact that this variety causes most human cryptococcal infections worldwide. A smaller proportion, 5.6% (n=19), were VNII (var. grubii, serotype A); 4.1% (n=14), VNIII (AD hybrid), with 9 isolates having a polymorphism in the URA5 gene; 1.8% (n=6), VNI (var. neoformans, serotype D); 3.5% (n=12), VG1; 6.2% (n=21), VGII; 9.1% (n=31), VGIII, and 1.5% (n=5) VGIV, with all four VG types containing var. gattii serotypes B and C isolates.

Cryptococcosis is among the most prevalent life-threatening mycoses and has a worldwide distribution. The etiologic agent is the basidiomycetous yeast Cryptococcus neoformans (1,2); three varieties are recognized: C. neoformans var. grubii (3), C. neoformans var. neoformans, serotype D, C. neoformans var. gattii, serotypes B and C, and the hybrid serotype AD (4).

Humans are infected by inhaling infectious propagules from the environment, which primarily colonize the lung and subsequently invade the central nervous system (4). C. neoformans var. grubii/neoformans has been isolated worldwide from soil enriched with avian excreta (4,5). More recently, decaying wood from certain species of trees has been proposed as environmental habitat for this variety (6). In contrast, the distribution in nature for C. neoformans var. gattii is geographically restricted to mainly tropical and subtropical regions (7,8). To date, specific host trees are represented by Eucalyptus species, Moquilea tomentosa, Cassia grandis, Ficus microcapra, and Terminalia catappa (7–11).

Worldwide, in immunocompromised hosts, most infections are caused by C. neoformans var. grubii (4,5). In contrast, C. neoformans var. gattii virtually always affects immunocompetent hosts (8).

In the last decade, a number of DNA typing techniques have been used to study the epidemiology of C. neoformans. These techniques include karyotyping, random amplification of polymorphic DNA, restriction fragment length polymorphism (RFLP), DNA hybridization studies, amplified fragment length polymorphism (AFLP), and polymerase chain reaction (PCR) fingerprinting (12–17).

PCR fingerprinting has been used as the major typing technique in the ongoing global molecular epidemiologic survey of C. neoformans (14,18), dividing >400 clinical and environmental isolates into eight major molecular types: VNI (var. grubii, serotype A), VNII (var. grubii, serotype A), VNIII (serotype AD), VNI (var. neoformans, serotype D), VG1, VGII, VGIII, and VGIV (var. gattii, serotypes B and C). No correlation between serotype and molecular type has been found for C. neoformans var. gattii. The molecular types were recently confirmed by RFLP analysis of the orotidine monophosphate pyrophosphorylase (URA5) gene and the phospholipase (PLBI) gene (19).

Globally, most of the isolates recovered from AIDS patients belong to the genotypes VNI and VNI, whereas the genotypes VNI and VG1 are predominant throughout the world for C. neoformans var. grubii and C. neoformans var. gattii, respectively. The larger number of genotype VNI isolates agrees with the fact that C. neoformans var. grubii causes most human cryptococcal infections worldwide (18,19).

The aims of this study were the following: 1) to extend the molecular epidemiologic survey to other parts of the world, 2) to establish a regional network of participating reference laboratories, and 3) to apply PCR fingerprinting and URA5 RFLP typing to investigate the genetic structure and possible epidemiologic relationships between clinical and environmental isolates obtained in Latin America and Spain. The results of this study permitted us to determine the major molecular types and their distribution within each participating country.

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RESEARCH

Materials and Methods

Study Design

During the 12th International Society for Human and Animal Mycoses meeting in Buenos Aires, Argentina, in March 2000, it was decided to establish an IberoAmerican Cryptococcal Study Group under the coordination of E. Castañeda and W. Meyer. Each of the participating laboratories was asked to submit 10–30 isolates. For the clinical isolates, the following data were requested: isolation date, demographic data (age and gender of patient), collection location, risk factors, source and variety, and serotype. For the environmental and veterinary isolates, the data collected included isolation date, source, collection location, variety, and serotype.

Fungal Isolates

An online appendix of cryptococcal isolates studied in this study is available at: URL: http://www.cdc.gov/ncidod/EID/vol9no2/02-0246_app.htm. The isolates were obtained by the participating laboratories of the IberoAmerican Cryptococcal Study Group and maintained on Sabouraud dextrose agar slants at 4°C and as water cultures at room temperature. Isolates were identified as \textit{C. neoformans} by using standard methods (20). The variety was determined by the color reaction test on L-canavanine-glycine-bromothymol blue medium (21), and the serotype was determined, in selected isolates, by the use of the Crypto Check Kit (Iatron Laboratories Inc., Tokyo, Japan).

The isolates were sent for molecular typing to the Molecular Mycology Laboratory at the University of Sydney at Westmead Hospital, Sydney, Australia, either as water cultures or on Sabouraud dextrose agar slants. For long-term storage, the isolates were maintained as glycerol stocks at –70°C.

Reference Strains

A set of laboratory standard \textit{C. neoformans} reference strains representing each molecular type were used in PCR fingerprinting and \textbf{URA5} RFLP as follows: WM 148 (serotype A, VNI), WM 626 (serotype A, VNI), WM 628 (serotype AD, VNI), WM 629 (serotype D, VNI), WM 179 (serotype B, VGII), WM 178 (serotype B, VGII), WM 161 (serotype B, VGIII), and WM 779 (serotype C, VGIV) (14).

DNA Extraction

High-molecular-weight DNA was isolated as described previously (14). Briefly, \textit{C. neoformans} isolates were grown on Sabouraud’s dextrose agar at 37°C for 48 h, a loopful of cells from the culture was mixed with sterile deionized water and centrifuged. The supernatant was discarded, and the tube containing the yeast cell pellet was frozen in liquid nitrogen. The pellet was ground with a miniature pestle. The cell lysis solution (100 mg trispropynaphthalene sulfonylic acid, 600 mg para-aminosalicylic acid, 10 mL sterile deionized water, 2.5 mL extraction buffer (1 M Tris-HCl, 1.25 M NaCl, 0.25 M EDTA, pH 8.0) and 7.5 mL phenol saturated with Tris-EDTA was preheated to 55°C, and 700 µL of this mixture was added to the frozen, ground cells. The tubes were incubated for 2 min at 55°C, shaken occasionally, and then 500 µL chloroform was added, and the mixture was incubated for a 2 min at 55°C and shaken occasionally. The tubes were centrifuged for 10 min at 14,000 rpm, and the aqueous phase was transferred to a new tube. Then, 500 µL of phenol-chloroform-isomyl alcohol (25:24:1) was added, shaken for 2 min at room temperature, and centrifuged as above. The aqueous phase was transferred to a new tube, 500 µL of chloroform was added, shaken, and centrifuged as above. To precipitate the genomic DNA, the aqueous phase was again transferred to a new tube, and 0.03 volumes 3.0 M sodium acetate (pH 5.2) and 2.5 volumes cold 96% ethanol were added, and the mixture was gently shaken and incubated at –20°C for at least 1 h or overnight. The solution was centrifuged for 30 min at 14,000 rpm to pellet the DNA. The DNA pellet was washed with 70% ethanol and centrifuged for 10 min at 14,000 rpm and air-dried. The DNA was resuspended in 200 µL sterile deionized water at 4°C overnight and stored at –20°C.

PCR Fingerprinting

The minisatellite-specific core sequence of the wild-type phage M13 (5′ GAGGTTGGCGTCTTC 3′) (22) was used as single primer in the PCR. The amplification reactions were performed in a volume of 50 µL containing 25 ng high-molecular-weight genomic DNA, 10 mM Tris-HCl. pH 8.3, 50 mM KCl, 1.5 mM MgCl, 0.2 mM each of the dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH, Mannheim, Germany), 3 mM magnesium acetate, 30 ng primer, and 2.5 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). PCR was performed for 35 cycles in a Perkin-Elmer thermal cycler (model 480) with 20 s of denaturation at 94°C, 1 min annealing at 50°C, and 20 s extension at 72°C, followed by a final extension cycle for 6 min at 72°C. Amplification products were removed, concentrated to approximately 20 µL and separated by electrophoresis on 1.4% agarose gels (stained with ethidium bromide, 10 mg/mL stock) in 1X Tris-borate-EDTA (TBE) buffer at 60 V for 14 cm, and visualized under UV light (14). Molecular types (VNI–VNIV and VGI–VGIV) were assigned, according to the major bands in the patterns. All visible bands were included in the analysis, independent of their intensity (14,18).

URA5 Gene RFLP

PCR of the \textbf{URA5} gene was conducted in a final volume of 50 µL. Each reaction contained 50 ng of DNA, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl; Applied Biosystems, Foster City, CA), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Roche Diagnostics GmbH), 3 mM magnesium acetate, 1.5 U AmpliTaq DNA polymerase (Applied Biosystems), and 50 ng of each primer URA5 (5′ ATGTCTCCT-CCAAAGCCCTCGACTCC 3′) and SJ01 (5′ TTAAGAC-CCTGTAACCCGGTACT 3′). PCR was performed for 35 cycles in a Perkin-Elmer thermal cycler (model 480) at 94°C for 2-min initial denaturation, 45 s of denaturation at 94°C, 1 min
annealing at 61°C, and 2-min extension at 72°C, followed by a
final extension cycle for 10 min at 72°C. Amplification products
were mixed with one fifth of volume of loading buffer (15% Ficoll
400, 0.25% orange G, MilliQ water), 15 μL of PCR products
were double digested with Sau96I (10 U/μL) and HhaI (20 U/
μL) for 3 h or overnight and separated by 3% agarose gel electro-
phoresis at 100 V for 5 h. RFLP patterns were assigned visually
by comparing them with the patterns obtained from the standard
strains (VNI-VNIV and VGI-VGIV) (Jackson et al. unpub.
data).

Statistical Analysis

Initially, individual PCR fingerprints were visually com-
pared to those of the standard strains, amplified in parallel, to
determine the major molecular type for each isolate. The com-
puter program GelComparII, version 1.01 (Applied Maths,
Kortrijk, Belgium) was used to determine the genetic relation-
ship of the strains. DNA bands of each fingerprint pattern were
defined manually with a band-position tolerance of 0.9%,
being the optimal settings needed to define the molecular size
marker bands as 100% identical. Similarity coefficients were
calculated by using the Dice algorithm, and cluster analyses
were performed by the neighbor-joining algorithms by using
the “Fuzzy Logic” and “Area Sensitive” option of the Gelcom-
parII program.

Results

During the course of this investigation, a network was estab-
lished with 15 laboratories from nine countries participating in
this study. The participating countries were: Argentina, Brazil, Chile,
Colombia, Guatemala, Mexico, Peru, Spain, and Venezuela.

A total of 340 C. neoformans isolates, comprising 266
clinical, 7 veterinary, and 67 environmental isolates were sub-
mitted for molecular typing. Of these, 57 were from Argentina
(53 clinical and 4 environmental), 66 from Brazil (56 clinical,
9 environmental, and 1 veterinary), 19 from Chile (15 clinical
and 4 environmental), 62 from Colombia (39 clinical and 23
environmental), 15 from Guatemala (all clinical), 69 from
Mexico (46 clinical and 23 environmental), 13 from Peru (all
clinical), 19 from Spain (9 clinical, 6 veterinary, and 4 envi-
ronmental), and 20 from Venezuela (all clinical). From the
total isolates investigated, 232 (68.2%) were molecular
type VNI (serotype A, var. grubii), 19 (5.6%) were molecular
type VNII (serotype A, var. grubii), 14 (4.1%) were molecular
type VNIII (serotype A/D, hybrid between the serotypes A and
D), with 5 having a RFLP pattern of the URA5 gene with seven
bands, indicated by VNIII in the online Appendix and Figure
3B and 4B, corresponding to a hybrid between VNI, VNII, and
VNI and 9 isolates having an RFLP pattern of the URA5 gene
with six bands, indicated by VNII* in the online Appendix and
Figure 4B and 5B, corresponding to a hybrid between VNI and
VNI, 6 (1.8%) were molecular type VNI (serotype D, var.
neofor-
mans), 12 (3.5%) were molecular type VGI (serotypes B
and C, var. gattii), 21 (6.2%) were molecular type VGII (sero-
types B and C, var. gattii), 31 (9.1%) were molecular type
VGIII (serotypes B and C, var. gattii), and 5 (1.5%) were
molecular type VGIV (serotypes B and C, var. gattii).

Figures 3A and 4A show examples of PCR fingerprints
obtained from Mexican and Spanish isolates; Figures 3B and
4B show the corresponding URA5 RFLP patterns for the same
isolates. The Mexican isolates were selected because they
were representative of the patterns observed from all the iso-
lates submitted by the Latin American participating laborato-
ries. The Spanish isolates were selected because they
represented a distribution of molecular types corresponding to
those observed in previous studies on European isolates (14).

From the 340 isolates studied 277, marked with “f” in the
online Appendix, have been included in the GelComparII anal-
ysis. Sixty-three isolates were excluded from the analysis
since their PCR fingerprinting patterns were not sharp or had
been run under slightly different electrophoresis conditions,
making the band positions impossible to compare. Cluster
analysis of the PCR-fingerprinting profiles by using the
GelComparII program grouped all isolates into three major
clusters according to variety and into eight major groups
according to the molecular type. The overall homology
observed was 50.4% among isolates of C. neoformans var.

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grubii, 50.9% for C. neoformans var. neoformans, and 51.2% for C. neoformans var. gattii (Figure 2). The homology within a given molecular type was as follows: 54.8% VNI, 57.3% VNII, 51.9% VNIII, 50.9% VNIV, 56.4% VGI, 56.4% VGII, 54.4% VGIII, and 68.3% VGIV.

Besides grouping all isolates into the eight major molecular patterns, the molecular type VNI could be subdivided into eight main subclusters, with most of these subclusters’ grouping isolates obtained from specific countries. The similarity between isolates obtained from any individual country varied from 65% to 82%. Most of the main subclusters within molecular type VNI also contained isolates from different countries, indicating gene flow and strain dispersal between South American countries, Spain, or both. However, all isolates could be separated by their unique PCR-fingerprinting pattern, with the highest homology being 84% between two unrelated environmental isolates from Mexico City (LA 22, budgerigar [parakeet] droppings, and LA 25, pigeon droppings).

Of the 266 clinical isolates, 177 (66.5%) were obtained from HIV-positive patients, with 139 (78.5%) being VNI, 14 (7.9%) VNII, 13 (7.4%) VNIII, 6 (3.4%) VNIV, 3 (1.7%) VGII, and 2 (1.1%) VGIII. Most (86.4%) isolates from HIV-positive patients belonged to the molecular types VNI and VNII, representing serotype A, C. neoformans var. grubii. Of these, 266 clinical isolates, 51 (19.2%) were recovered from patients with no reported risk factors. From those, 23 (45.1%) were var. grubii with the molecular type VNI (n=21) or VNII (n=2), and 28 (54.9%) were var. gattii with the molecular types VGI (n=3), VGII (n=10), VGIII (n=14), and VGIV (n=1). For 26 of the clinical isolates, no data concerning risk factors were available. Six veterinary isolates were molecular type VGI, and one was VGII. Most of the environmental isolates belonged to C. neoformans var. grubii with 73.1% being VNI (n=49) and 1.5% VNIII (n=1) AD hybrids. The remaining 17 (25.3%) isolates were C. neoformans var. gattii with the molecular type VGI (n=1), VGI (n=3), VGIII (n=12), and VGIV (n=1).

Cryptococcal isolates included in the IberoAmerican study were more frequently obtained from men than from women. The male-to-female ratio was 21:1 to 41, i.e., cryptococcosis was 5.1 times more common in men than in women. In the HIV-positive population alone, the incidence of cryptococcosis was 5.5 times more frequent in men than in women, based on the data obtained from the isolates investigated in this study. The age of the patients with clinically manifested cryptococcosis ranged from 4 to 73; 175 (65.9%) were between 21 and 40 years old.

The clinical isolates submitted to this study were collected over a period of 41 years, 1961–2001; most (92.5%) of the isolates were collected in the mid-1990s. The veterinary isolates were recovered from goats in Spain in 1995 and from a parrot in Brazil in 2000. The environmental isolates submitted were collected over a period of 7 years, 1993–2000.

Discussion

This retrospective study of cryptococcosis in IberoAmerica was set up in an effort to establish a network of medical
mycology laboratories to study the distribution of cryptococcal isolates, including the varieties and molecular types within the participating countries. The network was aimed at generating PCR-fingerprinting and URA5 RFLP patterns under standardized conditions in the Molecular Mycology Laboratory of the University of Sydney at Westmead Hospital, for a subset of clinical and environmental isolates from each participating country. These reference profiles are now available to each participating laboratory so they can set up the molecular typing techniques in their own laboratories, and to serve as internal controls in future extended studies of cryptococcal isolates in each country.

The data were obtained from a random selection of cryptococcal isolates from each participating country and laboratory, which do not necessarily reflect the true situation in IberoAmerica. Nonetheless, the data offer a general overview of molecular types and variety distribution of C. neoformans in IberoAmerica. For the first time, two different molecular typing techniques, PCR-fingerprinting with the minisatellite specific primer M13 and URA5 gene RFLP analysis, were applied simultaneously to the same set of cryptococcal isolates, demonstrating identical groupings to the eight major molecular types previously described (14,18).

Previous pilot studies that used PCR-fingerprinting at the University of Sydney at Westmead Hospital distributed more than 400 clinical and environmental isolates obtained from Argentina, Australia, Belgium, Brazil, Germany, Italy, New Zealand, Papua New Guinea, South Africa, Thailand, Uganda, and the United States in eight major molecular types, VNI and VNII (serotype A), VNIII (serotype AD), VNIV (serotype D), VGI, VGII, VGIII and VGIV (serotypes B and C) (14,18). At the time of this original work, the molecular types VNI and VGI were found to be the most common genotypes worldwide. The present study, which includes more isolates from Latin America, showed the same results as regards variety grubii, with VNI being the predominant molecular type, accounting for 68.2% of all isolates. However, the situation changed drastically for variety gattii; as in this study, the predominant molecular type was VGIII, accounting for 9.1% of all isolates, in contrast to previous studies which showed that the molecular type VGIII was geographically restricted to India and the United States (18,19). In the present study, VGIII was also found in Argentina, Colombia, Guatemala, Mexico...
and Venezuela, suggesting that it is not as limited as previously suggested. The same was true for the molecular type VGI, previously assigned only to India and South Africa (18,19); its presence in Colombia and Mexico, although in very low numbers, indicates a wider geographic distribution.

In general, the most common variety was C. neoformans var. grubii, 73.8% (n=251), followed by variety gattii, 20.3% (n=69). Much less common were the AD hybrids, 4.1% (n=14) and variety neoformans, 1.8% (n=6), which reflects the global distribution previously established (14,18,23).

The overall grouping of the isolates into eight major molecular types by PCR-fingerprinting with the minisatellite specific primer M13, obtained in this study and the previous pilot study by Meyer et al. 1999 (14) and Ellis et al. 2000 (18), agrees with the findings by Boekhout et al. 2001 (23) and by Cogliati et al. in 2000 (24). Boekhout et al. (23) used AFLP analysis to study 206 global isolates of C. neoformans, and grouped them into six major AFLP groups, whereas Cogliati et al. (24), using a slightly modified PCR-fingerprinting technique with the microsatellite specific primer (GACA)₄, grouped Italian isolates of C. neoformans var. grubii and var. neoformans into four major molecular types. Comparable molecular/genotypes, which where identified in the four cited independent studies, are VNI = AFLP1 = Cogliati VN6 (serotype A, var. grubii); VNII = AFLP1A (serotype A, var. grubii); VNIII = AFLP3 = Cogliati VN3 and VN4 (serotype AD, hybrid between var. grubii and var. neoformans); VNIV = AFLP2 = Cogliati VN1 (serotype D, var. neoformans); VGI = AFLP4, VGI1 = AFLP6, VGI11 = AFLP5 and VGI (all corresponding to serotypes B and C, var. gattii) (14,18,23,24).

The overall results show some clonality between isolates obtained from a certain country or even between different countries, suggesting partial clonal spread of the pathogenic yeast within South America. However, the approximately 50%, overall similarity between C. grubii isolates, with the highest being 82%, suggests that these South American isolates are more varied than those obtained in a previous study by Franzot et al. (25), in which they examined a limited number of isolates from Brazil by using less discriminatory molecular techniques (CNRE-1 RFLP analysis and URA5 sequencing). In Franzot’s study, the highest similarity was >94% between the Brazilian isolates, suggesting a higher clonality than observed in the isolates obtained from New York City studied in the same paper (25).

Interestingly, Chile and Spain share similar molecular types. Both countries have a large number of molecular type VNIII isolates (AD hybrids), 15.8% and 42.1%, respectively, although VNIV serotype D isolates were present only in Chile (26.3%). These groups are usually common in a number of European countries, such as France and Italy (26–28). However, only these two countries show two different URA5 RFLP patterns, one consisting of seven bands, indicating a hybrid between VNI, VNII, and VNIIV, and a second new hybrid URA5 RFLP pattern, consisting of six bands, indicating a hybrid between VNII and VNIIV. As a result of the IberoAmerican study, these hybrid patterns had recently been reported as part of Jackson’s honor’s thesis work (Jackson and Meyer unpub. data, 2000). The seven-band URA5 RFLP pattern was exclusively found in Spain (n=3) and Chile (n=2). These strains seem to be triploid, and cloning with subsequent sequencing of the PCR product showed that they contain three different copies of the URA5 gene. The six-band URA5 RFLP pattern found in Spain (n=5) and Chile (n=1), was also found in Mexico (n=3) and Argentina (n=2), possibly due to the presence of the molecular types VNII and VNIIV in these countries (14). These hybrid isolates are diploid at the URA5 locus and contain two different copies of the gene (Jackson and Meyer, unpub. data).

Further studies are needed to investigate the special relationship between isolates obtained from these two countries. The similarity in the molecular types obtained from Spanish and Chilean isolates provides further evidence, that the cryptococcal strains present today in South America could be introduced during the European colonization. This idea had been suggested by Franzot et al. (25) when investigating isolates obtained from Brazil. The authors argue that the pigeon (Columba livia), thought to provide a major reservoir of C. neoformans in pigeon excreta, is believed to have originated in southern Europe and northern Africa and has been dispersed worldwide by human travel (29).

Most of the cryptococcal isolates in this study were recovered from patients whose main risk factor was HIV infection. Overwhelming numbers of these isolates corresponded to the molecular type VNI, in accordance with previous findings, showing that isolates of this molecular type are the major source of infection in HIV-positive patients worldwide (18,19). This finding highlights the fact that most human cryptococcal infections are caused by C. neoformans var. grubii, serotype A (4,5). A distinct picture emerged in the group of isolates obtained from patients with no known risk factors, as most were C. neoformans var. gattii isolates (n=28), with the molecular types VGI (n=3), VGI1 (n=10), VGI11 (n=14), and VGI11 (n=1), compared to 23 isolates belonging to the overall most common molecular type, VNI (41.2%) of C. neoformans var. grubii. This finding supports the conclusion that variety gattii primarily infects immunocompetent patients as Chen et al. had found when investigating Australian isolates (30). These authors have proposed that aboriginal people living in rural areas of Australia’s Northern Territory have a higher risk of cryptococcosis because they live in close proximity to the potential natural host of C. neoformans var. gattii, the eucalyptus trees (30).

Despite the fact that isolates included in this study constituted a random sampling, the results show again that HIV infection is the most important risk factor for cryptococcosis (31). This conclusion is supported by the number of isolates recovered from HIV-positive patients (n=177), the age distribution, which peaks between 20 and 40 years of age, and the date of isolation with a peak corresponding to the 1990s.

Overall, the network of mycology laboratories established in IberoAmerica provided, for the first time, a baseline knowl-
edge of *C. neoformans* variety and molecular type distribution in the participating countries, placing the IberoAmerican isolates in the global picture of cryptococcosis.

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Dr. Meyer is the chief scientist of the Molecular Mycology Laboratory at the University of Sydney at Westmead Hospital, Sydney, Australia. His research is directed toward the phylogenetic analysis and development of molecular typing methods for epidemiologic studies of fungi; the early identification of pathogenic yeasts in pure culture and directly from clinical specimens; and molecular studies into virulence mechanisms and the pathogenicity of *Cryptococcus neoformans*.

**References**

Surgical site infections (SSIs), the second most common cause of nosocomial infection after urinary tract infections, cause approximately 17% of all hospital-acquired infections (1) and lead to increased costs and worse patient outcomes in hospital inpatients (2). The Centers for Disease Control and Prevention estimates that approximately 500,000 SSIs occur annually in the United States (3). Costs and outcomes secondary to SSIs can vary by location and surgery type. Infections in cardiac surgery have been estimated to add from US$8,200 (1982 dollars) to $42,000 (1985 dollars) to the cost of care after adjustments are made for preexisting illnesses and conditions, and these increased costs are likely attributable to excess hospital and intensive care unit stays (4–6). Overall, SSIs may result in $1–$10 billion in direct and indirect medical costs each year (3,7).

With the current trends favoring a shortened postoperative hospital stay, outpatient surgery, and same-day surgery, more SSIs are occurring after discharge from the hospital and, therefore, beyond the reach of most hospital infection control surveillance programs (8). Of all surgical procedures, 75% are now estimated to occur in the outpatient or ambulatory setting, and for those that do occur in the inpatient setting, postoperative length of stay is decreasing (9). An estimated 47% to 84% of SSIs occur after discharge; most of these are managed entirely in the outpatient setting (8,10).

Given the high costs and adverse patient outcomes associated with SSIs, quantifying the clinical and economic impact of SSIs recognized after discharge from the hospital is important. Several studies have focused on the direct medical costs borne by the hospital or insurer, but to our knowledge, no study has assessed the full societal impact of SSIs, which includes indirect costs, such as lost patient productivity and diminished functional status (11,12). Additionally, no study has addressed the costs of SSIs that arise from most of these infections which now occur in the postdischarge setting and for which patients are not readmitted to the index hospital. The magnitude of these costs might not be known if ascertainment were left solely to the index hospital’s information systems.

**Methods**

This study used a matched cohort design to compare the costs and illness of patients with an SSI to matched patients who had surgery during the same period but in whom an SSI did not develop. The study population was drawn from adult members of Harvard Vanguard Medical Associates, a 250,000-member multispecialty group practice, which at the time of the study was a staff model component of Harvard Pilgrim Health Care, a health maintenance organization. Study participants were those who had undergone a nonobstetric inpatient or outpatient operating room procedure at Brigham and Women’s Hospital from May 18, 1997, through October 31, 1998. Cases of SSI were identified prospectively by using an established method of automated medical record screening for 102 diagnostic, testing, or treatment codes that may have indicated the occurrence of an SSI in the outpatient setting (13). In addition, pharmacy records were screened for antibiotic dispensing, and claims were screened for hospital readmissions or emergency room visits pertaining to an SSI. Surgeries were identified in 2-week cycles, and a total of 38 cycles were completed. An investigator reviewed those records judged to indicate a postdischarge SSI by initial screening, using the National Nosoco-
mial Infections Surveillance criteria during the 30-day postoperative period to confirm infection (14). Patients who had an SSI that occurred during the index hospitalization were excluded. Case-patients were individually matched on surgery type, age and duration of surgical procedure in a ratio of one case-patient to two other members of the cohort.

**Questionnaire**

Participants were enrolled 5–7 weeks after surgery. All case-patients and matched pairs were mailed a 49-item questionnaire, an explanatory letter, and a consent form. The questionnaire contained three sections. The first section had questions designed to assess illness, which were taken from the National Health Interview Survey, and additional questions designed to quantify care and resource use during the 8-week postoperative period, including home visits, phone calls to practitioners, missed days from work, and family members’ missed days from work (15). The second and third sections were each designed to assess health-related quality of life by using the Medical Outcomes Study 12-Item Short-Form Health Survey (SF-12) during the 8 weeks after surgery and the 4 weeks before surgery, respectively (16). Patients were instructed to recall their overall health since surgery and their health before surgery. Patients who did not return questionnaires were followed up with phone calls and re-mailing of the survey. If they did not return the questionnaire within 90 days, they were considered nonresponders. If questionnaires were incomplete, the answers that were provided were included in the analyses. SF-12 mental and physical scores (MCS-12 and PCS-12, respectively) were normalized by using standard methods to obtain mean scores (16).

**Administrative Databases**

Four administrative databases were used to determine provider-level resource use associated with the 8 weeks after discharge from the operation that led to entry into the cohort. The Harvard Pilgrim Health Care demographic database was used to capture patient date of birth, gender, and zip code. This health maintenance organization maintains an automated administrative claims system that houses all charges from vendors, including hospitals, and outside the ambulatory-care centers. This database included the associated discharge date for index surgery, from which we calculated the 8 weeks’ postoperative time window for our analysis and from which we counted the resource utilization across all databases. This database provided all charges between the vendor or facility and the health maintenance organization, length of stay, procedure codes, diagnosis codes, and pharmacy codes for all encounters that occurred outside of the health plan. Thus, any readmission, emergency room visit, skilled nursing facility stay, or home health aide charge appeared in this database.

In addition, Harvard Pilgrim Health Care maintained an automated ambulatory medical record system that captured all ambulatory encounters and orders at its health centers. This database allowed determination of the number of outpatient visits, telephone calls, and most laboratory tests. This database also captures the number of inpatient physician encounters made by the health maintenance organization’s patients. Costs associated with outpatient visits at the health centers were imputed by using the costs for CPT Codes 99213–99215 from the 1998 National Physician Fee Schedule Relative Value File (available from: URL: http://www.hcfa.gov/stats/pufiles.htm). The first visit for each case-patient with an SSI was assumed to be an established-patient visit lasting 40 minutes (CPT 99215), and the first visit for those without an SSI was assumed to last 25 minutes (CPT 99214). All subsequent visits for all patients were assumed to last 15 minutes (CPT 99213). Costs in 1998 for CPT codes 99213, 99214, and 99215 were $41.46, $62.74, and $99.06, respectively.

Harvard Pilgrim Health Care also maintains a database that captures all pharmacy prescriptions dispensed in the outpatient setting (17). This database provided the standard wholesale costs for all antibiotic prescriptions for the 8-week postoperative period.

Chronic disease scores, as a marker for patient preexisting conditions and illnesses, have been shown to be predictors of SSI and also of death, hospitalization, and resource utilization (18–21). The chronic disease score, as used here, is a method for controlling for preexisting conditions on the basis of patient age, gender, and recent history of drug dispensing. This score predicts for hospitalization (22) and SSI (19) and thus would appear to be a useful adjuster for preexisting conditions in our cost analysis. For each patient, a chronic disease score was created by using patient age, sex, and presence or absence of 29 chronic diseases, calculated from the 6-month preoperative ambulatory pharmacy dispensing record (18,19).

Attributable charges of SSI recognized after discharge were calculated by taking the mean charges of case-patients and subtracting the mean charges of control patients. Mean charges were chosen for this comparison since the use of medians would negate the effect that even a moderately rare event (those that occur in <50% of the study population) would have on health-care costs. For those areas of resource utilization in which only charges were available, charges were converted to costs by using a cost-to-charges ratio. Since this study involved readmission and resource utilization at several different hospitals, conversion to costs would have required institution-specific ratios of costs to charge, to which we did not have access. We have, therefore, chosen to use a published ratio of costs to charges from a cohort of 4,108 patients admitted in the same city to two hospitals, one of which was the index hospital in this study, and during a similar period to this study (23).

**Statistics**

Student t test, Wilcoxon rank-sum test, or Fisher exact test were used, where appropriate, for univariate comparisons. Outcomes are presented as medians with interquartile range, means with standard deviations, or proportions. Cases and matched controls were compared by using the Wilcoxon
signed-ranks test for continuous outcomes with non-normal distributions, continuous linear regression by forcing the matching variable into the model for normally distributed variables, or the Cochran-Mantel-Haenszel for matched binary variables. Almost all assessed utilization outcomes, including all charges, were non-normally distributed so both medians with interquartile range and means with standard deviation are reported. Multivariable unconditional logistic regression was used to control for confounding variables in the analysis of the questionnaire data, and all matched variables were forced into the model to account for the matching process.

Since combined total costs and charges (ambulatory, pharmacy, and nonambulatory) of the entire cohort of 267 patients were log-normally distributed, the total cost variable was analyzed by using a log-transformation of total costs in a matched linear regression model. To estimate the effect that preexisting conditions or index surgery duration might have on the attributable effect of SSI on total costs, a matched linear regression with log-transformed total costs as the outcome was created with the predictors SSI/no SSI, chronic disease score (CDS), and index surgery duration entered as variables into the model. Results are given as β-estimates of effect, R-square statistic, and p value for five models (only SSI versus no SSI; only CDS; both SSI versus no SSI and CDS; both SSI versus no SSI and index surgery duration; and all three variables: SSI, CDS, and duration of index surgery). All statistical tests were two-tailed; p < 0.05 was considered statistically significant. Statistical analyses were performed with SAS v 8.01 for Windows (SAS Institute, Inc., Cary, NC).

During the anticipated study period, 3,000 surgeries would be estimated to be performed and, given a 2.8% risk for infection beginning after discharge from the hospital (based on our prior observations), 84 SSIs would be recognized after discharge. This gave a power of 0.89 to detect >5 days lost from usual activities. Our actual sample of SSIs recognized after discharge was 89 (1.9%) from a sample of 4,571 procedures.

All data collected were combined into one dataset for final analysis, after which all unique identifiers were removed. In addition, each patient provided a signed consent form before completing the questionnaire and being enrolled in the study. The Harvard Pilgrim Health Care institutional review board approved this study.

Results

SSI recognized after discharge was confirmed in 89 (1.9%) of 4,571 procedures. One hundred seventy-eight patients with similar age, procedure types, and surgical duration were matched to the SSI patients in a ratio of one case-patient to two controls (Table 1). No significant differences in age, gender, or surgery type between case-patients and matched controls were noted. Surgery duration was significantly longer for SSI patients, despite having been matched for procedure duration. This was expected because procedure duration is an important risk factor for infection.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case-patients N (% or SDa)</th>
<th>Controls N (% or SD)</th>
<th>p value</th>
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<td><strong>Demographics of complete cohort</strong></td>
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<tr>
<td>Age (yr)</td>
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<td>57.5 (+/-13.3)</td>
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<td>Male gender</td>
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<td>30.8%</td>
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<td>Homemaker</td>
<td>29.8%</td>
<td>28.2%</td>
<td>0.85c</td>
</tr>
<tr>
<td>Retired</td>
<td>42.9%</td>
<td>61.5%</td>
<td>0.07c</td>
</tr>
<tr>
<td>Student</td>
<td>2.1%</td>
<td>2.5%</td>
<td>1.0g</td>
</tr>
</tbody>
</table>

Preexisting medical conditions

Congestive heart failure 12.2% 2.5% 0.018c
Diabetes 24.5% 11.5% 0.057c
Arthritis 38.8% 21.5% 0.034c

Table 1. Descriptive characteristics of cohort in study of surgical site infections (SSI), Harvard Pilgrim Health Care, 1997–1998a

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aResults are shown as no. (%) or mean +/- standard deviation, along with p value for comparison of cases with SSIs to controls without SSIs.

bStudent t test.
cFisher exact test.
dWilcoxon rank-sum test.
eThirteen additional preexisting conditions were assessed, including chronic lung disease, vision or hearing impairment, asthma, peptic ulcer disease, chronic back pain, hypertension, angina, myocardial infarction, stroke, kidney disease, and cancer; all were not significantly different between cases and controls with p>0.05.
Impact on Health, Activities, and Perceived Care Needs

One hundred seventy-three (65%) of 267 questionnaires were returned. Those who completed the questionnaire (responders) were slightly older than those that did not respond (58.2 years vs. 54.6 years, p=0.05). No other differences between questionnaire responders and nonresponders were significant (Tables 2 and 3). Among patients who completed the questionnaire, no differences between case-patients and controls were significant for age, sex, and procedure types (Table 1), or in the baseline SF-12 assessment of mental and physical health (Table 3). Reported occupations of patients and controls did not differ, and few differences between case-patients and controls existed with respect to self-declared differences in pre-existing medical conditions (Table 1). Case-patients did experience longer duration of surgery than did controls. Case-patients were also more likely than controls to report a history of congestive heart failure (12% vs. 2.5%, p=0.02) and arthritis (39% vs. 22%, p=0.03). There was a trend towards more case-patients having diabetes than controls (24% vs. 12% p=0.06).

In assessing time and productivity costs, we found that case-patients (64%) were more likely than controls (42%) to have spent at least 1/2 day in bed, thus missing planned regular activities (p=0.04). However, differences between case-patients and controls in other areas of lost productivity, such as missed days of work and inability to complete regular activities, were not significant.

Case-patients with an SSI (69%) were more likely than controls (48%) to require home health provider visits (p=0.01). Similar results were found after controlling for age, procedure duration, and baseline SF-12 physical function. There were trends for patients with SSI wanting more home health visits than were provided and wanting a 24-hour hotline to contact a health-care practitioner. Patients, but not controls, reported significantly lower physical health and mental health component scores on the SF-12 after surgery, compared to their own baselines (p=0.003 and p=0.02, respectively).

Health Resource Use in 8 Weeks after Surgery

Patients with SSI recognized after discharge required significantly more resources within the outpatient setting than those without SSI (Table 4). Significantly more patients with SSI had at least one ambulatory-care visit, and their average number of visits (7.5) was more than twice the average of those without SSI (3.4). Additionally, case-patients were significantly more likely to call their provider and to make more phone calls to their provider than controls. The number of laboratory tests ordered did not differ between cases and controls. Estimated ambulatory outpatient visits costs generated were on average $365 per case with an SSI and $160 per control during the 8-week postoperative period (p<0.001).

Patients with an SSI recognized after discharge also used significantly more resources outside of the ambulatory-care centers. More case-patients (31%) had at least one visit to an emergency room compared to controls (9%), p<0.001, and they generated significantly more emergency room charges ($333 vs. $114, p<0.001).

Those with SSI were more likely to require a radiology test (40% vs. 28%, p=0.02) and had higher radiology test charges ($1,076 vs. $587, p=0.02) than those without SSI. More patients with an SSI received durable medical equipment than did controls (37% vs. 22%, p=0.008) and generated higher average durable medical equipment–related charges ($1,23 vs. $69, p=0.01). A greater proportion of case-patients (62%) than controls (47%) required home health services (p=0.009). Charges related to home health services were higher for those with an SSI ($827) than for those without an SSI ($579), p=0.007. Twice as many case-patients required a stay in a skilled nursing facility (9% vs. 4.5%, p=0.09). There was a nonsignificant trend towards higher average skilled nursing charges for case-patients ($460 vs. $204 p=0.14); however, the average number of days in a skilled nursing facility was the same for case-patients and controls.

Patients with an SSI recognized after discharge generated higher standard wholesale costs for antibiotics than did controls without an SSI. Case-patients had an average cost of $60 for antibiotics, while controls had costs of $13.60 per person (p<0.001). Patients with an SSI were more likely to be readmitted to the hospital (34%) than those without an SSI (12%), p<0.001. These readmissions led to $7,925 charges per person with an SSI compared with charges of $2,079 for those without an SSI (p<0.001). After the conversion of charges to costs, an SSI diagnosed after discharge was associated with excess costs of $2,573 ($3,489 minus $916) from rehospital-
as expected on the basis of the higher prevalence of selected
among case-patients (3,058) than controls (2,148) (p=0.005),
weeks after discharge from the hospital associated with the
regardless of readmission status.
ization across the entire population who developed an SSI,
room visits occurred at institutions other than the index hospi-
tal; such visits and admissions would not have been captured
by an infection control surveillance system limited to
had on average $307 additional costs that would not have been
SSI ($3,382), we found that the mean charge manifest outside
of the inpatient hospital setting attributable to SSI recognized
beyond the cost accounting systems of most index hospitals in
which the initial surgical procedure was performed. This 24%
would be the minimum fraction of the costs missed if all read-
ations occurred at the index hospital. In our study, 23% of
chronic diseases in those at risk for an SSI. To determine if
preexisting conditions could account for some of the costs
associated with SSI recognized after discharge, we used a
matched linear regression model; the calculated chronic dis-
ease score was the predictor for log-transformed total costs
(Table 5). Although the chronic disease score was a strong
independent predictor of postoperative resource use, even in
this matched cohort, it was not a meaningful confounder of the
impact of SSI on resource utilization. The parameter estimate
for being a case was 1.30 for log-transformed costs in the
unadjusted model and 1.20 for log-transformed costs in the
adjusted model when chronic disease score was included. This
finding suggests that, even after preexisting conditions are
adjusted for, SSIs recognized after hospital discharge are sig-
ificantly associated with higher total costs.

Even though we matched case-patients and controls on
duration of index surgery, patients with SSI recognized after
hospital discharge had significantly longer duration of surgery.
To measure if duration of index surgery could confound the
total attributable costs of SSI recognized after hospital dis-
charge, we used a matched linear regression model with dura-
tion of index surgery and SSI as predictors for log-transformed
total costs. The addition of duration of index surgery into the
model did not significantly confound the attributable impact
that SSI had on higher total costs (Table 5).

### Discussion

SSIs recognized after discharge from the hospital were
associated with significantly higher direct medical costs and
indirect costs. With respect to direct medical costs, SSIs diag-
nosed after hospital discharge incurred significantly more
attributable use of resources than matched controls in each of
the following categories: outpatient visits, inpatient care, phar-
cy, radiology, home health aide care, and durable medical
equipment. When all sources of direct medical costs were
combined, SSIs recognized after discharge were associated
with $3,382 in excess costs over those without SSI. This dif-
ference was significant after preexisting conditions and index
surgery duration were controlled for. Importantly, in the linear
regression models (Table 5), SSIs recognized after discharge
explained one-half the variation in total costs (R²=0.49), and
this finding was not altered by the addition of chronic disease
score or index surgery duration.

Direct medical costs have been postulated to be low in
patients who do not require readmission after a postdischarge
SSI has developed (10). When readmission costs attributable
to SSI ($2,573) were subtracted from total costs attributable
to SSI ($3,382), we found that the mean charge manifest outside
of the inpatient hospital setting attributable to SSI recognized
after discharge was $809. Therefore, 24% of costs attributable
to the SSI recognized after discharge would typically occur
beyond the cost accounting systems of most index hospitals in
which the initial surgical procedure was performed. This 24%
would be the minimum fraction of the costs missed if all read-
missions occurred at the index hospital. In our study, 23% of

### Table 3. Univariate analysis of questionnaire respondents, surgical
site infections (SSIs) study

<table>
<thead>
<tr>
<th></th>
<th>Case-patient (N=50)</th>
<th>Control (N=123)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRQOL with SF-12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoperative MCS-12</td>
<td>51.7 (+/-9.6)</td>
<td>51.5 (+/-9.9)</td>
<td>0.96b</td>
</tr>
<tr>
<td>Postoperative MCS-12</td>
<td>47.6 (11.6)</td>
<td>52.4 (+/-9.2)</td>
<td>0.025b</td>
</tr>
<tr>
<td>Preoperative PCS-12</td>
<td>41.1 (+/-12.7)</td>
<td>45.0 (+/-10.9)</td>
<td>0.058b</td>
</tr>
<tr>
<td>Postoperative PCS-12</td>
<td>33.9 (+/-10.0)</td>
<td>38.7 (+/-9.8)</td>
<td>0.003b</td>
</tr>
<tr>
<td>Change MCS-12 with surgery</td>
<td>-4.1 (+/-11.0)</td>
<td>0.9 (+/-9.6)</td>
<td>0.004b</td>
</tr>
<tr>
<td>Change PCS-12 with surgery</td>
<td>-7.2 (+/-10.6)</td>
<td>-6.3 (+/-13.3)</td>
<td>0.67b</td>
</tr>
<tr>
<td>Additional questions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time and productivity costs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>If employed, missed work</td>
<td>66.7%</td>
<td>62.3%</td>
<td>0.81c</td>
</tr>
<tr>
<td>Average no. missed days at work</td>
<td>61.2 (+/-38.6)</td>
<td>57.5 (+/-40.6)</td>
<td>0.95c</td>
</tr>
<tr>
<td>Unable to do regular activities</td>
<td>60.6%</td>
<td>69.5%</td>
<td>0.39c</td>
</tr>
<tr>
<td>Missed activities, in bed &gt;1/2 day</td>
<td>63.6%</td>
<td>41.8%</td>
<td>0.043c</td>
</tr>
<tr>
<td>Average no. days missed activities</td>
<td>49.6 (+/-41.3)</td>
<td>50.1 (+/-42.0)</td>
<td>0.90d</td>
</tr>
<tr>
<td>Additional costs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Provider made home visits</td>
<td>69.4%</td>
<td>47.5%</td>
<td>0.011c</td>
</tr>
<tr>
<td>Could have used home visits</td>
<td>30.8%</td>
<td>12.8%</td>
<td>0.068c</td>
</tr>
<tr>
<td>Used paid housekeeper</td>
<td>6.3%</td>
<td>5.8%</td>
<td>1.06c</td>
</tr>
<tr>
<td>Used 24-hr hotline</td>
<td>12.2%</td>
<td>5.7%</td>
<td>0.20c</td>
</tr>
<tr>
<td>Could have used 24-hr hotline</td>
<td>21.4%</td>
<td>8.9%</td>
<td>0.052c</td>
</tr>
</tbody>
</table>

aResults are shown as mean (+/- SD) or % of total responders, along with p value for comparison of cases with SSIs to controls without SSIs. Abbreviations used: HRQOL, Health Related Quality of Life; SF-12, Medical Outcomes Study 12-Item Short-Form Health Survey; MCS, Mental Health Component Score of SF-12; PCS, Physical Health Component Score of SF-12.
bStudent t test.
cFisher exact test.
dWilcoxon rank-sum test.
the readmissions occurred at settings other than the index hospital. Therefore, approximately $1,409 (42%) of all costs attributable to SSI were unknown to the index hospital. Kirkland et al. found that patients with an SSI had an increased risk of readmission and death associated with SSIs recognized during the initial hospitalization (11). No patients in our study died during the 8-week postdischarge follow-up period.

The matched cohort-design has been associated with selection bias when stringent matching criteria prevent some cases of SSI from being included in the study analysis (24,25). Selection bias was not a factor in this study because all cases of SSI were included.

We recognize that we were unable to assess all societal costs of SSI, such as individual patient transportation costs.

### Table 4. Univariate analysis of 8-week postoperative resource utilization, surgical site infections (SSIs) study

<table>
<thead>
<tr>
<th></th>
<th>Cases N=89</th>
<th>Controls N=178</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medians</td>
<td>Means</td>
<td>Medians</td>
</tr>
<tr>
<td><strong>Outpatient visit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Required outpatient visit</td>
<td>85 (96)</td>
<td>153 (86)</td>
<td>&lt;0.001b</td>
</tr>
<tr>
<td>Outpatient visits per patient</td>
<td>5 [4, 9]</td>
<td>7.5 (+/-6.3)</td>
<td>3 [1, 5]</td>
</tr>
<tr>
<td>Estimated outpatient visit costs</td>
<td>$265 [$223, $430]</td>
<td>$365 (+/-264)</td>
<td>$146 [$63, $229]</td>
</tr>
<tr>
<td>Lab test ordered by provider</td>
<td>69 (78)</td>
<td>143 (80)</td>
<td>0.66b</td>
</tr>
<tr>
<td>No. of lab tests ordered</td>
<td>1 [1, 3]</td>
<td>2.1 (+/-2.5)</td>
<td>1 [1, 2]</td>
</tr>
<tr>
<td>Patient phoned provider</td>
<td>77 (87)</td>
<td>125 (70)</td>
<td>0.002b</td>
</tr>
<tr>
<td>No. of phone calls made</td>
<td>3 [2, 6]</td>
<td>4.7 (+/-4.8)</td>
<td>1 [0, 4]</td>
</tr>
<tr>
<td><strong>Pharmacy use</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard wholesale costs per patient</td>
<td>$34.2 [$78.6, 10.6]</td>
<td>$60 (+/-71.6)</td>
<td>$0 [50, 50]</td>
</tr>
<tr>
<td><strong>Emergency room use</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient visits to emergency room</td>
<td>28 (31)</td>
<td>16 (9)</td>
<td>&lt;0.001b</td>
</tr>
<tr>
<td>Emergency room charges per patient</td>
<td>$0 [0, $370]</td>
<td>$333 (+/-729)</td>
<td>$0 [50, 50]</td>
</tr>
<tr>
<td><strong>Radiology services use</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients who had a radiologic test</td>
<td>36 (40)</td>
<td>49 (28)</td>
<td>0.023b</td>
</tr>
<tr>
<td>Radiology charges per patient</td>
<td>$0 [0, $242]</td>
<td>$1,076 (+/-3,845)</td>
<td>$0 [50, 124]</td>
</tr>
<tr>
<td><strong>Rehospitalization</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients rehospitalized</td>
<td>30 (34)</td>
<td>21 (12)</td>
<td>&lt;0.001b</td>
</tr>
<tr>
<td>Total rehospitalization charges</td>
<td>$0 [50, $4,370]</td>
<td>$7,925 (+/-22,321)</td>
<td>$0 [50, 50]</td>
</tr>
<tr>
<td>Total rehospitalization costs</td>
<td>$0 [50, $1,924]</td>
<td>$3,489 (+/-9,827)</td>
<td>$0 [50, 50]</td>
</tr>
<tr>
<td>Visited by provider in hospital</td>
<td>46 (52)</td>
<td>61 (34)</td>
<td>0.008b</td>
</tr>
<tr>
<td>Inpatient provider visits</td>
<td>1 [0, 6]</td>
<td>3.5 (+/-4.5)</td>
<td>0 [0, 3]</td>
</tr>
<tr>
<td><strong>Skilled nursing facility use</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skilled nursing facility used</td>
<td>8 (9)</td>
<td>8 (4.5)</td>
<td>0.09b</td>
</tr>
<tr>
<td>Days in skilled nursing facility</td>
<td>0 [0, 0]</td>
<td>0.21 (+/-0.83)</td>
<td>0 [0, 0]</td>
</tr>
<tr>
<td>Skilled nursing charges per patient</td>
<td>$0 [50, 50]</td>
<td>$460 (+/-2,198)</td>
<td>$0 [50, 50]</td>
</tr>
<tr>
<td><strong>Home health aide use</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Home health aide used</td>
<td>55 (62)</td>
<td>84 (47)</td>
<td>0.009b</td>
</tr>
<tr>
<td>Home health charges per patient</td>
<td>$110 [50, $605]</td>
<td>$827 (+/-1,765)</td>
<td>$0 [50, $275]</td>
</tr>
<tr>
<td><strong>Durable equipment use</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Durable medical equipment used</td>
<td>33 (37)</td>
<td>39 (22)</td>
<td>0.008b</td>
</tr>
<tr>
<td>Durable medical charges per patient</td>
<td>$0 [50, $102]</td>
<td>$123 (+/-436)</td>
<td>$0 [50, 50]</td>
</tr>
<tr>
<td>Total costsd</td>
<td>$1,240 [$445, $594]</td>
<td>$5,155 (+/-10,8570)</td>
<td>$300 [$146, $795]</td>
</tr>
</tbody>
</table>

aResults are shown as no. (%), mean (+/- standard deviation) or median [interquartile range] along with p value for comparison of cases with SSI to controls without SSI.  
bCohran-Mantel-Haenszel.  
c Wilcoxon signed-ranks test.  
dTotal costs encompass all emergency, radiology, readmission, skilled nursing, home health, and durable medical charges that have been converted to costs with a cost-to-charge ratio and all estimated outpatient visit and antibiotic costs.
However, in addition to the direct medical costs, we found that patients with SSI recognized after discharge had a significant decline in the mental health component of the SF-12. The magnitude of this drop, compared to results for controls, was significant. Patients with SSI recognized after discharge had a significant drop in the mental health component of the SF-12. These SSIs also incurred substantial excess length of hospitalization and extra costs. Infect Control Hosp Epidemiol 1997;18:54–8.

We conclude that SSIs diagnosed after hospital discharge were associated with significant impairment of physical and mental health. These SSIs also incurred substantial excess resource utilization across the spectrum of health care. These findings support the need to prevent SSIs that occur after discharge.

Funded by a grant from the Harvard Pilgrim Health Care Foundation and by the Centers for Disease Control and Prevention Eastern Massachusetts Prevention Epicenter cooperative agreement UR8/CCU115079.

Dr. Perencevich is an assistant professor in the Department of Epidemiology and Preventive Medicine, Division of Healthcare Outcomes Research at the University of Maryland, Baltimore. His research interests include the study of nosocomial infections and patient-to-patient transmission of resistant bacteria using mathematical models.

Table 5. Results of five separate matched linear regression models with log-transformed total costs as the outcome variable, surgical sites infection (SSI) study

<table>
<thead>
<tr>
<th>Model no.</th>
<th>Predictor variable</th>
<th>β parameter estimate</th>
<th>Standard error</th>
<th>p value</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SSI (case)</td>
<td>1.30</td>
<td>0.21</td>
<td>&lt;0.001</td>
<td>0.492</td>
</tr>
<tr>
<td>2</td>
<td>Chronic disease score</td>
<td>0.00018</td>
<td>0.00006</td>
<td>0.002</td>
<td>0.095</td>
</tr>
<tr>
<td>3</td>
<td>SSI (case)</td>
<td>1.20</td>
<td>0.21</td>
<td>&lt;0.001</td>
<td>0.507</td>
</tr>
<tr>
<td>4</td>
<td>Chronic disease score</td>
<td>0.00012</td>
<td>0.00005</td>
<td>0.03</td>
<td>0.514</td>
</tr>
<tr>
<td>5</td>
<td>SSI (case)</td>
<td>1.27</td>
<td>0.22</td>
<td>&lt;0.001</td>
<td>0.499</td>
</tr>
<tr>
<td></td>
<td>Index surgery duration</td>
<td>0.0017</td>
<td>0.0017</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chronic disease score</td>
<td>0.0001</td>
<td>0.00005</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Index surgery duration</td>
<td>0.0018</td>
<td>0.0017</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

References


27. Guadagnoli E, Cleary PD. How consistent is patient-reported pre-admission health status when collected during and after hospital stay? Med Care 1995;33:106–12.

Address for correspondence: Eli Perencevich, Division of Healthcare Outcomes Research, Department of Epidemiology and Preventive Medicine, University of Maryland, Baltimore, 10 N. Greene St., (BT111), V55D-150, Baltimore, MD 21201, USA; fax: 410-605-7914; e-mail:eperence@epi.umaryland.edu
Applying Network Theory to Epidemics: Control Measures for Mycoplasma pneumoniae Outbreaks

Lauren Ancel Meyers,*† M.E.J. Newman,*‡ Michael Martin,§ and Stephanie Schrag§

We introduce a novel mathematical approach to investigating the spread and control of communicable infections in closed communities. *Mycoplasma pneumoniae* is a major cause of bacterial pneumonia in the United States. Outbreaks of illness attributable to mycoplasma commonly occur in closed or semi-closed communities. These outbreaks are difficult to contain because of delays in outbreak detection, the long incubation period of the bacterium, and an incomplete understanding of the effectiveness of infection control strategies. Our model explicitly captures the patterns of interactions among patients and caregivers in an institution with multiple wards. Analysis of this contact network predicts that, despite the relatively low prevalence of mycoplasma pneumonia found among caregivers, the patterns of caregiver activity and the extent to which they are protected against infection may be fundamental to the control and prevention of mycoplasma outbreaks. In particular, the most effective interventions are those that reduce the diversity of interactions between caregivers and patients.

Mathematical modeling has a rich and growing tradition in epidemiology (1–3). Because experimental approaches to epidemic interventions are often impractical, and in some cases unethical, mathematical models can provide otherwise unobtainable insights on the spread and control of disease. Recently, considerable interest has been shown in the effect of contact networks on the spread of disease, and particularly in using the so-called percolation theory to model epidemics (4–10). Agent-based simulation is also being used increasingly to help epidemiologic investigations (11). In this paper, we use both of these tools to assess the effects of epidemic interventions in closed health-care facilities.

*Mycoplasma pneumoniae* is a major cause of bacterial pneumonia in the United States (12). This bacterium, the smallest self-replicating organism capable of cell-free existence, is spread both by direct contact between an infected person and a susceptible person, and by airborne droplets expelled when an infected person sneezes, coughs, or talks. Large, sustained outbreaks of *M. pneumoniae* have occurred in closed and semi-closed populations such as hospitals, psychiatric institutions, military and religious communities, and prisons (13–15). Public health officials and health-care providers struggle, often with little success, to control mycoplasma outbreaks because of the long incubation period of the organism, late detection of outbreaks, and an incomplete understanding of the effectiveness of various infection control strategies.

Effective measures to control mycoplasma outbreaks are needed to limit the associated illness and substantial costs. Previous work has addressed candidate strategies, including infection control practices to prevent the exchange of respiratory droplets between patients and caregivers, cohorting members of the community who display symptoms of a respiratory infection, and antibiotic prophylaxis of asymptomatic members of the community (14–16). The costs of these strategies include curtailed social interactions because of cohorting, undesirable side effects or allergic reactions to prophylactic antibiotics, and a potential increase in the risk for infections caused by antibiotic-resistant bacteria. Studies of these control measures have been limited by incomplete information and participation.

Using a network model approach, we show how data on interactions in real-world communities can be translated into graphs—mathematical representations of networks—and how to predict the course of an epidemic from the structure of a graph. We found that the assignment of caregivers to patient groups is more critical to the course of an epidemic than the cohorting of patients. Within our models, the most effective interventions are those that reduce the diversity of interactions that caregivers have with patients. For example, an institution with many wards can avoid a large outbreak by confining caregivers to work in only one or very few wards.

The Model

Here we model an institution with spatially disjointed wards. Patients are confined to a single ward, and caregivers work in one or more wards. Each person or ward is represented by a “vertex” in the graph. “Edges” connect people to the wards in which they reside or work. Figure 1 shows the graph for an institution with four wards, each with three or four patients and two to four caregivers.

A key property of graphs is their degree distribution. The degree of a vertex is the number of other vertices to which it is connected. In Figure 1, for example, the degree of all patients is one; the degree of each caregiver ranges from one to four;
and the degree of the wards ranges from six to seven, indicating the number of inhabitants and caregivers working there. Direct transmission of *M. pneumoniae* can only occur between two vertices if an edge connects them.

Throughout this model, we allow transmission to occur between people and places. We do not mean that bacteria actually infect a space by residing on inanimate objects or in the air. Rather, we mean that the person has transmitted the bacteria to another person who resides or works in that place. Conversely, when a place transmits to a person, we mean that the bacterium is transmitted to an uninfected person living or working in that place.

We begin by considering only the caregivers and wards. Later we add the patients to the model. (All notations are defined in the Table.) A probability generating function (pgf) is a mathematical quantity that describes a probability distribution, and thereby summarizes a large amount of useful information about the network architecture. We can define pgfs that capture the distribution of the number of wards assigned to each caregiver and the distribution of the number of caregivers working in each ward.

Pgfs can be mathematically manipulated to give many useful results. For example, the derivative gives the average of the distribution, e.g., the mean number of wards assigned to a caregiver, or the mean number of caregivers working in a ward. We can also answer the following question using pgfs: If an infected caregiver exposes a ward, how many other caregivers,

<table>
<thead>
<tr>
<th>Notation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$W$</td>
<td>Number of wards in the facility</td>
</tr>
<tr>
<td>$C$</td>
<td>Number of caregivers working in the facility</td>
</tr>
<tr>
<td>$\mu_w$</td>
<td>Average no. of caregivers working in a ward</td>
</tr>
<tr>
<td>$\mu_c$</td>
<td>Average no. of wards in which a caregiver works</td>
</tr>
<tr>
<td>$r$</td>
<td>Probability that a given caregiver works in a given ward</td>
</tr>
<tr>
<td>$p_k$</td>
<td>Probability that a caregiver works in $k$ wards</td>
</tr>
<tr>
<td>$q_k$</td>
<td>Probability that a ward has $k$ caregivers working in it</td>
</tr>
<tr>
<td>$f_0(x)$</td>
<td>Probability generating function (pgf) for the degree distribution of caregivers</td>
</tr>
<tr>
<td>$g_0(x)$</td>
<td>pgf for the degree distribution of wards</td>
</tr>
<tr>
<td>$f_1(x)$</td>
<td>First select a random ward, and then select a random caregiver working there. This expression represents the pgf for the number of other wards in which that caregiver works.</td>
</tr>
<tr>
<td>$g_1(x)$</td>
<td>First select a random caregiver, and then select a random ward associated with that caregiver. This expression represents the pgf for the number of other caregivers working in that ward.</td>
</tr>
<tr>
<td>$\tau_w$</td>
<td>Probability of transmission from a ward to a caregiver</td>
</tr>
<tr>
<td>$\tau_c$</td>
<td>Probability of transmission from a caregiver to a ward</td>
</tr>
<tr>
<td>$\Phi_0(x)$</td>
<td>pgf for the number of wards affected by transmission from a random caregiver</td>
</tr>
<tr>
<td>$\Phi_1(x)$</td>
<td>First select a random ward and assume that it is affected by the bacterium, then select a random caregiver working there. This expression represents the pgf for the number of other wards affected by that caregiver.</td>
</tr>
<tr>
<td>$\Gamma_0(x)$</td>
<td>pgf for the number of caregivers affected by transmission from a random ward</td>
</tr>
<tr>
<td>$\Gamma_1(x)$</td>
<td>First select a random caregiver and assume he/she is infected, then select a random ward in which that caregiver works. This expression represents the pgf for the number of other caregivers infected by individuals working/living in that ward.</td>
</tr>
<tr>
<td>$\langle s \rangle$</td>
<td>Average number of wards affected in an outbreak</td>
</tr>
<tr>
<td>$1-S_c$</td>
<td>The size of the caregiver giant component—the largest set of infected caregivers that are all connected through work in common wards</td>
</tr>
<tr>
<td>$1-S_w$</td>
<td>The size of the ward giant component—the largest set of affected wards that are all connected through common caregivers</td>
</tr>
<tr>
<td>$\beta_w(x)$</td>
<td>pgf for the number of patients in affected ward $w$ who contract the bacterium</td>
</tr>
<tr>
<td>$B(x)$</td>
<td>pgf for the total number of patients in the facility who are infected during an epidemic</td>
</tr>
</tbody>
</table>
on average, will be vulnerable to infection because they also work in that ward? Appendix A (online only) defines our pgfs and describes the derivations that answer this question.

Transmission through the Graph

Transmission of M. pneumoniae occurs when people occupy the same physical space for some period of time. Therefore, in our model, transmission can occur between persons if the vertices representing them are connected to the same ward.

We derive two complementary estimates for the size of an outbreak. The first is appropriate for conditions not conducive to large outbreaks, such as a pathogen with low transmissibility, or an institution with few interpersonal interactions. The second applies to conditions that favor large outbreaks.

Transmission through the Graph

We begin with two questions. If a healthy caregiver works in an infected ward, how many other wards will eventually become infected as a result of that caregiver’s interaction with that ward? Similarly, if an infected caregiver works in a yet uninfected ward, how many other caregivers will eventually become infected as a result of that caregiver’s activity in that ward? Answers to these questions vary from ward to ward and from caregiver to caregiver. Therefore, we calculate probability distributions for the spread, which we represent by using pgfs.

First, consider an edge linking an infected ward to a caregiver. Figure 2 breaks down the possible scenarios. First, the caregiver may not become infected. Second, the caregiver might become infected but not transmit to any other wards. Third, the caregiver might transmit infection to one or more other wards in which he or she works. In Appendix B (online only), we construct a pgf by summing up the probabilities of these different outcomes.

Next, we start with an edge from an infected caregiver to a ward. As shown in Figure 3, there may be no transmission along the edge in question to the ward, no further transmission from the ward to other people, or transmission to one or more other people who spend time in the ward.

With these two pgfs, we derive the average size of a small outbreak, starting from a single infection:

\[
\langle s \rangle = 1 + \frac{\tau_c \tau_w f_w'(1) g_c'(1)}{1 - \tau_c \tau_w f_w'(1) g_c'(1)} [1]
\]

where \( f' \) denotes the first derivative of \( f \) with respect to its argument. Thus, the average size of the outbreak is 1 (the original patient) plus a function of the two transmission rates (from caregivers to wards, \( \tau_c \), and from wards to caregivers, \( \tau_w \)), and the average number of wards assigned to a caregiver \( \langle g_c'(1) \rangle \).

The term \( f_w'(1) \) assumes that we choose any ward at random from the entire network, then choose one of the edges connected to that ward at random, then follow that edge to a caregiver, and finally calculate the number of other wards assigned to the caregiver. On average, that will be \( f_w'(1) \). Likewise \( g_c'(1) \) is the average number of other caregivers working in a ward that we reach by first choosing a caregiver at random and then randomly choosing one of the wards in which the caregiver works. These terms contain information not only about the average degrees of caregivers and wards but also about the probability that a given caregiver or node will become infected in the first place.

The expression for \( \langle s \rangle \) diverges when

\[
\tau_c \tau_w f_w'(1) g_c'(1) = 1.
\]

This expression represents the transition between a regime in which only small isolated outbreaks of disease can occur and one in which a full-blown community-wide epidemic can occur. A community will cross that transition point if transmission rates are sufficiently high (\( \tau_c \) and \( \tau_w \)) or the interactions among wards and caregivers are sufficiently dense (\( f_w'(1) \) and \( g_c'(1) \)). Equation no. 1 provides an estimate of the epidemic size below the threshold only. It is based on the assumption that interactions are rare enough that a person or a place only encounters the infection once. When interactions are more common and the community lies above the epidemic transition, we must use a different estimate for the size of the outbreak.

The “giant component” of the graph is the largest connected set of vertices that have all been infected. The size of the outbreak above the epidemic transition is exactly equal to the number of vertices in this giant component. We calculate the size of the giant component (the number of caregivers affected) by calculating the fraction of vertices not contained in it:

\[
1 - S_c = \Phi_c(1), \quad [3]
\]

where \( \Phi_c(1) \) is the probability that an infected caregiver will produce no further infections (Appendix B, online only). A similar expression describes the number of wards affected in an epidemic:

\[
1 - S_w = \Gamma_w(1). \quad [4]
\]

These expressions reflect both the fraction of the population infected and the probability that an outbreak will reach epidemic proportions in the first place. Since \( S_c \) and \( S_w \) are often much less than 1, not all outbreaks turn into epidemics, even above the epidemic transition.

Degree Distributions

Equation nos. 3 and 4 allow us to estimate the size of an epidemic on the basis of transmission probabilities and the degree distribution of caregivers to wards. To make specific numerical predictions, we must first calculate pgfs for the
degree distributions. Here we make the simple assumption that the degree distributions follow a Poisson distribution for both the number of wards associated with a given caregiver and the number of caregivers associated with a given ward. This assumption is equivalent to requiring that all caregivers have an equal likelihood of working in any ward and that a caregiver is assigned to any given ward independent of his or her other ward assignments. In the absence of more specific information about assignment to wards, this assumption seems a reasonable first step. This distribution assumes an infinite population and is generally applied to very large populations. Although perhaps not the ideal model for small institutions, this distribution is used here because it yields pgfs with convenient mathematical properties (see Appendix C, online only).

Case Study

Data gathered by the Centers for Disease Control and Prevention (CDC) during a recent mycoplasma outbreak allowed us to extract values for the parameters in our theory. In 1999, an outbreak of mycoplasma pneumonia occurred in a psychiatric institution (14). All 15 wards at the institution were affected, with 60 of 257 residents and 82 of 440 employees diagnosed with mycoplasma-like illness. In the following sections, we predict the epidemic threshold for this institution. The threshold is a function of the degree distribution of caregivers and transmission rates, the size of the epidemic above the threshold, and a range of realistic transmission rates for \( M. \text{pneumoniae} \) in this outbreak.

We assumed that each patient was confined to a single ward. While this was not true for all patients at the institution, it simplified the mathematics and allowed us to make a reasonable approximation of the epidemiology. Interactions between patients in separate wards will increase the threat of a full-blown epidemic and make early intervention all the more critical. Including such interactions in the model is possible by adding edges to the graph that connect patients to multiple wards. This scenario can be solved exactly by using techniques similar to those presented here.

Epidemic Threshold

If we assume that the degree distributions for wards and caregivers are Poissonian, the epidemic threshold (equation no. 2) is equivalent to

\[
\tau_c \tau_w \mu_c = 1.
\]

In other words, when the product of the transmission rates, the average number of caregivers per ward, and the average number of wards per caregiver exceeds 1, epidemics become possible. In the psychiatric institution, \( W = 15 \) and \( C = 440 \), hence

\[
\mu_c = \frac{440}{15} \mu_c \text{ and the threshold becomes } \frac{440}{15} \mu_c \tau_w \tau_c = 1.
\]

Figure 4 illustrates the epidemic threshold for five different demographic scenarios (\( \mu_c = 1,2,3,4,5 \)). For the most densely connected case, when each caregiver works in five wards on average, the epidemic threshold is crossed at very low rates of transmission. When the community is less densely connected, it can withstand much higher infectivity without giving rise to epidemics.

Calculating the Size of the Epidemic

Combining equation no. 2 with equations 5, 6, 7, and 8 from online Appendix C, we derived the following:

\[
\Phi_0(1) = \exp[\mu_c(1 - \tau_c + \tau_c \mu_c \Phi_0(1) - 1) - 1]. \tag{9}
\]

Given values for demographic parameters \( \mu_c \) and \( \mu_w \), we search for the value of \( \Phi_0(1) \) that satisfies equation no. 9 numerically. Then, the predicted number of caregivers infected during an epidemic is \( S_c = 1 - \Phi_0(1) \). (The number of affected wards is similarly derived.) Since we know neither the exact distribution of caregivers in wards nor the transmission rates between caregivers and wards, we solve for the size of the epidemic outbreak in a range of values of the three independent parameters \( \mu_c \), \( \tau_c \), and \( \tau_w \).

Figure 5 shows both the fraction of wards and caregivers infected in our model as a function of the number of wards per caregiver (\( \mu_c \)), and the fraction of wards and caregivers...
infected in the actual outbreak. We assume transmission rates of $\tau_c = 0.6$ and $\tau_w = 0.6$ (discussed below). The top dashed line indicates that 100% of the wards were affected during the actual epidemic. The lower horizontal lines depict the upper and lower bound empirical estimates for the number of caregivers affected (TB Hyde, unpub. data). As $\mu_c$ increases, so does the possibility of transmission from one ward to another through caregivers that work in both. The number of wards affected climbs sharply to 100% (as actually occurred in this outbreak), whereas the number of caregivers climbs more gradually, passing through the realistic range at relatively low values of $\mu_c$.

This analysis suggests that the likelihood of an epidemic and the eventual size of an epidemic, should one occur, are highly sensitive to the degree distribution for caregivers. Transmission of $M. pneumoniae$ is limited, and the extent and duration of the outbreak are reduced if each caregiver’s activities are confined to just a few wards.

The derivations given here are exact in the limit of large network size. To assess their accuracy on networks like these with a few hundred vertices, we have constructed specific graphs that realize these distributions and performed computer simulations of the spread of epidemics on them. Each simulation constructs a network with 15 wards and 440 caregivers, where the degree distribution of each caregiver is binomial with $n = 15$, and $p$ such that $np = \mu_c$. We assume constant infection periods of $\delta_c = 14$ days (for caregivers) and $\delta_w = 21$ days (for wards) and that contact between a caregiver and a ward occurs independently of any other such contact. Initially a single, randomly chosen caregiver is infected. Every day, transmission occurs from an infected caregiver to a connected ward with probability $\tau_c$. Thus, the daily transmission rate from an affected ward to a healthy caregiver that works there is $1 - (1 - \tau_c)^{\delta_c}$. Likewise, the daily transmission rate from an affected ward to a healthy caregiver that works there is $1 - (1 - \tau_w)^{\delta_w}$.

Figure 6 shows a frequency distribution of the sizes of epidemics for 1,000 runs of the simulation. Figure 7 compares these results with the predictions of our analytic theory. As the figure clearly shows, the agreement between simulation and theory is excellent.

**Inferring Transmission Rates**

Our numeric method also allows us to pinpoint transmission rates that are consistent with the empirical observations. Assuming the average caregiver works in one to four wards, we identify transmission rates that predict the observed numbers of affected caregivers and wards. We find that $\tau_c \in [0.2,1]$ and $\tau_w \in [0.03,0.1]$. Transmission from an infected caregiver to at least one patient in a ward must therefore be about 10 times more likely than transmission from a ward with sick patients to a caregiver who works in that ward. Remarkably, caregivers are not likely to become infected, yet when they are infected, they become the primary vehicles for spreading bacteria from ward to ward. Hence the most effective interventions will be those that prevent transmission to caregivers.

**The Patients**

Based on the outbreak data, the probability that a particular patient will become infected if at least one other patient in the ward is infected is 0.15 (0.02) for confirmed cases or 0.23 (0.02) when probable cases are included. Figure 8 shows the within-ward transmission rates and ward size for the 15 wards.

---

1We calculate these rates by averaging the fraction of infected patients per ward across the 15 wards and compute the error by taking the standard deviation of these fractions, divided by the square root of the sample size.
Although not shown, ward size and the transmission rate are not correlated.

We simulate the spread of \textit{M. pneumoniae} among patients, assuming the ward size distribution shown in Figure 8, and assuming that the number of patients infected per ward follows a binomial distribution with probability parameter \( p \). (The Poisson approximation is inappropriate as it only applies to very large wards with small transmission rates.) That is, all 15 wards are assumed to be affected, and each patient in a ward becomes infected with probability \( p \). Figure 9 shows frequency distributions for the fraction of patients infected in 100,000 simulations at three values of \( p \) (\( p = 0.2, 0.25, 0.3 \)). These distributions resemble the actual frequency distribution shown in Figure 8, and thereby support the binomial approximation.

**Discussion**

Network theory enables epidemiologists to model explicitly and analyze patterns of human interactions that are potential routes for transmission of an infectious disease. The statistical properties of an epidemic graph determine the extent to which an infectious agent can spread. By manipulating the structure of a graph, we can identify interventions that may dramatically alter the course of an epidemic, or even prevent one altogether, and translate them into measures that make sense in a real community. In this paper, we have used network methods to model the spread of a respiratory tract infection in a health-care facility.

How might this be applied to a real outbreak? We have considered data from a recent investigation of an outbreak of \textit{M. pneumoniae} in a residential psychiatric institution (14). In that investigation, standard infection control practices, including strict respiratory droplet precautions, cohorting of ill patients, and employee education about mycoplasma illness and symptoms were instituted at the facility. Unfortunately, \textit{M. pneumoniae} has a long incubation period (1–4 weeks), during which time an asymptomatic, infected person can transmit the bacterium to an uninfected person. This long incubation period limits the beneficial effect of cohorting, since infected persons are only identified and taken out of the community after they have passed through the incubation period.

In both the outbreak and our model (assuming parameters based on this particular institution), caregivers are less likely to become infected than are patients. This observation may mislead investigators and lead to inappropriate recommendations. Although caregivers are less likely to become ill, they are the primary vectors of infection in the facility. Our model suggests that transmission rates from patients to caregivers are lower than transmission rates from caregivers to patients. Therefore, once a caregiver is infected with \textit{M. pneumoniae}, the likelihood is high that they will transmit the infection to their patients. These data support infection control strategies that limit transmission of \textit{M. pneumoniae} to caregivers.

We suggest two complementary strategies: limit the number of wards with which caregivers interact, and reduce the probability that caregivers become infected through, for example, respiratory droplet precautions. This strategy limits the time and cost of laboratory testing as well as the risks for antibiotic use in uninfected persons. The activity of some ancillary staff (e.g., physical therapists and nutritionists) cannot be limited to a select number of wards. In these cases, alternative precautions against transmission of \textit{M. pneumoniae} are required.

We conclude with three caveats. First, the epidemic model includes all infections, even those that do not result in symptoms. Most persons with \textit{M. pneumoniae} infections have relatively mild disease, only a cough or sore throat or no symptoms at all (17). When applying the model to the outbreak investigation, we considered only symptomatic carriers. While including asymptomatic carriers would change the estimates for the rates of transmission, our qualitative recommendations for intervention would remain the same.

Second, for mathematical tractability, our model assumes random (Poissonian) assignment of caregivers to wards. The quantitative (but probably not qualitative) results would differ under different degree distributions. In the future, we hope to analyze distributions taken from actual health-care institutions, when available.

Third, because of the long incubation period of \textit{M. pneumoniae} infection, interventions are often initiated well into the
outbreak. Since epidemics can last months, and in the psychiatric institution at least half of the wards were not affected until 6 weeks after the first case-patient was diagnosed, we are optimistic that intervention of the type proposed will have a positive impact.

The theoretical tools are in place for building community-specific networks and analyzing the transmission of infectious diseases on these networks. Our approach enables mathematical experiments, in which the inputs are interventions—structural reorganization, cohorting, treatment, and the like—and the output is predictions about the spread of a disease (or lack thereof) on the network. This approach can both aid the development of general measures and lend insight into specific scenarios in which intervention is still possible.

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References


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Antimicrobial resistance to penicillin and macrolides in *Streptococcus pneumoniae* has increased in the United States over the past decade. Considerable geographic variation in susceptibility necessitates regional resistance tracking. Traditional active surveillance is labor intensive and costly. We collected antibiogram reports from North Carolina hospitals and assessed pneumococcal susceptibility to multiple agents from 1996 through 2000. Susceptibility in North Carolina was consistently lower than the national average. Aggregating antibiogram data is a feasible and timely method of monitoring regional susceptibility patterns and may also prove beneficial in measuring the effects of interventions to decrease antimicrobial resistance.

*Streptococcus pneumoniae* is a leading cause of community-acquired illness, resulting in an estimated 3,000 cases of meningitis, 50,000 cases of bacteremia, 500,000 cases of pneumonia, and 7 million cases of otitis media each year in the United States (1). Even with appropriate antimicrobial therapy, case-fatality rates for high-risk patients can be as high as 40% for bacteremia and 55% for meningitis (1). Surveillance systems have shown decreasing antimicrobial susceptibility among pneumococci (2–13). A comparison of susceptibility among combined respiratory and invasive isolates from respiratory seasons 1994–1995 and 1999–2000 showed a decrease in both penicillin susceptibility (from 76% to 66%) and erythromycin susceptibility (from 90% to 74%) (8). An examination of only invasive isolates from 1997 and 2000 also showed a decline in isolates’ susceptibility to penicillin (from 75% to 73%) and to erythromycin (from 85% to 78%) (2,5).

Many of the current surveillance systems monitoring emerging drug resistance detect susceptibility patterns over large areas, such as an entire country (2,6,9,13). Results may not be generalizable to all locations within the study area (1). For example, although the overall proportion of penicillin-nonsusceptible pneumococci within a seven-region, population-based, active surveillance program was 25%, the proportion ranged from 15% in Maryland to 38% in Tennessee (14). Data are typically gathered from a limited number of medical establishments within a specified region; national surveillance systems may not collect data from every state. Monitoring trends in pneumococcal susceptibility over smaller geographic areas is necessary to aid clinicians in choosing the best drug treatment for empiric therapy (1). This local information can also help evaluate efforts to decrease resistance rates through judicious antibiotic use.

Chin et al. compared antimicrobial susceptibility results from active and antibiogram surveillance of pneumococcal isolates at 12 hospitals in the Portland, Oregon, area in 1996 (15). Active surveillance was defined as collecting isolates and patient data from participating hospitals and performing susceptibility testing at a centralized laboratory (15,16). Antibiogram surveillance is quite different. Clinical laboratories assess the antimicrobial susceptibilities of bacterial isolates and summarize all susceptibility results for a specified period on an antibiogram report. Antibiograms conform to the susceptibility testing practices of individual laboratories, include information on both sterile and nonsterile isolates, may include duplicate isolates from a single patient, and lack an epidemiologic characterization of the patient or isolate. The data contained on laboratory-specific antibiograms, however, can be compiled to assess regional susceptibility, monitor trends over time, and assess effects of interventions designed to reduce antibiotic resistance through judicious antibiotic use.

Chin et al. found no statistically significant difference in results obtained by the two methods for any of the four drugs tested at the 12 Portland area hospitals, except for a single drug at a single hospital where erythromycin susceptibility was reported at 97% by active surveillance and 84% from the antibiogram (chi square p=0.01) (15). The cost difference between the active and antibiogram surveillance systems was substantial: $52,000 for active surveillance and $700 for antibiogram surveillance. The authors concluded that although antibiogram surveillance produced less information than active surveillance, “antibiograms provided accurate, community-specific drug-resistant *S. pneumoniae* data.”

We examined the practicability of collecting hospital antibiogram data in North Carolina, a state with a population of 8,049,313 people and a land area of 48,711 square miles divided into 100 counties (17). We also assessed pneumococcal susceptibility to multiple antimicrobial agents using the aggregated antibiogram data.

**Methods**

This study was conducted in North Carolina from April to September 2001. A study packet was mailed in April 2001 to the directors of clinical microbiology laboratories at all 114...
North Carolina hospitals identified by the Centers for Medicare and Medicaid Services (CMS). Hospitals subsequently identifying themselves as specialty hospitals (e.g., psychiatric, drug treatment) were excluded from all analyses. The packet included a letter describing the project, a questionnaire on hospital characteristics and laboratory testing methods, a request for submission of *S. pneumoniae* antibiogram data for each year from 1996 to 2000, and a prepaid, preaddressed, return express mail envelope. The nature of the information provided on hospital antibiograms does not necessarily allow for identification of duplicate specimens from the same patient, differentiation of susceptibility results by source of specimen, or determination of conformance to National Committee for Clinical Laboratory Standards (NCCLS) guidelines for susceptibility cut points.

From the antibiograms, the numbers of pneumococcal isolates tested and numbers of isolates testing susceptible were added across all hospitals for each antimicrobial agent for each year of data to create statewide summary totals. Data for drugs that predictably elicit the same susceptibility result were combined: penicillin/oxacillin, cefotaxime/ceftriaxone, and levofloxacin/ofloxacin. Antibiogram data from each hospital for each year of data were assessed for inclusion. Data from antibiograms reporting 1) testing results cumulative over >1 year, 2) percentages of susceptible isolates without providing the total number of isolates tested, 3) more than one susceptibility value for the same drug for the same period, or 4) results by nursing unit or named patient were all excluded for the year and drug in question. We also excluded data for drugs other than penicillin if more isolates were tested for penicillin susceptibility than for the other drugs, and the antibiogram did not clearly indicate that the subgroup selected for additional testing was based on the source of the specimen (i.e., bloodstream). Testing only penicillin-non susceptible isolates for susceptibility to other drugs could yield misleading results because penicillin-resistant isolates are more likely to be resistant to other drugs as well. The aggregated statewide summary totals were used to calculate yearly susceptibility proportions for nine different antibiotics for the entire state. Nonsusceptible isolates encompassed those identified as either intermediate- or high-level resistant. Susceptibility proportions for penicillin were categorized into three regions (west, central, east) by the county the respondent listed on the questionnaire. The Committee on the Protection of the Rights of Human Subjects, University of North Carolina School of Medicine, granted Institutional Review Board approval.

North Carolina's pneumococcal susceptibility pattern from 1997 through 2000 was compared to patterns shown by national surveillance systems tracking *S. pneumoniae* susceptibility. Data from published reports were included if they covered a period of no more than 12 months and identified the source of isolates as respiratory, invasive, or both. If the surveillance period overlapped two calendar years while covering one respiratory season, the data were classified by the latter year. For instance, isolates collected from October 1999 through April 2000 were labeled as year 2000 data.

We used the Cochran-Armitage trend test, which tests for trends in binomial proportions across levels of an ordinal covariate, to evaluate temporal patterns in the data. A two-sided p-value \( \leq 0.05 \) was considered statistically significant. Trend tests were performed by using SAS version 8.1 (SAS Institute, Inc., Cary, NC). Exact binomial 95% confidence intervals were calculated for proportions by using Stata version 7.0 (Stata Corporation, College Station, TX).

### Results

Overall, 60 of the 110 (55%) potentially eligible hospitals replied to the survey, although ultimately fewer hospitals were able to contribute pneumococcal susceptibility data. Thirty of the 114 CMS-identified hospitals responded to the initial request for information within the first month. After follow-up telephone calls to the remaining 84 hospitals, 30 additional hospitals responded. Four hospitals were excluded: one listed under two different names, one psychiatric hospital, one orthopedic hospital, and one alcohol treatment center. North Carolina hospitals not enumerated on the CMS list, and hence not invited to participate in this study, included five military, four Veterans Affairs, and two prison hospitals, as well as several long-term care and rehabilitation centers. Additionally, small hospitals that were part of a health-care system dominated by one large hospital were frequently omitted from the CMS list. These noninvited hospitals were primarily rural, community facilities.

The proportion of responding hospitals was similar across the three regions of the state: 17/30 (57%) hospitals in the west, 27/51 (53%) in the central region, and 16/29 (55%) in the east. The average number of beds was 257 (range 40 to \( \geq 1,000 \)). The central region contained most of the state’s large, academic hospital centers as well as most of its urban counties. No discernable difference was evident between the 50 potentially eligible hospitals that did not participate and the 60 that did, except that all major academic centers participated.

The primary source of pneumococcal isolates was specimens from hospitalized patients in 74% of hospitals and outpatients in 12%. The remaining specimens came from emergency departments, nursing homes, and physicians’ offices. Among the hospitals describing susceptibility testing methods, 51% used E-test, 47% oxacillin screening, 36% MIC broth dilution, and 20% disk diffusion. Many hospitals performed more than one type of susceptibility testing. Although 11 hospitals reported differentiating sterile from nonsterile isolates, only 7 hospitals provided this stratification on their antibiograms.

Eleven of the 60 (18%) hospitals responding to the study request did not provide antibiogram data. Of these hospitals, nine did not perform on-site susceptibility testing, and two gave no explanation for not submitting antibiograms. One additional hospital reporting off-site testing made antibiogram information available through a reference laboratory. Although
49 hospitals contributed antibiogram data for at least one drug for at least 1 year, not all of the data from these antibiograms qualified for inclusion in the analyses. Susceptibility data were excluded for at least one class of drugs for ≥12 months from 1996 to 2000 for the following reasons: 1) seven antibiograms provided data for a period of more than 12 months, 2) four antibiograms reported susceptibility rates without numbers of isolates tested, 3) one antibiogram listed more than one susceptibility result for the same drug for the same period, 4) two antibiograms provided results by nursing unit or named patient, and 5) 12 antibiograms reported more isolates tested for penicillin susceptibility than for susceptibility to other drugs without explaining the drop-off in isolate number, therefore, requiring exclusion of the nonpenicillin data. After accounting for the aforementioned exclusions, the number of antibiograms with usable data for any single drug for a given 1-year period ranged from 1 (levofloxacin, 1996 and 1997) to 42 (penicillin, 2000).

Although most hospitals submitted susceptibility testing results for 2000, fewer did so for earlier years. The numbers of hospitals providing data on penicillin susceptibility were 18 hospitals (1,854 isolates) for 1996, 24 hospitals (2,406 isolates) for 1997, 33 hospitals (2,827 isolates) for 1998, 36 hospitals (3,562 isolates) for 1999, and 42 hospitals (3,497 isolates) for 2000 (Table 1). The numbers of hospitals submitting data on macrolide susceptibility also increased over the years: four hospitals (488 isolates) for 1996, 11 hospitals (786 isolates) for 1997, 17 hospitals (1,095 isolates) for 1998, 20 hospitals (1,397 isolates) for 1999, and 27 hospitals (1,762 isolates) for 2000 (Table 1).

From 1996 to 2000, the proportion of S. pneumoniae isolates testing susceptible to penicillin decreased (p<0.001) (Figure 1). Although 65% of isolates were reported as susceptible to penicillin in 1996, only 52% were susceptible in 2000 (p<0.001). This pattern of decreasing susceptibility was also evident when stratifying by region of the state (p<0.001 for each region) (Figure 2). From 1997 on, no statistically significant difference in susceptibility was found between either the west and eastern regions or the west and central regions. However, penicillin susceptibility was significantly lower (10%) in the east than in the central region during this time period.

A subanalysis of the 15 hospitals for which usable information on penicillin susceptibility was available for each year produced a comparable trend. Although with this reduced amount of data susceptibility was higher in 1996 (69%) than in the full analysis (65%) (chi square p=0.03), the trend over time remained consistent (p<0.001). By 2000, 54% of the isolates reported at these 15 hospitals were susceptible to penicillin, compared to 52% in the entire study group (chi-square p=0.13).

Among penicillin-nonsusceptible isolates, the proportions intermediate and resistant were available from 5 hospitals (419 isolates) in 1996, 6 hospitals (592 isolates) in 1997, 11 hospitals (849 isolates) in 1998, 12 hospitals (1,184 isolates) in 1999, and 11 hospitals (1,055 isolates) in 2000. These represented between one-quarter and one-third of all isolates tested for penicillin susceptibility, depending on the year. During the 5-year period, the proportion of susceptible isolates appeared to decrease, the proportion of resistant isolates increased, and the proportion of intermediate isolates showed little change (Figure 3).

Macrolide susceptibility decreased from 78% in 1996 to 61% in 2000 (p<0.001). From 1996 to 2000, the proportion of S. pneumoniae isolates susceptible to cefotaxime decreased 8%. Although third-generation cephalosporins did not show a consistent decrease in susceptibility each year, the decline during the 5-year period was still significant (p<0.001). Susceptibility to quinolones and vancomycin remained high, despite the fact that two hospitals, one for two different years, reported a total of five isolates as vancomycin-resistant. The low level of levofloxacin susceptibility in 1998 (92%) was based on only 237 isolates. Larger numbers of isolates available in sub-

- **Table 1. Streptococcus pneumoniae susceptibility to nine antimicrobial agents, North Carolina, 1996–2000**

<table>
<thead>
<tr>
<th>Year</th>
<th>Penicillin</th>
<th>Erythromycin</th>
<th>Cefotaxime</th>
<th>Levofloxacin</th>
<th>Tmp-smx</th>
<th>Tetracycline</th>
<th>Clindamycin</th>
<th>Vancomycin</th>
<th>Chloramphenicol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996</td>
<td>65 (18; 1,854)</td>
<td>78 (4; 488)</td>
<td>85 (9; 985)</td>
<td>100 (1; 205)</td>
<td>64 (5; 626)</td>
<td>96 (2; 254)</td>
<td>90 (4; 492)</td>
<td>100 (7; 580)</td>
<td>93 (2; 381)</td>
</tr>
<tr>
<td>1997</td>
<td>63 (24; 2,406)</td>
<td>69 (11; 786)</td>
<td>80 (13; 1,272)</td>
<td>100 (1; 283)</td>
<td>57 (11; 786)</td>
<td>79 (4; 666)</td>
<td>76 (7; 655)</td>
<td>100 (11; 903)</td>
<td>95 (3; 117)</td>
</tr>
<tr>
<td>1998</td>
<td>56 (33; 2,827)</td>
<td>64 (17; 1,095)</td>
<td>83 (20; 1,970)</td>
<td>92 (4; 237)</td>
<td>51 (16; 975)</td>
<td>83 (10; 402)</td>
<td>88 (11; 606)</td>
<td>100 (19; 1,202)</td>
<td>89 (10; 520)</td>
</tr>
<tr>
<td>1999</td>
<td>54 (36; 3,562)</td>
<td>61 (20; 1,397)</td>
<td>80 (22; 2,062)</td>
<td>94 (5; 525)</td>
<td>51 (16; 1,068)</td>
<td>84 (10; 406)</td>
<td>85 (13; 1,017)</td>
<td>100 (21; 1,308)</td>
<td>92 (9; 540)</td>
</tr>
<tr>
<td>2000</td>
<td>52 (42; 3,497)</td>
<td>61 (27; 1,762)</td>
<td>77 (27; 2,296)</td>
<td>98 (12; 822)</td>
<td>50 (20; 1,292)</td>
<td>81 (14; 717)</td>
<td>88 (18; 1,238)</td>
<td>100 (26; 1,648)</td>
<td>94 (12; 730)</td>
</tr>
</tbody>
</table>

Values in parentheses are number of hospitals contributing data and total number of isolates reported, respectively.

Tmp-smx, trimethoprim-sulfamethoxazole.

![Image](image-url)

Figure 2. Streptococcus pneumoniae penicillin susceptibility by geographic region, North Carolina, 1996–2000. Error bars represent 95% confidence intervals.

Discussions

Aggregating hospital antibiogram data from the state of North Carolina appears to be a feasible, practical method for monitoring trends in pneumococcal susceptibility. Large numbers of isolates are available for annual comparisons with consistent reporting on penicillin, albeit less consistent reporting on other drugs. Susceptibility to clinically important antibiotics was shown to decrease significantly.

The observed progression of both penicillin and macrolide resistance is of particular concern. The increase in penicillin resistance appears to correlate with an increase in high-level resistance to antibiotic susceptibility tests, which then increased higher levels of resistance and high-level penicillin resistance has been associated with worse outcomes for pneumococcal infections (18). The increased macrolide resistance is most likely mediated by a low-level efflux pump since clindamycin susceptibility remained stable over the study period (19). Erythromycin susceptibility generally predicts that of azithromycin and clarithromycin (20). Increased macrolide resistance is disturbing since erythromycin, azithromycin, and clarithromycin are some of the most commonly prescribed antibiotics for outpatient treatment of community-acquired pneumonia and low-level macrolide resistance has been associated with clinical failure (21–23).

Pneumococcal resistance rates tend to increase moving along the spectrum of isolates obtained from bloodstream to lower respiratory tract to upper respiratory tract (8). This fact potentially confounds point comparisons of resistance rates since a marked increase in resistance can result from not testing a preponderance of upper respiratory isolates, rather than reflecting a true increase in the burden of resistant pneumococci. We were unable to assess the extent to which the source of the specimen may have produced spurious results since only seven hospitals identified the specimen source on their antibiograms. If the relative distribution of isolates remained the same, however, the trend would not be altered.

Although Chin et al. showed that antibiogram surveillance and active surveillance yield comparable results, national data may not be directly comparable to our findings (15). The national data used for comparison to this study result from active surveillance use different reporting periods, and in some cases focus solely on invasive isolates that have consistently higher susceptibility than respiratory isolates (8). Pneumococcal susceptibility in North Carolina is nonetheless lower than reported national averages and appears to be decreasing more quickly (Table 2). This finding is consistent with comparatively low antibiotic susceptibilities previously described for the Southeast. Active surveillance for year 2000 susceptibility in the southeastern United States ranged from 56% to 57% for penicillin and from 61% to 67% for erythromycin (8,12,13).

Antibiogram surveillance is cost efficient. Expenses for collecting and analyzing five years worth of data from an entire state were limited to mailings and paper materials (<$1,000) and the support of one graduate student. Chin et al. spent $52,000 for 1 year of active surveillance in 12 hospitals. The antibiogram surveillance had several potential limitations, however. First, a 55% response rate may be adequate for certain studies, but full participation from all N.C. microbiology laboratories would be the best way to ensure that surveillance data accurately reflect susceptibility patterns. Furthermore, all participating hospitals did not submit an antibiogram for each year nor did all data on each antibiogram meet inclusion criteria. Yet our data included many more hospitals in this study locale than any previously published surveillance system. Second, many hospitals were unable to access data from past years for a variety of reasons, including changes in testing and computer systems. Collecting antibiogram reports on a yearly basis should allow more hospitals to more easily contribute their data. Third, specimens are increasingly sent to referral hospitals or reference laboratories. For instance, 9 of the 60 participating hospitals did not have antibiogram data, and we were able to get results from a reference laboratory only for a single hospital. The lack of availability of antibiograms at hospitals that use reference laboratories is disconcerting since information needed to guide local antibiotic decisions is not accessible. Lastly, testing and reporting procedures were inconsistent, such as drugs tested, identification of specimen source, breakdown of intermediate and high-level resistance, and period covered by the antibiogram. We hope that providing N.C. microbiology laboratories with these study findings would allow more hospitals to more easily contribute their data.
will encourage participation in continued surveillance activities as well as more uniform testing and reporting procedures.

The submitted data appeared to be consistent despite the fact that the population under study changed from year to year as more data became available. The sub-analysis, reflecting solely those hospitals providing information for each year from 1996 to 2000 yielded results comparable to those found in the overall study. Additionally, the observed susceptibility results generally support known resistance patterns, such as the correlation of penicillin and ceftriaxone susceptibility, lower levels of macrolide than clindamycin susceptibility, and near universal vancomycin susceptibility (24).

Combining hospital antibiogram data appears to be an effective method of tracking antimicrobial susceptibility among Streptococcus pneumoniae, both in North Carolina and within regions of the state. To further develop this antibiogram surveillance system, we are partnering with the North Carolina State Laboratory of Public Health to establish an electronic network of microbiology laboratories to enhance interlaboratory communication. We hope to share practices, encourage testing consistent with current NCCLS guidelines, and support standardized, efficient, annual, electronic submission of antibiograms. We also hope that knowledge of N.C. resistance patterns will both guide treatment decisions and motivate judicious antibiotic prescribing behavior.

Judicious use of antibiotics is essential for their continued effectiveness. Not only have regional trends in antibiotic resistance been linked to antibiotic use (25–29), but decreasing antibiotic use has resulted in declining levels of resistance (30). After an aggressive campaign to educate its population on the need for shrewd use of antibiotics, the rate of penicillin-resistant pneumococci in Iceland declined from nearly 20% in 1993 to 16.9% in 1994 (31,32). Regional surveillance can identify areas most in need of interventions aimed at decreasing resistance and can monitor the progress of these interventions. Aggregating antibiogram data appears to be an easy, inexpensive, effective way of accomplishing these goals.

Acknowledgments
We thank Peter Gilligan for his assistance in developing the questionnaire and all of the North Carolina microbiology laboratories that participated in this study.

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Ms. Stein is a doctoral student in the Department of Epidemiology at the School of Public Health the University of North Carolina at Chapel Hill. Her research interests include emerging drug resistance.

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Influence of Role Models and Hospital Design on Hand Hygiene of Health Care Workers

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We assessed the effect of medical staff role models and the number of health-care worker sinks on hand-hygiene compliance before and after construction of a new hospital designed for increased access to handwashing sinks. We observed health-care worker hand hygiene in four nursing units that provided similar patient care in both the old and new hospitals: medical and surgical intensive care, hematology/oncology, and solid organ transplant units. Of 721 hand-hygiene opportunities, 304 (42%) were observed in the old hospital and 417 (58%) in the new hospital. Hand-hygiene compliance was significantly better in the old hospital (161/304; 53%) compared to the new hospital (97/417; 23.3%) (p<0.001). Health-care workers in a room with a senior (e.g., higher ranking) medical staff person or peer who did not wash hands were significantly less likely to wash their own hands (odds ratio 0.2; confidence interval 0.1 to 0.5); p<0.001. Our results suggest that health-care worker hand-hygiene compliance is influenced significantly by the behavior of other health-care workers. An increased number of hand-washing sinks, as a sole measure, did not increase hand-hygiene compliance.

One of the key components for limiting spread of healthcare–associated infectious disease is adequate infection control practice. A cornerstone of infection control is ensuring that health-care workers wash their hands at appropriate times. The Association for Professionals in Infection Control and Epidemiology (APIC), the Guidelines for Handwashing and Hospital Environmental Control (1985, 2001) from the Centers for Disease Control and Prevention (CDC), and the Hospital Infection Control Practices Advisory Committee each highlight specific indications for handwashing compliance (1–4). Although CDC guidelines state that handwashing is the single most important procedure to prevent nosocomial infection (2,4), studies continue to report unacceptable health-care worker hand-hygiene compliance rates (5–12). Efforts to improve hand-hygiene behavior that have focused on broad-based educational and motivational programs have had minimal sustained success (11–14).

Factors perceived as contributing to poor hand-hygiene compliance include unavailability of handwashing sinks, time required to perform hand hygiene, patient’s condition, effect of hand-hygiene products on the skin, and inadequate knowledge of the guidelines (10,15–21). In addition, some reports suggest that role models, group behavior, and the level of managerial support influence reported levels of compliance (17,21–24). One measure recommended to improve the hand-hygiene rate is enhanced access to hand-hygiene facilities (15–17,25). However, few studies have prospectively evaluated the association between hand-hygiene compliance and building design (16,26). We assessed the effect of medical staff role models and the number of health-care worker sinks on hand-hygiene compliance before and after construction of a new hospital designed for increased access to handwashing sinks. We also evaluated whether the frequency of health-care worker hand hygiene was influenced by the behavior of senior medical-care providers.

Methods

Setting and Study Participants

The old hospital had 683 private and semi-private rooms. Observations were made in the 33-bed hematology/oncology unit, the 23-bed solid organ transplant unit, the 16-bed surgical intensive-care unit (SICU), and the 11-bed medical intensive-care unit (MICU). Sink-to-bed ratios in the units were 8:33 in the hematology/oncology unit, 4:23 in the solid organ transplant unit, and 1:1 in both ICUs.

Sinks were located in various sites in the old hospital. The non-ICUs had a limited number of handwashing sinks for health-care worker use located on walls in the middle of each hallway, in clean storage rooms, and in soiled-linens utility rooms. The hematology/oncology unit had a single handwashing sink located in each of three hallways, two handwashing sinks located in each corridor for the bone marrow transplant patient rooms, and a handwashing sink in the anteroom to the bone marrow transplant suite. The solid organ transplant unit had a single handwashing sink located in each of two hallways. ICUs had private rooms with a sink located inside the entrance of every patient room but no hallway sinks.

The new hospital opened with 492 individual (private) patient rooms. Observations in the new facility were done in
the 30-bed hematology unit, the 30-bed oncology unit, 30-bed solid organ transplant unit, the 12-bed SICU, and the 17-bed MICU. A sink dedicated for hospital personnel use is located inside every patient room. No sinks are available in the hallways.

**Hand-Hygiene Definition**

We defined hand hygiene as any duration of washing with soap and water. No waterless alternatives were available for other types of hand hygiene during the study. We recorded hand-hygiene compliance on room entry and after each hand-hygiene opportunity. The definitions of hand-hygiene opportunities, patient contact, and invasive procedures used for this analysis are consistent with APIC or CDC guidelines (Table 1). Inanimate objects considered likely to be contaminated included endotracheal tubes, suction equipment, urinary collection devices, rectal tubes, thermometers, bed linens, and biohazardous waste containers.

**Periods of Observation**

During the two study periods, 1-hour observation periods were conducted weekdays between 8:00 a.m. and 5:00 p.m. The first observation period (period I; 25 weeks) took place from October 8, 1998, to April 29, 1999, in the old facility. The second observation period (period II; 24 weeks) took place in the new building from July 7 to December 23, 1999. A physician, two infection-control professionals, and a microbiologist were trained to individually observe, as follows: 1) protocol guidelines and study definitions were explained in detail from a printed handout that could be carried to the floor for reference during the study, and the data collection form was discussed; 2) the new observer accompanied the physician to a study unit and observed how to perform surveillance and complete the data collection form; 3) after observing several handwashing opportunities, the new observer made and recorded observations along with the physician; 4) the physician and new observer compared observations, and discrepancies were discussed to assess understanding of the protocol guidelines and study definitions; and 5) side-by-side comparisons were performed on a subsequent day, and the training was considered complete.

After entering the nursing unit, observers followed the initial worker or group of health-care workers they encountered who went into a patient room. To maintain health-care worker anonymity, individual identities were not recorded; therefore, we could not eliminate or control for repeat observations during analysis. The first persons entering a room were observed until departure from the room. We then went back into the hallway, and the next new persons entering a room were followed for the next observation, thus avoiding repeat observations of an individual health-care worker on any single observation day. Although health-care workers were not informed regarding the purpose of this study, if questioned during hand-hygiene observations, the investigators replied that infection-control measures were being monitored. No immediate feedback was provided to the health-care workers regarding hand-hygiene behavior.

Data gathered during observations included time of day, type and number of health-care workers entering the room, patient or equipment contact, compliance with hand-hygiene practices, glove use, invasive procedures, nursing unit and hospital, and whether isolation precautions had been posted. Health-care workers were designated as one of the following categories: physician; registered nurse; patient-care technician; respiratory, physical, or occupational therapist; pharmacist; radiology, electrocardiogram, or ultrasound technician; dietician; food service worker; unit secretarial staff; housekeeping staff; transportation staff; student; chaplain; volunteer staff; or technical sales support. Each physician was further classified as an attending or fellow, resident, or medical student.

A hierarchy was defined to assess the effect of other medical staff on hand-hygiene compliance. Health-care workers were ranked in the following order: 1) attending physician or fellow, 2) resident or intern, 3) nurse, 4) technical staff consisting of respiratory therapists, physical, or occupational therapists, radiology, electrocardiogram, ultrasound technicians, pharmacists, or dieticians, 5) patient-care technicians, and 6) housekeeping or transportation staff. Because the number of observations of medical students was small and determining the proper rank for medical students was difficult, we did not include students in the analysis of the influence of other personnel in the room. During analysis of the influence of other personnel in the room, medical students were not included in the study. When observations of medical students were small and determining the proper rank for medical students was difficult, we did not include students in the analysis of the influence of other personnel in the room.

<table>
<thead>
<tr>
<th>Hand-hygiene opportunities</th>
<th>Patient contact</th>
<th>Invasive procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient contact</td>
<td>Contact with patient’s skin</td>
<td>Phlebotomy</td>
</tr>
<tr>
<td>Performance of an invasive procedure</td>
<td>Contact with blood or body fluids</td>
<td>Intravenous or intramuscular injection of a medication</td>
</tr>
<tr>
<td>Placement of an intravascular device or urinary catheter</td>
<td>Contact with mucous membranes</td>
<td>Wound care</td>
</tr>
<tr>
<td>Visible soiling of hands</td>
<td></td>
<td>Urinary catheterization</td>
</tr>
<tr>
<td>Contact with body fluids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glove removal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Patient contact and invasive procedure are not mutually exclusive categories.
health-care workers present in the room, each health-care worker room entry was placed in one of the following categories: single person, highest ranking person in the room, in the room with a higher ranking person who performed hand hygiene, in the room with a higher ranking person who did not perform hand hygiene, in the room with a peer who performed hand hygiene, or in the room with a peer who did not perform hand hygiene. Peer status was defined as two or more health-care workers in the same category listed above (e.g., two nurses in the room together would be classified as peers). Hierarchy status was related to a purely theoretical belief of clinical expertise and knowledge held by the health-care worker.

Changes in accessibility and availability of hand-hygiene products and supplies between the two study periods included product modifications of soap, hand lotion, towels, and gloves. In general, products were more accessible in the new hospital. A para-chloro-meta-xylene soap, (Medi-Scrub, Huntington Laboratories/Ecolab, St. Paul, MN) was the hand-hygiene agent in the ICUs and hematology/oncology unit in the old hospital. A 5-chloro-2-[2,4-dichlorophenoxy], triclosan product (Healthstat, Richmond Laboratories, Huntington, IN) was used in the solid organ transplant unit in the old hospital and is now used in all units of the new hospital. An aloe vera lotion with triclosan was available in clean utility rooms of the old hospital (Accent Plus 1, Huntington Laboratories/Ecolab). In the new hospital, an amino lotion product is mounted at every health-care worker sink next to the soap dispenser (Tender Touch, Richmond Laboratories). Single-unit paper towel dispensers were available at each handwashing sink in both hospitals (Big Fold, Fort James Corp., Deerfield, IL, and Kleenex, Kimberly-Clark, Irving, TX, respectively). In the old hospital, various glove sizes were kept in clean utility rooms. In the new hospital, three sizes of gloves are located in wall-mounted dispensers next to towels and sinks. Powder-free gloves were available only for staff requiring them in the old hospital. Powder-free gloves are used exclusively in the new hospital by all personnel.

**Statistical Analysis**

Data were collected on standardized forms and entered in Microsoft Excel (Microsoft Corp., Redmond, WA). To evaluate predictors for hand-hygiene compliance after a hand-hygiene opportunity, we compared categorical variables using chi-square or Fisher’s exact test; odds ratios (OR) and 95% confidence intervals (CI) were calculated by using Epi Info version 6.04c (27). All variables with p<0.1 by univariate analysis were evaluated by stepwise logistic regression for inclusion in the final logistic regression model. To evaluate the effect of group behavior on individual health-care workers, we performed a separate analysis. Using stepwise logistic regression, we constructed a model and entered all health-care worker groups into the final model. Single-person room entry was the referent group to which all other groups were compared. SAS software was used for all multivariate analyses (SAS Institute, Inc., Cary, NC).

**Results**

**Observation Data**

Observations were performed on 49 separate occasions for a total of 45 hours (range 9.6–13.5 h/U). A total of 560 health-care worker–patient interactions were observed, resulting in 729 hand-hygiene opportunities. A total of 305 (41.8%) hand-hygiene opportunities were observed in the old hospital and 424 (58.2%) in the new hospital. Of the 560 health-care worker–patient interactions observed, 237 (42.3%) of the workers were registered nurses, 190 (33.9%) were physicians, and 133 (23.8%) were other health-care workers. The old and new hospitals were similar in performance of invasive procedures and health-care worker type. In the older facility, health-care workers were more likely to wear gloves or touch a patient during the hand-hygiene opportunity.

Hand-hygiene compliance on room entry was significantly greater in the old hospital at 12% (36/304) compared to the new hospital at 6% (26/424) (p=0.006). After all hand-hygiene opportunities were assessed, we found that hand-hygiene compliance was significantly better in the old hospital compared to the new hospital (161/304 [53%] vs. 97/417 [23%]; p<0.001). Hand-hygiene compliance was significantly better after a hand-hygiene opportunity (258/721; 35.7%) compared to before a hand-hygiene opportunity (62/727; 8.5%; p<0.001). By univariate analysis, characteristics significantly associated with hand-hygiene compliance after a hand-hygiene opportunity included working at the old hospital, having patient contact, performing an invasive procedure, using gloves, and performing hand hygiene on room entry. A key finding was that when a higher ranking person in the room did not perform hand hygiene, other health-care workers were significantly less likely to wash their hands (Table 2).

During multivariate analysis, we identified the following independent predictors of hand-hygiene compliance: using gloves, performing an invasive procedure, working at the old hospital, performing hand hygiene on room entry, and having patient contact. Again, health-care workers present in the room with a higher ranking person or peer who did not perform hand hygiene were significantly less likely to wash their hands (Table 3).

When we further evaluated group behavior, we found that compared to single person room entry, health-care workers in a room with a higher ranking person who did not wash were significantly less likely to wash their own hands. In each of these episodes, the higher ranking person was a physician or nurse. Surprisingly, if either a higher ranking person or peer was in the room and performed hand hygiene, then the frequency of hand hygiene for others in the group was no better than that of a room which only one person entered (Table 4). This observation suggests that the effect of a role model is highly signifi-
Discussion

Despite construction of a new hospital with an increased number of sinks, we found that hand-hygiene compliance in the new facility decreased substantially. We demonstrated that health-care workers were significantly less likely to wash their hands if they were in a room with a peer or higher ranking person who did not perform hand hygiene. Not unexpectedly, hand-hygiene compliance was better after patient contact, performing an invasive procedure, and removing gloves.

Health-care workers were much less likely to perform hand hygiene if a peer or a higher ranking person in the room did not perform hand hygiene. Compared to health-care workers who entered a room alone, group behavior did not seem to improve if the higher ranking person or peer did wash their hands. Although these findings suggest that hand-hygiene behaviors can be affected by role model or peer hand-hygiene compliance, learned behaviors or time constraints may negatively influence group compliance with hand-hygiene procedures.

As suggested by some studies, physician hand-hygiene compliance has an impact on peer and group behaviors (25,28). A recent evaluation of learned physician behaviors found that only 8.5% medical student candidates washed after patient contact (28). Since medical students may someday be influencing future hand-hygiene compliance behaviors of other health-care workers, the importance of hand hygiene should be incorporated into the medical school curriculum.

Our observations also suggest that health-care worker hand-hygiene compliance may improve when health-care providers perceive risk for their own health. In particular, hand hygiene before patient contact in our study was significantly worse than hand hygiene after patient contact. Whereas patients may be protected from acquisition of pathogenic organisms if health-care workers perform hand hygiene before patient contact, health-care workers may perceive a risk to themselves after patient contact; they respond by washing their hands. In addition, health-care workers were more likely to perform hand hygiene after an invasive procedure, which does not benefit the individual patient, but rather the health-care worker, who may be concerned about acquiring a pathogen present in body fluids. Finally, glove use could be a marker for hand-hygiene compliance if health-care workers are concerned about the personal risk from transmission of pathogens, and thus are more likely to wear gloves and cleanse their hands.

The hand-hygiene compliance we observed (finding that nearly 50% of our workers washed their hands after patient contact) was similar to the frequency of hand-hygiene compliance reported by other investigators (5,7,10–12). Even though we saw no improvement, our baseline rate was comparable to that of a recent report by Bischoff and associates after they improved hand hygiene compliance by using accessible alcohol-based antiseptics and increased hand-hygiene compliance (41% to 48%) after patient contact (5).

Table 2. Comparison of characteristics for health-care workers who performed hand hygiene to those who did not perform hand hygiene, Northwestern Memorial Hospital

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hand hygiene</th>
<th>Odds ratio (95% confidence interval)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glove use</td>
<td>Yes (n=258) (%)</td>
<td>No (n=463) (%)</td>
<td>5.7 (4.0 to 8.0)</td>
</tr>
<tr>
<td>Hand hygiene on room entry</td>
<td>42 (16)</td>
<td>18 (3.9)</td>
<td>4.8 (2.6 to 8.9)</td>
</tr>
<tr>
<td>Invasive procedure performed</td>
<td>34 (13)</td>
<td>25 (5.4)</td>
<td>4.4 (2.3 to 8.7)</td>
</tr>
<tr>
<td>Old hospital</td>
<td>161 (62)</td>
<td>143 (31)</td>
<td>3.7 (2.7 to 5.2)</td>
</tr>
<tr>
<td>Patient contact</td>
<td>130 (50)</td>
<td>132 (29)</td>
<td>2.6 (1.8 to 3.5)</td>
</tr>
<tr>
<td>Nurse</td>
<td>135 (52)</td>
<td>219 (47)</td>
<td>1.2 (0.9 to 1.7)</td>
</tr>
<tr>
<td>Physician</td>
<td>60 (23)</td>
<td>127 (27)</td>
<td>0.8 (0.6 to 1.2)</td>
</tr>
</tbody>
</table>

In room with a higher ranking person who did not perform hand hygiene | 12 (4.7) | 77 (17) | 0.2 (0.1 to 0.5) | <0.001 |

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio (95% confidence interval)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glove use</td>
<td>3.5 (2.4 to 5.1)</td>
<td>0.003</td>
</tr>
<tr>
<td>Invasive procedure performed</td>
<td>2.7 (1.4 to 5.1)</td>
<td>0.003</td>
</tr>
<tr>
<td>Hand hygiene performed on room entry</td>
<td>2.4 (1.2 to 4.5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Patient contact</td>
<td>2.1 (1.4 to 3.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Health-care workers with a higher ranking health-care worker or peer who did not wash hands</td>
<td>0.4 (0.2 to 0.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hospital units</td>
<td>1.0</td>
<td>--</td>
</tr>
<tr>
<td>Old hospital, non-ICU</td>
<td>1.0 (0.6 to 1.8)</td>
<td>0.89</td>
</tr>
<tr>
<td>New hospital, non-ICU</td>
<td>0.4 (0.2 to 0.7)</td>
<td>0.002</td>
</tr>
<tr>
<td>New hospital, ICU</td>
<td>0.4 (0.2 to 0.7)</td>
<td>&lt;0.001</td>
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<tr>
<td>New hospital, ICU</td>
<td>0.4 (0.2 to 0.7)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The hand-hygiene compliance we observed (finding that nearly 50% of our workers washed their hands after patient contact) was similar to the frequency of hand-hygiene compliance reported by other investigators (5,7,10–12). Even though we saw no improvement, our baseline rate was comparable to that of a recent report by Bischoff and associates after they improved hand hygiene compliance by using accessible alcohol-based antiseptics and increased hand-hygiene compliance (41% to 48%) after patient contact (5).
While some studies (5,15), and health-care worker surveys (29) suggest that sink access is an important determinant of hand-hygiene compliance, we found access is not the sole requirement needed to increase hand-hygiene compliance. Few reports address the impact of hospital design on hand-hygiene compliance. Kaplan and McGuckin (15) compared two units and demonstrated a greater hand-hygiene frequency among nurses in an MICU having a 1:1 sink-to-bed ratio compared with an SICU having a 4:1 sink-to-bed ratio (76% vs. 51%; p<0.01). However, a study by Preston and colleagues evaluating hand hygiene after the number of sinks in an ICU was increased found that improved sink access had no effect on hand-hygiene frequency (26). Possible explanations for the decreased hand-hygiene compliance we observed include: 1) more patient-days (5.2%) and more admissions (11.7%) per month occurred for study period II compared to study period I; 2) disrupted work flow because of the new and unfamiliar environment of the new hospital; 3) removal of hallway sinks; and 4) addition of new or temporary nursing staff because of the increased number of patients. Patient:nurse ratios are considered an important determinant in hand-hygiene compliance (6). In our study, we believe the ratios were similar in the old and new hospitals, but these data were only formally available for the ICUs. The average patient:nurse ratio during the observation periods in the new MICU was 1.42 (standard deviation [SD] 0.15) and in the old MICU, the ratio was 1.43 (SD = 0.05). We found similar data in the SICU areas, where average patient:nurse ratio during our observation periods in the new unit was 1.03 (SD = 0.24); in the old unit, the ratio was 1.22 (SD = 0.14). Thus, the ICU staffing patient:nurse ratio was similar during the observation shift for the ICUs in the old and new hospitals (p>0.2).

Because of changes in unit size throughout the new hospital, the study units used nursing staff from areas of the hospital with decreased staff requirements. Nursing personnel were reassigned to appropriately staff the newly configured larger patient-care areas. Specifically, nurses were transferred to units requiring the same education and skill sets (i.e., medical unit to medical unit, surgical unit to surgical unit, and intensive care to intensive care). To ensure adequacy of training, institutional education activities are systematic. All new employees are required to attend a hospital orientation that includes general information on infection-control issues. On the first day of work in the department, new and transferred employees also participate in individual departmental orientation. The orientation includes a review of policies and procedures, job-specific responsibilities, performance expectations, and unit-specific infection control measures (Northwestern Memorial Hospital Orientation Program, Human Resources Policy 4.96). For agency and float pool nursing staff, a 3-hour orientation is provided, which includes a review of policies, procedures, and paperwork. Float pool nurses have at least 1 year of experience before being hired and are required to complete annual competencies that incorporate infection-control measures. While they are used throughout the hospital, only nurses having cardiac-care certification may work in the ICUs.

Our study had several limitations. First, the two study periods were in different seasons and involved different house staff. A greater proportion of observations in the older hospital were conducted in the winter months, which we postulated would decrease hand-hygiene compliance because of increased skin dryness (30,31). However, hand-hygiene compliance was better in the old hospital, despite more observations being performed during the winter. Hand-hygiene rates were consistently lower in the new hospital for all types of health-care workers; a change in house staff (residents) was unlikely to have influenced our overall results. While nurse-to-patient ratios were not specifically measured on all units, we noted no obvious change in staffing levels, and when we compared ICUs, the staffing ratios were similar (p>0.2). Since we did not perform observations at night or on the weekends, and duration or efficacy of hand hygiene was not evaluated during our study, we cannot comment on hand-hygiene compliance for these shifts or on the effectiveness of health-care workers’ hand-hygiene technique.

Most units changed soap products between the old and new facilities, with the exception of the solid organ transplant unit. On this unit, the handwashing compliance dropped from 62% in the old hospital to 23% in the new hospital (data not shown); however, the decrease was similar to that observed in other monitored units, which suggests any change in soap product was not the major factor. Additionally, a change from powdered to nonpowdered gloves may have negatively influenced hand hygiene. Reviewing our data set for the potential influence of this factor, we found that if gloves were worn and removed, then hand-hygiene frequency in the old hospital was 131/176 (74%), whereas in the new hospital, the frequency was 76/128 (59%) (p=0.005). If gloves were not worn, then hand-hygiene frequency in the old hospital was 30/116 (26%), and in the new hospital 21/260 (8%) (p<0.001). Thus, both the absolute and relative decreases in hand-hygiene frequency

### Table 4. Effect of behavior of other health-care workers in the room on health-care workers’ hand-hygiene compliance, by multivariate analysis, Northwestern Memorial Hospital

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio (95% confidence interval)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room entry alone (n=291)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>In a room when a peer performs hand hygiene (n=48)</td>
<td>1.1 (0.6 to 2.3)</td>
<td>0.7</td>
</tr>
<tr>
<td>In a room when a higher ranking person performs hand hygiene (n=64)</td>
<td>0.8 (0.4 to 1.3)</td>
<td>0.3</td>
</tr>
<tr>
<td>Highest ranking person in the room (n=144)</td>
<td>0.6 (0.4 to 1.0)</td>
<td>0.07</td>
</tr>
<tr>
<td>In a room when peer does not perform hand hygiene (n=41)</td>
<td>0.4 (0.2 to 1.0)</td>
<td>0.05</td>
</tr>
<tr>
<td>In a room when higher ranking person does not perform hand hygiene (n=111)</td>
<td>0.2 (0.1 to 0.5)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Adjusted for variables significantly associated with increased hand-hygiene compliance, i.e., health-care worker glove use, hand hygiene on room entry, invasive procedures, patient contact, and old versus new hospital.

*Nurses and physicians accounted for most observations for all categories.
were greater for the nongloved health-care workers, which suggests that powder in the gloves was not the reason for diminished hand hygiene.

Pittel has posed the problem of hand-hygiene compliance: How can we change the behavior of health-care workers and how can we maintain such a change (32)? We strongly agree and believe the time has come to “think outside the box” for solutions to poor hand hygiene by health-care workers. Obtaining simple feedback by measuring soap and paper towel levels was recently shown not to have an impact (33). Our observations also show that another straightforward measure—improving health-care worker access to sinks—used as a sole measure, does not result in increased hand-hygiene compliance. However, the new facility is now ideally equipped to determine what is needed to improve hand-hygiene performance among health-care workers (34). To substantially improve hand-hygiene compliance, additional factors must be considered, including improving health-care workers’ skin conditions and using alcohol-based alternatives (a factor recently demonstrated to improve hand-hygiene compliance [35]), focusing on educational interventions, and providing administrative support. Since hand-hygiene compliance was significantly worse in groups where a ranking member of the group did not perform hand hygiene, a greater focus on improving compliance among physicians and nurses who are important role models may also result in better hand-hygiene compliance among all health-care workers.

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Ms. Lankford, a research nurse at Northwestern Memorial Hospital, began her career working in intensive care during the late 1970s and became a nurse investigator in the late 1990s, first in cardiology and later in infection control. She has presented or published six reports and is an active member of the Academy of Medical-Surgical Nurses.

References


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Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.
Aeromonas Isolates from Human Diarrheic Stool and Groundwater Compared by Pulsed-Field Gel Electrophoresis

Mark A. Borchardt,* Mary E. Stemper,† and Jon H. Standridge‡

Gastrointestinal infections of Aeromonas species are generally considered waterborne; for this reason, Aeromonas hydrophila has been placed on the United States Environmental Protection Agency Contaminant Candidate List of emerging pathogens in drinking water. In this study, we compared pulsed-field gel electrophoresis patterns of Aeromonas isolates from stool specimens of patients with diarrhea with Aeromonas isolates from patients’ drinking water. Among 2,565 diarrheic stool specimens submitted to a Wisconsin clinical reference laboratory, 17 (0.66%) tested positive for Aeromonas. Groundwater isolates of Aeromonas were obtained from private wells throughout Wisconsin and the drinking water of Aeromonas-positive patients. The analysis showed that the stool and drinking water isolates were genetically unrelated, suggesting that in this population Aeromonas gastrointestinal infections were not linked with groundwater exposures.

The Safe Drinking Water Act amendment of 1996 requires the United States Environmental Protection Agency (EPA) to establish a list of contaminants of public health concern that are known or anticipated to occur in drinking water systems and may require future regulation under the Safe Drinking Water Act. The list, known as the Contaminant Candidate List, is intended to generate scientific research that will assist the EPA in creating new regulations to protect the public from health risks associated with drinking water. Currently, the putatively emerging enteric pathogen, Aeromonas hydrophila, is included in the list because it has the potential to grow in water distribution systems, especially in biofilms, where it may be resistant to chlorination (1). However, the role of drinking water consumption in Aeromonas infections is unclear.

Three phenotypically defined species, A. hydrophila, A. caviae, and A. veronii biotype sobria, constitute 85% of all clinical isolates involved with gastrointestinal and extraintestinal infections (2). Whether Aeromonas is indeed a causative agent of gastroenteritis has been debated. Numerous case reports have described isolating Aeromonas from patients with acute diarrhea, but the bacterium can also be isolated from stool of healthy persons (3). Determining the enteropathogenicity of Aeromonas has been inconclusive, probably because of differences in diarrheagenic potential among strains. A consensus appears to be growing that certain strains are likely human enteric pathogens (2,4).

Aeromonas is ubiquitous in water, including chlorinated drinking water (5–7). In surface water, Aeromonas abundance peaks in the warm summer and fall months (8,9). In one municipality, the seasonal increase in Aeromonas detection in the drinking water supply matched the peak occurrence of clinical isolates (8). Aeromonas also occurs in groundwater (6,10,11), and in a single well, the same strain can persist for years (11). Some strains of Aeromonas isolated from water have been shown to possess virulence traits, such as adhesions, hemolysins, and cytotoxic enterotoxins, presumably involved with human pathogenicity (3,12,13).

If Aeromonas enteric infections are transmitted by drinking water and symptomatic infections are strain-specific, then the same strains isolated from patients with acute gastroenteritis should be found in drinking water. The objective of this study was to isolate Aeromonas from patients with acute diarrhea and, by using pulsed-field gel electrophoresis (PFGE), compare the molecular fingerprints of these isolates with isolates from the patients’ drinking water.

Methods

Fecal Specimens

This study was reviewed and approved by the Institutional Review Board of Marshfield Clinic. All diarrheic stool specimens submitted by physicians to Marshfield Laboratories, a clinical reference laboratory, for routine microbiologic analysis were screened for Aeromonas during two periods, July 28–November 13, 1998, and June 2–October 18, 1999. Specimens were plated for Aeromonas within 2–3 days after submission. Stool in Cary-Blair transport media was directly streaked to sheep blood agar containing 10 µg/mL ampicillin (14) (Remel, Lenexa, KS) and incubated at 35°C. Presumptive Aeromonas isolates were screened for standard phenotypic traits (β-hemolysis, oxidase positive, indole positive), and species iden-
tity was determined by using the API-20E identification system (10th edition, analytical profile index, bioMérieux, Marcy-‘Etoile, France).

**Drinking Water Samples**

Patients with positive results for *Aeromonas* were asked to allow a trained technician to collect a water sample from their residence. Samples were collected within 1–3 weeks after the clinical isolate was identified. *Aeromonas* was directly cultured from two 100-mL water samples by using ampicillin dextrin agar in a membrane filtration technique (15). One sample was incubated at 30°C and the other at 35°C. Yellow oxidase-positive colonies were streaked for purity and confirmed as *Aeromonas* by using the API-20E (bioMérieux) identification system. Stool and water isolates were stored in Microbank cryovials (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada) at –70°C for subsequent PFGE.

**PFGE**

The PFGE procedure for *Aeromonas* was modified from methods previously described (16,17). Isolates were grown overnight in 5 mL of brain heart infusion broth at 37°C, harvested by centrifugation, and washed with 1 mL resuspension buffer (10 mM Tris-HCl [pH 7.6], 1 M NaCl). Pelleted cells were adjusted to a concentration of 1 x 10⁹ CFU/mL in resuspension buffer by using a Vitek colorimeter (Hach Co., Loveland, CO), mixed with an equal volume of 2% low melt agarose (FMC Bioproducts, Rockland, ME), dispensed into plug molds (Bio-Rad Laboratories, Hercules, CA), and allowed to solidify 10 min at room temperature. Plugs were incubated in 3 mL lysis buffer (6 mM Tris-Cl, 1.0 M NaCl, 0.1 M EDTA, 0.5% Brij 58, 0.5% sarkosyl, 0.2% deoxycholate, 1 mg/mL lysozyme) at 37°C for 4 h. Lysis buffer was replaced with proteinase K solution (0.5 M EDTA, 1% N-lauroyl sarcosine, 1mg/mL proteinase K) followed by incubation at 55°C overnight. Plugs were washed 3 times in Tris-EDTA buffer (10 mM Tris-HCl, 0.1 M EDTA [pH 8.0]) and stored at 4°C. Genomic DNA was digested with 30 U *XbaI* (Promega Corp., Madison, WI) at 37°C overnight. Electrophoresis was performed in 1% Seakem agarose (FMC Bioproducts) by using the CHEF-DRIII system (Bio-Rad Laboratories) in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.0]) at 14°C. The running parameters were 150 V for 12 h with 20-sec pulses and 17 h with 5- to 15-sec pulse times. One *A. hydrophila* isolate (isolate 1320) was run in multiple lanes of each gel as a DNA global reference for standardizing runs. DNA band size was determined from *Staphylococcus aureus* strain NCTC 8325 DNA, digested with *SmaI*. DNA banding patterns were visualized with 0.1% ethidium bromide and digitally photographed. Molecular Analyst Fingerprinting Plus software (version 1.12, Bio-Rad Laboratories) was used to compare the genetic similarity among isolates and construct a similarity dendrogram by using the Dice coefficient and the UPGMA algorithm (unweighted pair-group method with arithmetic mean) with a position tolerance of 1.5%.

**Results**

Cultures for *Aeromonas* were performed on 2,565 diarrheic stool specimens from 2,310 patients. The median age of the patient population was 37 years (range 4 days to 97 years), and 55% were female. Most specimens (97.6%) were from patients residing in Wisconsin, primarily the central portion of the state, where groundwater, either from a municipal system or private well, is the source of drinking water (Figure 1). Some specimens came from communities along Lake Michigan where lake water is the source of drinking water.

Seventeen specimens (0.66%) from 17 patients (0.74 %) tested positive for *Aeromonas*. Three stool isolates were identified as *A. hydrophila* and 14 isolates were *A. caviae*. All positive specimens were from Wisconsin residents. The median age of *Aeromonas*-positive patients was 27 years (range 1 month to 87 years) and 59% were male. Five *Aeromonas*-positive patients were coinfected with one other enteric pathogen (two patients with *Campylobacter*, one with *Salmonella*, one with *Cryptosporidium*, and one with *Clostridium difficile* toxin A), suggesting that in these patients *Aeromonas* may have been a transient colonizer.

Fourteen of the *Aeromonas*-positive patients agreed to have their drinking water sampled. Five patients resided in a household with a private well, eight were served by municipal wells, and one lived in a municipality that used Lake Michigan for its drinking water. Except for one system, all municipal water was chlorinated. One drinking water source, a private well, tested positive for *A. hydrophila*. Designing this study, we assumed that the fecal carriage rate of *Aeromonas* would be

![Figure 1. Location of Wisconsin residents who submitted diarrheic stool specimens to Marshfield Laboratories. The symbol ⋄ indicates the location of Marshfield, WI. Symbol size is proportional to the number of specimens. (For reference, the symbol for Marshfield = 208 specimens.)](image-url)
similar to its carriage rate in another study conducted in the Midwest (18) and, based on the ubiquitous occurrence of *Aeromonas* in water, we anticipated that a number of isolates would be collected from patients’ drinking water. Since only one was collected, additional drinking water isolates were obtained by combining samples from 1,500 private wells throughout Wisconsin that had been submitted to the Wisconsin State Laboratory of Hygiene between September and November 1998 for routine coliform testing. The composite samples (composed of 4–10 well samples) were membrane filtered and cultured for *Aeromonas* as described above. This process yielded an additional 37 *A. hydrophila* and 17 *A. caviae* isolates.

PFGE of the stool and groundwater *Aeromonas* isolates yielded 10–20 well-resolved genomic DNA bands, ranging in size from approximately 10–400 kb (Figure 2). Six isolates (one stool, five groundwater) were not amenable to *Xba*I digestion, resulting in poorly resolved DNA fragments. PFGE patterns indicated extensive genetic diversity. The 65 isolates analyzed by PFGE had 58 distinct patterns. Five patterns grouped two or more identical isolates. Three of those groups included only isolates derived from the same composite water sample, suggesting multiple isolations of the same strain. Analyzing all pairwise comparisons among the 65 isolates, the median similarity was 59% (range 16% to 100%, n=2,080). Isolates from the same ecologic source also exhibited high genetic diversity. The median similarities of stool and groundwater isolates were 58% (range 30% to 76%, n=120) and 60% (range 25% to 100%, n=1,176), respectively. Among the 12 isolates from diarrheic stool specimens that were negative for other enteric pathogens, the median similarity was 58% (range 30%–76%, n=66).

None of the stool isolates was genetically indistinguishable from the groundwater isolates (Figure 2). The two isolates that appeared epidemiologically related, the patient stool isolate (isolate 0209) and the isolate from his private well (isolate 1320), were 72% similar by the Dice coefficient and differed by nine bands, which, following the criteria of Tenover et al. (19), would be generally interpreted as being genetically unrelated. The highest similarity between a stool and water pair of isolates was 88%, between isolates 1251 and 2094, which differed by four bands (Figure 2). However, the stool isolate was from a patient simultaneously positive for *Cryptosporidium* oocysts, suggesting that the *Aeromonas* was transient. The second highest similarity was 86%, a four-band difference between isolates 0949 and 1294 (Figure 2). Of the 784 paired comparisons between stool and water isolates, 776 (99%) had similarities <80%, and the median similarity was 58% (range 16% to 88%).

**Discussion**

The prevalence rate in this study was lower than rates from other large surveillance studies in the United States for *Aeromonas* in stool. *Aeromonas* was cultured from 2,848 diarrheic stool specimens submitted to a Los Angeles, California, hospital in the early 1980s and 80 (2.8%) were positive (20). Among 1,821 patients with diarrhea visiting a clinic in La Crosse, Wisconsin, during an 18-month period, Agger et al. (21) identified 20 (1.1%) that were positive for *A. hydrophila*. Moyer (18) examined 3,334 diarrheic stool specimens submitted by physicians over a 2-year period to an Iowa public health laboratory and found 238 (7.1%) positive for either *A. caviae*, *A. hydrophila*, or *A. sobria*. Isolation in the latter study included an alkaline peptone water enrichment step, which may explain the higher prevalence rate. In our study, the specimen prevalence rate was 0.66%, and this rate was likely biased upwards because stool specimens were collected only in the summer and fall months when the incidence of *Aeromonas* gastrointestinal infections is reportedly highest (18,21). The data, albeit limited, do not suggest that the prevalence of *Aeromonas* enteric infections is increasing in the United States. Worldwide, the isolation rate of *Aeromonas* from diarrheic stool has been reported as high as 10.8% (22) and as low as 0% (23). In the latter study, recently conducted in Melbourne, Australia, during a 68-week observation period, 795 fecal specimens were collected from city residents with highly credibl gastroenteritis. *Aeromonas* was not detected in any of the fecal specimens, even though 50% of water samples drawn weekly from the drinking water distribution mains serving the study participants were *Aeromonas*-positive (23).

The *A. hydrophila* and *A. caviae* species designations were equivocal in this study. Except for stool isolate 0858, all iso-
lates from stool identified by the API-20E system as A. caviae were β-hemolytic, suggesting that they were A. hydrophila instead. When the Vitek automated microbe identification system (bioMérieux) was used, all stool and water isolates were identified as the A. hydrophila/caviae group. If the most recent edition of the API analytical profile index were used with the A. caviae profiles derived during the study, the new species designation would be A. hydrophila group 1. Some profiles determined in this study are not listed in the most recent index and, given the ever-changing taxonomy of aeromonads (4), we opted for a consistent one-index approach, reporting the species designations for all stool and water isolates on the basis of the index available at the time of the study.

The high level of genetic diversity observed in our study among clinical and environmental strains of Aeromonas has been corroborated by other nucleic acid–based subtyping methods, such as amplified fragment length polymorphisms (11) and ribotyping (24). Talon et al. (17) subtyped 10 epidemiologically unrelated strains of A. hydrophila by PFGE and reported that the median similarity (calculated by using the Pearson correlation coefficient) was 28.4% (range 9.3% to 44.3%). The variation is not likely due to genetic lability, because the PFGE patterns of Aeromonas reportedly do not become unstable during frozen storage and long-term laboratory culture (17,25). As a control in this study, isolate 1320 was digested and underwent electrophoresis five independent times, and each time yielded the same PFGE pattern. The PFGE patterns also did not correspond to phenospecies. Millersh et and Want (26) reported a similar finding based on whole-cell protein fingerprinting.

The capacity for human enteropathogenicity among clinical isolates may be derived from a unique set of genes that were acquired or evolved in a common ancestor. Alternatively, enteropathogenicity may have arisen independently among several genotypes. In either scenario, one might expect the subset of Aeromonas strains that are pathogenic to have less genetic variation than the environmental strains. In this study, the level of genetic diversity was similar between environmental and clinical strains, even when the clinical strains were restricted to the subset from stool specimens that were negative for other enteric pathogens. One explanation is that some clinical strains were transient gastrointestinal colonizers, and only a few strains are truly diarrheagenic. This issue will likely remain unresolved until the pathogenicity mechanisms of Aeromonas are better understood.

PFGE is a reproducible, highly discriminatory subtyping method capable of identifying the transmission source of bacterial infections (19). If the Aeromonas-positive patient with the positive well acquired the infection from his drinking water, the molecular fingerprints of the stool and water isolates should at least have been closely related (i.e., ≤3-band difference). Likewise, if drinking water is a frequent source of Aeromonas infections in this study population, one would expect at least a few of the stool and water isolates to be more closely related than a four-band difference. The analysis for this study was weighted towards identifying similar stool and water isolates. The 1.5% position tolerance that was selected for band calling resulted in matching bands that differed by as much as 17 kb, and the Dice coefficient gives greater weight to matching bands compared to other similarity coefficients.

The study had several limitations, however, that need to be considered when interpreting the data. Only one isolate per stool specimen or water sample was analyzed by PFGE. Multiple strains may have been present in water, and by chance the enteropathogenic strains were not selected for PFGE analysis. Kühn et al. found multiple Aeromonas strains in a single drinking water source, although only one or a few strains were numerically dominant, and these could persist for years (6,11). When duplicate water samples (incubated at 30°C and 35°C) both yielded Aeromonas, then PFGE was performed on a colony from each plate. Some of these isolate pairs were indistinguishable by PFGE, and some were unrelated, showing that some composite water samples had at least two different strains. Both strains were included in the genetic similarity analysis. Another study limitation was that if diarrheagenic Aeromonas strains are very rare in groundwater, the sample size might have been insufficient to find those strains, even though the composite water samples tested represented 1,500 wells. The stool and water isolates were collected from the same geographic area and during the same period, which should have increased the odds of finding genetically similar isolates if the two ecologic sources are linked. Finally, the drinking water sample volume for Aeromonas isolation was 100 mL. Since the study was conducted, the EPA has developed and validated Method 1605 for detecting Aeromonas in drinking water, which specifies a minimum sample volume of 1 L (27). Possibly, if the sample volume had been 1 L, more patient wells would have been Aeromonas positive.

To our knowledge, this is the first study to use PFGE to compare Aeromonas strains from human stool with strains found in groundwater. Other studies have compared the relatedness of strains from drinking water and stool by fatty acid methyl ester profiles (28), ribotyping (29), and randomly amplified polymorphic DNA (30), all highly discriminatory subtyping methods, and found little similarity between clinical and environmental isolates. Aeromonas isolates from stool and drinking water have been linked by biotyping (31) and whole-cell protein fingerprinting (25), but the discriminatory power of these methods with Aeromonas is questionable (28,30). Thus, the evidence to date from using highly discriminatory subtyping methods suggests that human enteropathogenic strains are rare in drinking water. In the group of primarily Wisconsin residents in this study, Aeromonas was identified infrequently in diarrheic stool specimens and drinking water from a groundwater source did not appear to be an Aeromonas transmission route.

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We thank Phillip Bertz, Sharon Kluender, Jeremy Olstadt, Susan Spencer, and Amy Witte for their technical assistance; Carla Finck for
providing data management support; Alice Stargardt for assisting with manuscript preparation; and Lynn Iavic for preparing the graphics.

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Dr. Borchardt is a research scientist at Marshfield Medical Research Foundation in Marshfield, Wisconsin. His research interests include waterborne infectious diseases and agricultural zoonoses.

References


Risk Factors for Sporadic Giardiasis: A Case-Control Study in Southwestern England

James M. Stuart,* Hilary J. Orr,* Fiona G. Warburton,† Suganthiny Jeyakanth,† Carolyn Pugh,‡ Ian Morris,‡ Joyshri Sarangi,§ and Gordon Nichols¶

To investigate risk factors for sporadic infection with *Giardia lamblia* acquired in the United Kingdom, we conducted a matched case-control study in southwest England in 1998 and 1999. Response rates to a postal questionnaire were 84% (232/276) for cases and 69% (574/828) for controls. In multivariable analysis, swallowing water while swimming (p<0.0001, odds ratio [OR] 6.2, 95% confidence intervals [CI] 2.3 to 16.6), recreational fresh water contact (p=0.001, OR 5.5, 95% CI 1.9 to 15.9), drinking treated tap water (p=0.0001, OR 1.3, 95% CI 1.1 to 1.5 for each additional glass per day), and eating lettuce (p=0.01, OR 2.2, 95% CI 1.2 to 4.3) had positive and independent associations with infection. Although case-control studies are prone to bias and the risk of *Giardia* infection is minimized by water treatment processes, the possibility that treated tap water is a source of sporadic giardiasis warrants further investigation.

*Giardia lamblia*, a flagellate waterborne protozoan parasite, is a common cause of gastrointestinal disease in industrialized and unindustrialized countries (1). Most information on risk factors for giardiasis has come from investigation of outbreaks. Water is the most frequently identified route of transmission (1,2), through drinking contaminated tap water (3,4) or recreational exposure in lakes, rivers, or swimming pools (5). Person-to-person spread is well documented in day-care centers and among male homosexuals (6,7), and food may also be a vehicle of infection (8).

The relative contribution of these routes of transmission to sporadic cases of giardiasis is unknown, and further studies have been recommended in the United States (9). In the U.K., recreational exposure to fresh water and swimming pools has been identified as a possible risk factor (10–12), as well as travel to developing countries (11). We set out to determine risk factors for giardiasis in residents of southwest England who had not recently traveled outside the U.K.

Methods

Our study was conducted from April 1, 1998, to March 31, 1999 in southwest England, including Cornwall and Isles of Scilly, Devon, Somerset, Dorset, Wiltshire, Hampshire, Isle of Wight, Avon, Gloucestershire, and Herefordshire. Laboratories used for the study were the microbiology laboratories (managed by National Health Service trust or Public Health Laboratory Service) in the study area that routinely screened for *Giardia* cysts in all stool specimens submitted from persons with history of diarrhea. Study laboratories were located in Torbay, Exeter, Yeovil, Dorchester, Poole, Bournemouth, Salisbury, Basingstoke, Winchester, Portsmouth, Newport (Isle of Wight), Weston-super-Mare, Bristol/Bath, Frenchay, Cheltenham, and Hereford. All microbiology laboratories in the study area that fulfilled the study criteria (16/24) agreed to participate. Ethical approval for the study was obtained from the Public Health Laboratory Service Ethics Committee, the Southwest Multicentre Research Ethics Committee, and 16 Local Research Ethics Committees.

Case-patients were defined as residents of the study area with a history of diarrhea and *Giardia* cysts in their stool specimen seen by light microscopy (13) in the study period. Case-patients were excluded if they had traveled outside the U.K. in the 3-week period before onset of diarrhea or if a household member had diarrhea in the 3-week period before the patient’s onset of diarrhea. Controls were persons registered at the same general practice as patients, of the same gender, and in the same broad age band (0–5 years, 6–15 years, ≥16 years). Controls were excluded if they had traveled outside the U.K. during the 3 weeks before interview or if they had diarrhea in the 3 weeks before interview.

The study questionnaire was derived from an instrument used in a case-control study of cryptosporidiosis in the north of England (S. Goh, pers. comm.). The questionnaire covered personal details, recent illness, travel, water contact, water and food consumption (food history focusing on dairy produce, salads, fruit), and contact with animals, farms, and day nurseries. Adults were asked for details of occupation. The questionnaire was modified from a pilot study; the wording of some questions was simplified, and a question about previous infection or treatment of *Giardia* infection was added. Patients were asked about exposures in the 3 weeks before onset of illness.
and controls were asked about exposures in the 3 weeks before completion of the questionnaire. In questions about drinking unboiled tap water and bottled water, patients and controls were both asked about usual consumption patterns.

Cases were reported on identification to the principal researcher by fax or post. Health authorities, through the consultant in communicable disease control, randomly selected three controls for each case selected from the Health Authority population age-sex register. If the general practitioner had no objection to contacting patients and controls, the questionnaire was sent out with a covering letter. One month after the first questionnaire was posted, nonresponders were sent one reminder.

A required sample size of 192 cases was estimated by using Generalised Linear Interactive Modelling (GLIM) (14). This size was based on a case-control ratio of 1:3, with 95% confidence and 80% power to detect an odds ratio (OR) of at least 3 for the variable under investigation. Data were double entered onto a microcomputer, and single variable conditional logistic regression was carried out by using GLIM (15). Variables with p<0.2 in the single variable analysis were then included in a multivariable conditional logistic regression analysis. A series of models were fitted; clearly nonsignificant terms were eliminated to maximize the number of subjects that could be used in the regression modeling. As a large number of observations were “don’t know” or “missing” for the “swallowing water while swimming” variable, an extra level was included for this variable. This addition resulted in a final main effect model that included 655 subjects (95% of the 689 available). All the 2-way interactions were tested, including those involving age and sex.

Results

Questionnaires were returned from 232/276 case-patients and 574/828 controls, response rates of 84% and 69%, respectively. Two additional patients were not included because their general practitioners did not agree to participate in the study. After the exclusion criteria were applied, data from 192 cases and 492 controls were available for analysis, a ratio of 1:2.5. Of the 40 case-patients excluded, 18 had traveled outside the U.K., 15 were possible secondary case-patients, and 7 did not report diarrhea. Of the 82 excluded controls, 43 reported diarrhea, 38 had traveled outside the U.K., and reasons for exclusion were missing for one. The incidence of giardiasis fulfilling the study criteria was 4.7/100,000 during the 12-month study period. Seasonal variation was not marked, and no outbreaks were detected during the study. Cases were most frequent in the 30- to 39-year age group. Although frequency matching had been used to obtain controls with similar age distribution to the case-patients, some differences were observed in those remaining for analysis: 17% of the patients and 22% of the controls were <16 years of age. Eighty-seven (44%) of the case-patients were male, and 109 (56%) were female. Among the nonresponders, a higher proportion were male (33/44,) but the age distribution was similar. Social class distribution was similar in adult case-patients and controls (p=0.1).

By definition, all case-patients (192) had diarrhea. One hundred and forty-eight (77%) case-patients had abdominal pain, 73 (38%) reported vomiting, 53 (28%) had a fever, 12 (6%) noted blood in stools, and 90 (47%) reported other symptoms, including tiredness, weight loss, nausea, and headache. The median reported duration of illness was 21 days (range 1–305). Thirteen patients reported that they were still unwell at the time of completing the questionnaire.

In the single variable analysis, a dose-response relationship existed between reported number of glasses of tap water usually drunk per day and risk of illness (p=0.0001, Table 1). No evidence of a dose-related response existed for those who drank only bottled water. The homes of >98% of patients and controls were on public water supply (provided by one of the seven statutory water companies in the study area). Most case-patients (73%) were supplied by the same water treatment works as their matched controls. Home water supply was purely surface water for 40% of cases and 41% of controls, and purely ground water for 39% of cases and 33% of controls; data on water supply at work were not available. Several exposures relating to swimming in chlorinated water and fresh water contact were significantly associated with illness (Table 2). In a wide range of questions on food consumption, eating lettuce was the only variable significantly (p<0.05) associated with infection (OR 2.3, 95% confidence interval [CI] 1.3 to 4.1, p=0.003). Visiting a farm was more frequent among case-patients (OR 2.2, 95% CI 1.2 to 4.1, p=0.01), but none of the associations with specified animal exposures (dogs, cats, horses, cows, sheep) was statistically significant. Case-patients were more likely to have contact with a nursery (OR 2.2, 95% CI 1.2 to 4.2, p=0.01).

In the multivariable analysis, swallowing water while swimming, recreational contact with fresh water, dose response to drinking tap water, eating lettuce, and not eating ice cream were independently associated with illness (Table 3). For swallowing water while swimming, the “don’t know/missing” category had an OR of 5.3 (95% CI 1.9 to 14.8). If analysis of the water swallowing variable was restricted to those who specified chlorinated water, the association remained highly significant in the model (p=0.006, OR 3.9, 95% CI 1.3 to 11.4). Attributable fractions were estimated as 14% for swallowing water, 9% for fresh water contact, 35% for drinking three or more glasses of tap water per day, and 40% for eating lettuce.

Discussion

We found an association between drinking tap water and giardiasis in persons who had not recently traveled outside the U.K. This finding had a high level of statistical significance and was unexpected. U.K. public water supplies are thought to be at low risk for transmitting Giardia infection because of the lower resistance of Giardia cysts to chlorination compared with Cryptosporidium oocysts (16,17), their easier removal by
filtration (18), and the few outbreaks of giardiasis attributed to drinking water (19). A causal link cannot, however, be excluded, as *Giardia* cysts have been frequently found in U. K. surface waters (D. Sartory, pers. comm.), and outbreaks attributed to chlorinated water in the United States are well documented (3,20). The association with drinking water might have been overestimated because of changes in water consumption after illness or because of bias. Information bias is a recognized problem in case-control studies; such bias was not found, however, in a case-control study conducted during and after a giardiasis outbreak in Nevada (21). The matched design of our study reduced the risk of error from confounding factors but also resulted in close matching on home water supply such that assessing the effect of drinking water quality indicators on outcome was not possible.

Another new finding was an increased risk for giardiasis associated with eating lettuce (green salad). A true risk is certainly plausible, as irrigation and fertilization practices may lead to contamination with *Giardia* and other organisms (22,23). Outbreaks of giardiasis in the United States have been attributed to eating salad contaminated by food handlers (24). The negative association between illness and eating ice cream is unlikely to represent a true protective effect. This finding may have arisen from study biases, but preference for ice cream could simply be a marker for those who are less likely to eat salads, which are at higher risk of *Giardia* contamination. An apparently false protective effect of soft drinks has been observed in outbreaks of cryptosporidiosis attributed to tap water (25).

The association between giardiasis and swallowing water while swimming is not surprising; several outbreaks of giardiasis have been linked with exposure to chlorinated water in swimming pools (3,5). If those who did not know if they swallowed water were more likely to have actually swallowed

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**Table 1. Single variable analysis of risk factors for giardiasis: drinking water exposures**

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Cases (n=192)</th>
<th>Controls (n=492)</th>
<th>Matched odds ratio (95% CI)*</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consumed tap water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>17</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>174</td>
<td>410</td>
<td>2.3 (1.1 to 4.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>Usual no. of glasses of tap water consumed per day (glass=approx 1/3 pint)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>17</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5–1.0</td>
<td>22</td>
<td>71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5–2.0</td>
<td>52</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5–3.0</td>
<td>23</td>
<td>46</td>
<td></td>
<td></td>
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<tr>
<td>3.5–4.0</td>
<td>23</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5–5.0</td>
<td>20</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5.0</td>
<td>33</td>
<td>61</td>
<td>1.2 (1.1 to 1.4)*</td>
<td>&lt;0.0001</td>
</tr>
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<td>Consumed bottled water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>154</td>
<td>406</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>38</td>
<td>83</td>
<td>1.6 (0.9 to 2.9)</td>
<td>0.1</td>
</tr>
<tr>
<td>Usual no. of glasses of bottled water consumed per day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>154</td>
<td>406</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>8</td>
<td>1.2 (0.9 to 1.6)*</td>
<td>0.3</td>
</tr>
<tr>
<td>Used water filter at home</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>157</td>
<td>387</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>34</td>
<td>104</td>
<td>0.9 (0.5 to 1.7)</td>
<td>0.8</td>
</tr>
<tr>
<td>Type of water supply</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Public</td>
<td>187</td>
<td>483</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Private</td>
<td>3</td>
<td>3</td>
<td>0.05 (0.002 to 1.11)</td>
<td>0.04</td>
</tr>
<tr>
<td>Had a disruption in the water supply</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>124</td>
<td>447</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>11</td>
<td>3.6 (0.6 to 20.8)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*a* CI, confidence intervals.

*b* For each additional glass.
water, this could explain the high OR in this category. A higher risk of exposure to recreational fresh water accords with results of other studies (10,12,26). By contrast with studies from the United States (26,27), contact with children’s nurseries was not found to be an independent risk factor. In New Zealand, where the attack rate among young children is relatively high, an increased risk was found in adults who changed infants’ diapers (28), and information on household contact with young children should be sought in further studies. No risk was identified from contact with animals. One case-control study in the east of England showed associations with exposure to farm animals and pets, particularly pigs, dogs, and cats (29); other studies have not found such associations (26,30). The microbiologic evidence for zoonotic transmission is similarly conflicting (2). Although no questions were included on sexual behavior, the similar numbers of male and female cases in the study does not support a hypothesis that male homosexual behavior was an important method of transmission in this population.

The incidence of laboratory-reported cases of Giardia infection in England and Wales was 8.2/10^5 in 1998–2000 (31), and a study of infectious intestinal disease in England in 1993–95 suggested that the true incidence rate of giardiasis was around 50/10^5 (32). Giardia infection may be asymptomatic in children, but infection in adults usually leads to acute severe gastrointestinal illness that may be prolonged (median duration 3 weeks in our study) and may lead to chronic malabsorption and weight loss. In marked contrast to most other gastrointestinal infections, giardiasis can be successfully treated with drugs such as metronidazole (1). Higher awareness of non–travel-associated infection is needed in primary care and diagnostic microbiology laboratories so that correct treatment

### Table 2. Single variable analysis of risk factors for giardiasis: recreational water exposures

<table>
<thead>
<tr>
<th>Swimming and related activities by location and frequency:</th>
<th>Cases (n=192)</th>
<th>Controls (n=492)</th>
<th>Matched odds ratio (95% CI)^a</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swimming anywhere</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>123</td>
<td>406</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>66</td>
<td>85</td>
<td>3.9 (2.2 to 7.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Swimming while swimming</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>132</td>
<td>428</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>26</td>
<td>33</td>
<td>6.6 (2.5 to 17.6)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Frequency of swimming</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>123</td>
<td>406</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–2</td>
<td>29</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3–4</td>
<td>22</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5–6</td>
<td>6</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7–8</td>
<td>4</td>
<td>11</td>
<td>1.3 (1.1 to 1.5)^b</td>
<td>0.002</td>
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<tr>
<td>Chlorinated water exposure</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swimming</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>149</td>
<td>434</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>43</td>
<td>58</td>
<td>2.7 (1.4 to 5.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Swallowed water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>174</td>
<td>464</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18</td>
<td>28</td>
<td>2.4 (0.9 to 6.0)</td>
<td>0.07</td>
</tr>
<tr>
<td>Head immersion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>156</td>
<td>443</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>36</td>
<td>49</td>
<td>2.5 (1.3 to 5.0)</td>
<td>0.009</td>
</tr>
<tr>
<td>Fresh water exposure</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>172</td>
<td>469</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20</td>
<td>23</td>
<td>5.1 (2.0 to 12.5)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Swallowed water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>191</td>
<td>491</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1</td>
<td>1</td>
<td>3.9 (0.1 to 136)</td>
<td>0.4</td>
</tr>
<tr>
<td>Head immersion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>188</td>
<td>489</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3</td>
<td>2</td>
<td>26.6 (1.2 to 572)</td>
<td>0.02</td>
</tr>
<tr>
<td>Sea water exposure</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>187</td>
<td>486</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
<td>6</td>
<td>1.2 (0.2 to 8.4)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

^a CI, confidence intervals.

^b For each additional visit.

### Table 3. Multivariable analysis of risk factors for giardiasis

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Odds ratio (95% CI)^a</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swallowed water while swimming</td>
<td>6.2 (2.3 to 16.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Recreational fresh water contact</td>
<td>5.5 (1.9 to 15.9)</td>
<td>0.001</td>
</tr>
<tr>
<td>Each additional glass of tap water consumed per day</td>
<td>1.3 (1.1 to 1.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ate lettuce</td>
<td>2.2 (1.2 to 4.3)</td>
<td>0.01</td>
</tr>
<tr>
<td>Ate ice cream</td>
<td>0.4 (0.2 to 0.7)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

^a CI, confidence intervals.
can be given and disease incidence measured more accurately. A causal link between exposure to treated drinking water and sporadic giardiasis would have considerable public health importance but cannot be established on the basis of this study. Further case-control studies, in conjunction with assessment of drinking water quality that includes examination for *Giardia* cysts, would help to resolve this issue.

**Acknowledgments**

We are grateful for the help and cooperation received during this study from laboratory, health authority, and primary care staff in southwest England. We particularly thank Nick Andrews for statistical support; case-patients and controls for their kind assistance in completing questionnaires, and the water companies in the study area for supplying water quality data.

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Dr. Stuart is a regional epidemiologist in southwest England, working for the national Communicable Disease Surveillance Centre. He has a research interest in the epidemiology of infectious diseases.

**References**


Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.
Katy L. Davison,* Natasha S. Crowcroft,* Mary E. Ramsay,* David W.G. Brown,† and Nick J. Andrews*

We analyzed hospitalizations in England from April 1, 1989, to March 31, 1998, and identified approximately 700 cases, 46 fatal, from viral encephalitis that occurred during each year; most (60%) were of unknown etiology. Of cases with a diagnosis, the largest proportion was herpes simplex encephalitis. Using normal and Poisson regression, we identified six possible clusters of unknown etiology. Over 75% of hospitalizations are not reported through the routine laboratory and clinical notification systems, resulting in underdiagnosis of viral encephalitis in England. Current surveillance greatly underascertains incidence of the disease and existence of clusters; in general, outbreaks are undetected. Surveillance systems must be adapted to detect major changes in epidemiology so that timely control measures can be implemented.

Routine laboratory reports and statutory clinical notifications of infectious diseases are a source of viral encephalitis surveillance in England; however, these methods are considered to be incomplete. Hospital episode statistics record hospital admissions of viral encephalitis; although not timely enough for surveillance, these statistics can be used to monitor the distribution of admissions attributed to viral encephalitis by hospital physicians.

Most cases of viral encephalitis in the U.K. are thought to be rare complications of common infections. Herpes simplex virus (hereafter referred to as herpes) is the virus most often associated with encephalitis (1–3). Other viruses known to cause encephalitis include varicella-zoster virus 1 (VZV), measles virus, rubella virus, Lymphocytic choriomeningitis virus, cytomegalovirus, Epstein-Barr virus, and the arboviruses (4–6). Cases of encephalitis attributed to arthropod-borne viruses (arboviruses) are common in certain areas of the world, but only rare, imported cases have been reported in the U.K. (7,8).

Clinical diagnosis of viral encephalitis is based on symptoms such as fever, headache, and altered mental state; however, a definitive diagnosis of viral encephalitis relies on detecting the virus in cerebrospinal fluid or brain. The use of virologic investigation has been inconsistent in England. Virus isolation, formerly the standard criterion for diagnosis, is steadily being replaced by polymerase chain reaction (PCR) (9–11), which is more sensitive and provides a more rapid result. However, PCR was not widely used at the time of this study (9,12,13).

The demonstration of specific intrathecal antibody, either by detecting a raised antibody index or by detecting specific oligoclonal bands in cerebrospinal fluid, provides evidence of recent neurologic infection. When combined with an appropriate clinical picture, this demonstration is considered diagnostic (14). A noninvasive investigation such as magnetic resonance imaging (MRI) is also diagnostic (15). Serologic confirmation based on a fourfold rise in the level of antibody to the virus in the acute- and convalescent-phase blood samples or the demonstration of an intrathecal antibody response also provides evidence of recent infection.

The accurate diagnosis of viral encephalitis is important, particularly so for herpes and VZV encephalitis because several effective antiviral drugs are widely available for treatment (1,16,17). Accurate diagnosis is also required to increase the usefulness of surveillance so that the pattern of viral encephalitis cases can be monitored, especially since concern is increasing about the potential problems posed by new and reemerging infections (18). As new treatments and vaccines for existing viral infections become available, good surveillance data are required to accurately describe the public health cost of viral encephalitis, develop appropriate vaccination strategies, and perform treatment algorithms (available from: URL: http://www.isabel.org.uk/) (19,20).

This study describes the epidemiology of viral encephalitis in England from 1989 to 1998 by using hospital episode statistics (available from: URL: http://www.doh.gov.uk/hes/) and evaluates routine surveillance systems in terms of their ability to quickly and accurately monitor sporadic cases and clusters. We also consider possible future surveillance methods.

Methods

Data Sources

Hospital episode statistics provide information on hospital care in National Health System hospitals in England. No case definition is available for viral encephalitis, but diagnosis is generally based upon clinical evidence of viral encephalitis and available confirmatory laboratory data as recorded in the patient’s medical record at the time of discharge. The diagnosis is recorded by using the World Health Organization Inter-
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national Classification of Disease (ICD) codes. These codes allow for specific (clinical and laboratory evidence) and non-specific (only clinical evidence) diagnoses. We analyzed reports of hospital admissions for all adults and children (<17 years of age) from April 1, 1989, to March 31, 1998. Cases of viral encephalitis were identified by ICD ninth edition (ICD-9) codes for admissions in 1989–1995 and 10th edition (ICD-10) codes for admissions in 1995–1998. Patient records were also extracted if any of seven diagnostic codes mentioned a diagnosis of viral encephalitis (Table 1). Since no ICD-9 code has been defined for VZV or adenovirus encephalitis, any VZV or adenovirus infection code accompanied by a code for nonspecific viral encephalitis before 1995 was included in the analysis. Multiple episodes for one patient were considered to be a single infection if <1 month elapsed between episodes.

The second data source was laboratory reports sent to the Communicable Disease Surveillance Centre from the Public Health Laboratory Service (PHLS), National Health System, and private laboratories in England. All laboratory reports of isolates of viruses known to cause encephalitis from January 1, 1990, to December 31, 1998, were extracted from LabBase (the PHLS laboratory network electronic database). From these reports, infections thought to be associated with encephalitis were identified. Data extracted included laboratory reports in England with specimen date between January 1, 1990, and December 31, 1998, of adenoviruses, herpes, VZV, cytomegalovirus, lymphocytic choriomeningitis virus, Epstein-Barr virus, measles, mumps, or rubella detected in the cerebrospinal fluid, or adenovirus, herpes, VZV, cytomegalovirus, lymphocytic choriomeningitis virus, Epstein-Barr virus, measles, mumps, or rubella in any specimen with an encephalitis diagnosis in the feature or comment field.

An additional data source was the Notifications of Infectious Disease System (NOIDS) (available from: URL: www.phls.co.uk/facts/NOIDS/noid.htm). Clinically diagnosed cases of viral encephalitis are reported weekly as acute, infectious encephalitis to consultants in Communicable Disease Control and collated by the Communicable Disease Surveillance Centre. Data collected included the week of the report and patients’ age, sex, and local health authority of residence. Reports from 1989 through 1998 were available for analysis.

The final source was certified deaths reported to the Office of National Statistics. Information about deaths attributed to viral encephalitis from January 1, 1993, to December 31, 1998, in England was available from Office of National Statistics data held by the Communicable Disease Surveillance Centre. All deaths were coded with an ICD-9 designation.

Analysis

We investigated trends in hospitalizations of viral encephalitis by fiscal year, using 1995 mid-year resident population estimates for England to calculate rates. Single- and multivariable regression was used to compare rates by fiscal year, age group (<1, 1–4, 5–9, 10–16, 17–24, 25–34, 35–44, 45–54, 55–64, and >65 years of age), sex, and regional health authority of hospital (Anglia and Oxford, North West, South and West, Trent, North Thames, Northern and Yorkshire, South Thames, and West Midlands). We initially investigated evidence of clustering of case-patients with a nonspecific diagnosis by comparing weekly totals of hospitalizations of nonspecific viral encephalitis to the weekly overall mean number during the study period. Possible clusters were considered to have occurred when the weekly total was ≥2.58 and ≥3.3 standard deviations from the mean, corresponding to 99.5% and 99.95% upper prediction limits, respectively. In addition, we used Poisson regression to calculate an upper weekly and monthly prediction limit (at 99.5% and 99.95%) for each hospital’s district health authority. Any weekly or monthly count greater than or equal to the limit in each district was flagged as a possible outbreak. We compared NOIDS and laboratory reports to hospital episode statistics to determine the level of underreporting in each.

Results

Hospital Episode Statistics

From April 1, 1989, to March 31, 1998, a total of 6,464 adults and children were hospitalized in England with a diag-

Table 1. Diagnostic codes used to identify cases of viral encephalitis in hospital episode statistics

<table>
<thead>
<tr>
<th>Encephalitis diagnosis</th>
<th>ICD-9</th>
<th>ICD-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific diagnosis: Exotic virus</td>
<td>0620–0629, 0630–0638, 064, 0661, 0622, 3233</td>
<td>A830–A839, A840–A849, A852</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>0543</td>
<td>B004</td>
</tr>
<tr>
<td>Varicella-zoster virus 1</td>
<td>Undefined</td>
<td>B011, B020</td>
</tr>
<tr>
<td>Measles virus</td>
<td>0550</td>
<td>B050</td>
</tr>
<tr>
<td>Mumps</td>
<td>0722</td>
<td>B262</td>
</tr>
<tr>
<td>Rubella</td>
<td>0560, 0567</td>
<td>B060</td>
</tr>
<tr>
<td>Lymphocytic choriomeningitis virus</td>
<td>0490, 3126</td>
<td>A872</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Undefined</td>
<td>A851</td>
</tr>
<tr>
<td>Other</td>
<td>0498, 3234</td>
<td>A858</td>
</tr>
<tr>
<td>Nonspecific diagnosis: Unspecified</td>
<td>0499, 3239</td>
<td>A86, G051</td>
</tr>
</tbody>
</table>

*ICD, International Classification of Disease.
nosis of viral encephalitis (Table 2), a figure that corresponds to an estimated annual rate of 1.5 cases per 100,000 population. Most of these patients had a nonspecific infection. A specific diagnosis was recorded for 2,574 patients, the largest proportion of which (52%) had herpes infection. The other diagnoses included 18% with “other” and 13% with VZV. The remaining 223 included patients with encephalitis associated with measles, mumps, rubella, exotic viruses (arboviruses), and Lymphocytic choriomeningitis virus infections.

A total of 2,734 cases were diagnosed in children (including 35 in neonates), corresponding to an estimated annual rate of 2.8 per 100,000 children and accounting for 43% of all hospitalizations of viral encephalitis. An estimated 21% declining trend occurred in the rate over the 9 years, although this decline is not significant (p=0.065). Over half (51%) of the hospitalizations of children occurred for those <5 years of age, with the highest rate (8.7/100,000) for infants <1 year of age. The rate remained highest for infants throughout the 9 years of the study; however, we found some variation between the age groups in the time trends that approached significance (p=0.063), with the largest decline in adolescents ages 10–16 years (estimated to be 37%) and the smallest in toddlers 1–4 years of age (estimated to be 4%). Little difference appeared in the rate in male children compared to female children overall (2.9 and 2.8/100,000 children; p=0.23); furthermore, the trend over the 9 years did not differ by sex (p=0.63).

In adults, 3,680 patients diagnosed with encephalitis were hospitalized, which approximates an annual rate of 1.1 per 100,000 adults with no overall significant trend over time (p=0.17). No significant variation appeared in the rate between the age groups overall, but the highest rate occurred in those aged 17–24 years at approximately 1.2 per 100,000. The change in the incidence of hospitalized case-patients over time varied between age groups (p=0.0016); this change was mostly attributable to an increase of 46% in the rate in elderly case-patients (>65 years of age) with no significant changes in other age groups. We found no difference in the overall rate of male case-patients compared to female case-patients (1.1 and 1.0/100,000 adults; p=0.12); furthermore, the trend over the 9 years did not differ by sex (p=0.74).

The rate between the regional health authority of hospitals did not vary significantly overall (p=0.55); however, the largest proportion of cases (16%) and highest rate (1.63/100,000 population) were hospitalized in the North Thames region. The trend over the 9 years did not significantly vary by regional health authority (p=0.13).

The proportion of cases with a specific diagnosis was significantly lower in children (33%) than adults (45%) (p<0.001). In children, this proportion varied throughout the study (p<0.001); it was at a minimum of 23% during 1992–1993 and a maximum of 50% during 1996–1997. Children without a specific diagnosis were significantly younger than those with (mean age of 5 vs. 6.1 years; p<0.001) but did not differ in terms of sex. In adults, the proportion with a specific diagnosis was not significantly different by year. Those with a specific diagnosis were significantly older compared to those without (mean age 49 vs. 43 years; p<0.001) but did not differ in terms of sex. The proportion of all cases with a specific diagnosis varied significantly by region, ranging from 34% in Trent to 47% in Northern and Yorkshire (p<0.001). The regions of Northern and Yorkshire had the highest proportion of herpes encephalitis (28.35%); in comparison, Trent had

<table>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Exotic</td>
<td>3</td>
<td>3</td>
<td>7</td>
<td>7</td>
<td>5</td>
<td>9</td>
<td>20</td>
<td>7</td>
<td>3</td>
<td>64</td>
</tr>
<tr>
<td>Herpes viruses</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Herpes simplexa</td>
<td>138</td>
<td>163</td>
<td>170</td>
<td>175</td>
<td>172</td>
<td>168</td>
<td>120</td>
<td>147</td>
<td>166</td>
<td>1,419</td>
</tr>
<tr>
<td>Varicella zoster</td>
<td>8</td>
<td>17</td>
<td>13</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>94</td>
<td>99</td>
<td>80</td>
<td>333</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measles</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>23</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>86</td>
</tr>
<tr>
<td>Mumps</td>
<td>12</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>Rubella</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>LCMVb</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Adenoviruses</td>
<td>19</td>
<td>15</td>
<td>21</td>
<td>14</td>
<td>17</td>
<td>14</td>
<td>12</td>
<td>10</td>
<td>7</td>
<td>129</td>
</tr>
<tr>
<td>Otherc</td>
<td>47</td>
<td>75</td>
<td>56</td>
<td>51</td>
<td>62</td>
<td>48</td>
<td>58</td>
<td>46</td>
<td>37</td>
<td>480</td>
</tr>
<tr>
<td>Total specified viral infection (%)</td>
<td>248 (40)</td>
<td>289 (40)</td>
<td>282 (37)</td>
<td>266 (37)</td>
<td>275 (33)</td>
<td>273 (36)</td>
<td>318 (47)</td>
<td>320 (48)</td>
<td>303 (47)</td>
<td>2,574 (40)</td>
</tr>
<tr>
<td>Unspecified viral infection (%)</td>
<td>379 (60)</td>
<td>422 (60)</td>
<td>488 (63)</td>
<td>461 (63)</td>
<td>551 (67)</td>
<td>480 (64)</td>
<td>365 (53)</td>
<td>351 (52)</td>
<td>343 (53)</td>
<td>3,840 (60)</td>
</tr>
<tr>
<td>Total (%)</td>
<td>627 (100)</td>
<td>711 (100)</td>
<td>770 (100)</td>
<td>727 (100)</td>
<td>826 (100)</td>
<td>753 (100)</td>
<td>683 (100)</td>
<td>671 (100)</td>
<td>646 (100)</td>
<td>6,414 (100)</td>
</tr>
</tbody>
</table>

*aHerpes simplex virus is undefined after 1995–1996 and recorded thereafter as herpes.*

bLCMV, Lymphocytic choriomeningitis virus.

cBoth “other” and “unspecified” are included because both groups existed from 1995 to 1996 onward.
21.4%, and North West (the region with the lowest proportion) had 18%.

In hospitalized case-patients, 417 deaths were identified during the 9 years of study. The severity of cases was compared by using a crude case-fatality rate and mean length of stay (Table 3). The overall case-fatality rate was 6.5 per 100 cases, and the mean length of stay was 17.5 days. Considerable variation appeared in case-fatality rate and mean length of stay among the different diagnoses. Herpes encephalitis had the highest overall case-fatality rate and mean length of stay and was considered to be the most severe diagnosis. In children, the overall case-fatality rate was 2.3 per 100 cases (95% confidence interval [CI] 1.8 to 3), 1.9 per 100 cases of herpes (95% CI 0.7 to 4), and 2.1 per 100 cases of viral encephalitis without a specific diagnosis (95% CI 1.5 to 2.8). The mean length of stay for children was 10.9 days, increasing to 11.6 days for patients without a diagnosis specified and 13.2 days for patients with herpes encephalitis. In adults, the case-fatality rate was higher than children at 9.7 per 100 cases (95% CI 8.8 to 10.7), 12.5 in herpes encephalitis (95% CI 10.4 to 14.3), and 8.5 per 100 cases without a diagnosis specified (95% CI 7.2 to 9.7). The mean length of stay for adults was 22.4 days, which increased to 30 days for those with herpes or “other” diagnoses compared with 17 days for cases without a diagnosis specified.

Evidence of Clustering

For nonspecific cases of viral encephalitis, the frequency of hospitalizations (noted by week) indicated that some clustering of undiagnosed viral encephalitis had occurred during the 9 years of study since the upper prediction limits at 99.5% and 99.95% were exceeded in 6 weeks. Three of the six clusters occurred closely together in 1993 (weeks 45, 47, and 51), which suggests an overall period of higher incidence during this time. The Poisson regression model suggested clustering of encephalitis of unknown cause in district health authorities, and the total number of cases occurring in a week was greater than the prediction limit at 99.95% in three districts. The model did not identify any clusters when the period was extended to those occurring within 1 month.

Comparison of Data Sources

Significant underreporting of viral encephalitis was seen in the routine systems when compared to hospital episode statistics. A total of 215 cases of acute infectious encephalitis were identified through NOIDS between April 1, 1989, and March 31, 1998, compared to 6,414 identified in hospital episode statistics. This disparity implies that almost all (97%) hospitalized cases were not formally reported.

A total of 599 cases of viral encephalitis were identified through the laboratory reporting system from January 1, 1990, to December 31, 1998. Fifty nine per cent of these cases were herpes (86 herpes simplex virus 1, 48 herpes simplex virus 2, and 219 subtype not known), 21% varicella and 4% adenovirus. The remaining 16% of cases included 26 cytomegalovirus, 15 Epstein-Barr virus, 20 measles virus, and 13 mumps. Of herpes encephalitis, 19% (66/353) of cases occurred in children and 4.8% (17/353) in neonates. Of herpes encephalitis cases in neonates that were typed, seven were herpes simplex virus 2 and two were herpes simplex virus 1.

The number of laboratory reports of viral encephalitis increased from 52 in 1990 to 80 in 1991, falling to 55 in 1993, and rising again to a peak of 104 in 1998. Excluding those cases for which no specific ICD-9 or ICD-10 code exists and which could not have been identified in hospital episode statistics, 558 cases of viral encephalitis were reported through the laboratory system from January 1, 1990, to March 31, 1998. This total represents approximately 71% underreporting when compared to 1,867 hospitalizations of viral encephalitis with a specific infection reported (Table 4).

Table 3. Hospitalizations of viral encephalitis patients, deaths, and duration of stay, England, April 1, 1989–March 31, 1998a

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
<th>No. of deaths (%)</th>
<th>Case-fatality rate/100 cases</th>
<th>95% CI</th>
<th>Length of stay in days</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exotic</td>
<td>64</td>
<td>1 (0.2)</td>
<td>1.6</td>
<td>0.01 to 8.6</td>
<td>16.5</td>
<td>7.5 to 25.5</td>
</tr>
<tr>
<td>Herpes viruses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>1,419</td>
<td>141 (33.8)</td>
<td>10</td>
<td>8.4 to 11.6</td>
<td>26.6</td>
<td>23.6 to 29.6</td>
</tr>
<tr>
<td>VZV</td>
<td>333</td>
<td>25 (6.0)</td>
<td>7.6</td>
<td>4.9 to 10.9</td>
<td>11.8</td>
<td>10.0 to 13.4</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measles</td>
<td>86</td>
<td>1 (0.2)</td>
<td>1.2</td>
<td>0.01 to 6.3</td>
<td>6.8</td>
<td>3.1 to 10.5</td>
</tr>
<tr>
<td>Mumps</td>
<td>30</td>
<td>1 (0.2)</td>
<td>3.3</td>
<td>0.01 to 15.8</td>
<td>4.1</td>
<td>2.9 to 5.3</td>
</tr>
<tr>
<td>Rubella</td>
<td>26</td>
<td>2 (0.5)</td>
<td>7.7</td>
<td>0.9 to 25.1</td>
<td>76.0</td>
<td>0.0 to 174.0</td>
</tr>
<tr>
<td>LCMV</td>
<td>7</td>
<td>0 (0.0)</td>
<td>0</td>
<td>7.2</td>
<td>2.1 to 12.3</td>
<td></td>
</tr>
<tr>
<td>Adenoviruses</td>
<td>129</td>
<td>6 (1.4)</td>
<td>4.7</td>
<td>1.8 to 9.8</td>
<td>8.5</td>
<td>6.8 to 10.2</td>
</tr>
<tr>
<td>Other</td>
<td>480</td>
<td>33 (7.9)</td>
<td>6.8</td>
<td>4.7 to 9.4</td>
<td>18.8</td>
<td>12.4 to 25.1</td>
</tr>
<tr>
<td>Unspecified viral infection</td>
<td>3,840</td>
<td>207 (49.6)</td>
<td>5.5</td>
<td>4.7 to 6.2</td>
<td>14.8</td>
<td>13.7 to 15.9</td>
</tr>
<tr>
<td>Total</td>
<td>6,414</td>
<td>417 (100)</td>
<td>6.5</td>
<td>5.9 to 7.1</td>
<td>17.5</td>
<td>16.4 to 18.6</td>
</tr>
</tbody>
</table>

aCI, confidence interval; VZV, varicella-zoster virus 1; LCMV, Lymphocytic choriomeningitis virus.
This underreporting varied by virus, ranging between 28% and 100%.

Hospital episode statistics showed an underascertainment of deaths. Viral encephalitis was given as the final underlying cause of 622 deaths certified from January 1, 1993, to March 31, 1998. In that comparative period, hospital episode statistics showed 259 deaths, only 42% of those formally certified and reported to the Office of National Statistics. Most (483/622) certified deaths from viral encephalitis did not have a specific diagnosis; however, 139 deaths did. We compared the 139 deaths to the 112 identified in hospital episode statistics for which a specific diagnosis was recorded (Table 4).

## Discussion

The public health impact of viral encephalitis in England cannot be clearly defined with existing data sources. During our study of viral encephalitis in England, we found that more cases of viral encephalitis were identified in hospital episode statistics than in routine surveillance systems. Most cases requiring hospitalization are viral encephalitis with unknown etiology; of those cases with a specific diagnosis recorded, herpes is the most common cause. Adults account for the majority of case-patients, and rates have increased in those aged >65 years. Our analysis of cases of viral encephalitis without a specific diagnosis in hospital episode statistics identified clusters that would have otherwise gone undetected. Timely hospital episode statistics would provide a more comprehensive surveillance than any existing system.

The coding system used for the hospital diagnoses recorded in hospital episode statistics changed from ICD-9 to ICD-10 beginning on April 1, 1995. In some cases, diagnoses became less specific and in some cases more specific (e.g., herpes simplex virus was coded to herpes while VZV encephalitis had a specific ICD-10 code from 1995). In this study, we made some effort to compensate by including hospitalized cases with a diagnosis of chickenpox and an unspecified viral encephalitis infection. The change in coding did not appear to cause any major changes in the total number of hospitalized viral encephalitis cases.

Why are so few diagnoses in hospital episode statistics specific? Whether an appropriate investigation has been carried out, the extent to which a specific diagnosis is sought, and the quality of medical records affect the recording of specific diagnoses in hospital episode statistics. Appropriate investigation is limited by the diagnostic techniques available; virus isolation from cerebrospinal fluid or the brain can delay a diagnosis because culturing is slow, sensitivity may be poor, and obtaining specimens may require special techniques. Determining the virus causing the encephalitis may not be seen as essential if all patients diagnosed with viral encephalitis are routinely given acyclovir, regardless of the virologic diagnosis. Regional variation in the proportion of hospitalizations without a specific diagnosis suggests that local laboratory practice or investigation by clinicians may differ. A nonspecific diagnosis is recorded in hospital episode statistics if the laboratory results arrive after discharge. During the period of study, the rapid and highly sensitive PCR test was not widely used in England; however, as the test becomes more widely available, the proportion of cases with a specific diagnosis in hospital episode statistics may increase. In this study, we found that adults with a specific diagnosis were older than those without a diagnosis, which is likely to reflect the age distribution of herpes, VZV, and other specific viral encephalitis diagnoses. A lower case-fatality rate, however, and mean length of stay in hospital suggests that these infections without a specific diagnosis are less severe.

With the emergence of new diseases and availability of vaccines for some viruses, determining the cause of these infections is increasingly important (19,21–25). Information on etiology would also increase the specificity of cluster detection. In the past, routine data sources have been used alongside hospital episode statistics to determine the likely cause of hos-
pitalizations without a specific diagnosis (26). These methods depend on seasonality of the viruses and are thus inappropriate for encephalitis.

To minimize overestimating the public health cost of viral encephalitis, multiple consultant episodes of care for a patient were counted as a single infection if <1 month elapsed between each. Hospital episode statistics do not include strict case definitions, which could also lead to overestimating. For example, between January 1, 1990, and March 31, 1998, a total of 71 cases of measles were reported in hospital episode statistics, most in children <5 years of age. These cases are unlikely to be true measles encephalitis since measles was rare in the 1990s following the introduction of the combined measles, mumps, and rubella vaccine in 1988 (27). Furthermore, none of the laboratory reports of measles encephalitis in this study were in infants, and only three were in children (aged 7, 11, and 14 years). The laboratory reports may also be overestimated. Cases have been included in the laboratory data based on a single high serum antibody titer. Had a stricter case definition been used, as has been suggested for measles (28), even fewer cases of viral encephalitis would have been identified in the laboratory system.

Hospital episode statistics identified a lower number of deaths from viral encephalitis than the number reported routinely to the Office of National Statistics. Deaths in hospital episode statistics are known to be incomplete since patients with encephalitis may die after discharge, and the death may be recorded in an episode that does not include encephalitis as a diagnosis and was thus missed in the data extraction (29).

Some epidemics of encephalitis in England are undetected in routine surveillance systems and undiagnosed in hospital episode statistics. This situation suggests the potential for another emerging infectious disease, such as West Nile infection, to occur in the U.K.; existing routine surveillance systems would be incapable of detecting this (30–32). Levels of disease in England are not as high as reported in a similar study in the United States (33); however, the proportion without a specific diagnosis is comparable. To improve surveillance, clinicians must be encouraged to report viral encephalitis, although notifications are not specific. Other existing surveillance systems, such as the British Pediatric Surveillance Unit (available from: URL: http://www.bpsu.iopsum.com/), could be modified to include a specific diagnosis of viral encephalitis. Alternatively, a sentinel surveillance system using existing laboratory networks and offering screening for a wide range of viruses could provide more accurate and timely data.

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Preparing for a Bioterrorist Attack: Legal and Administrative Strategies

Richard E. Hoffman*

This article proposes and discusses legal and administrative strategies for a bioterrorist attack. To perform the duties expected of public health agencies during a disease outbreak caused by bioterrorism, an agency must have a sufficient number of employees and providers at work and a good communications system between staff in the central offices of the public health agency and those in outlying or neighboring agencies and hospitals. The article proposes strategies for achieving these objectives as well as for removing legal barriers that discourage agencies, institutions, and persons from working together for the overall good of the community. Issues related to disease surveillance and special considerations regarding public health restrictive orders are discussed.

This article proposes and discusses legal and administrative strategies that state and local public health officers and attorneys should consider when preparing for a bioterrorist attack. Through thoughtful preparation, intelligent and enlightened leadership can maximize coordination of available resources in the community.

Two predictable factors will dictate the manner in which state and local governments respond to a bioterrorist attack: 1) the exposure will be covert, and an incubation period will occur before ill persons seek medical care, i.e., there will not be a single location for emergency response by emergency medical teams, law enforcement officers, and firefighters as there was in New York City and Washington, D.C., on September 11, 2001; and 2) the attack will be treated not only as an epidemic but also as an emergency, a crime, and a matter of national security. Because of the second factor, elected political leaders will be in charge of response to the attack, rather than the health commissioner or the state epidemiologist, either of whom would normally manage an epidemic or outbreak control activities. The president, governor, or mayor will assume leadership roles, and public health agencies will need to carry out their duties within an incident command structure.

The magnitude of a bioterrorist attack (i.e., how many persons are exposed to the agent and how many become ill) and the characteristics of the bioagent (e.g., contagious or not) employed by the terrorists are not predictable, but these factors will affect virtually all of the response activities. Nonetheless, “generic” public health duties during a major bioterrorist attack are predictable: providing accurate information to health-care providers and the public about the status of the epidemic and protective measures; conducting disease surveillance and contact tracing; administering vaccines or prophylactic antibiotics; implementing restrictive measures; analyzing human and environmental laboratory specimens; and maintaining the quality of air, water, and food.

To perform these public health duties, two basic objectives must be achieved: 1) a sufficient number of employees and providers in state and local health agencies, hospitals, clinics, and laboratories must show up for work; and 2) a good communications system must exist between staff in the central offices of the public health agency and those in outlying or neighboring agencies and hospitals. Legal and administrative strategies should be developed in advance of an attack with these objectives in mind.

“A sufficient number of employees and providers” includes not only previously trained medical-care providers, laboratory technicians, and public health epidemiologists but also all untrained workers and volunteers who participate in the response to the outbreak. Untrained does not mean unskilled or untrainable. Untrained persons could come from unrelated programs and departments within a public health agency or hospital, from physicians practicing in the community, and from volunteers within and outside the community.

If the number of ill or exposed persons were large, the bioagent were contagious, or both, an epidemic could last for weeks, and the demands on staff could be enormous. For example, in response to a small number of smallpox cases in New York City in 1947, the New York City Department of Health gave smallpox vaccine to over 6 million residents by operating 179 clinics from 9:00 a.m. to 10:00 p.m. 7 days a week for more than 3 weeks and by gaining the support of private physicians, unions, and businesses (1). Before October 2001, no one in the United States had experience with anthrax transmitted through the mail or in the air of postal offices. Many epidemiologic questions arose in the course of responding to that anthrax outbreak, and any future bioterrorist attack will probably result in unpredictable or unimaginable issues despite preparations and training that have been undertaken in the past few years. Thus, the persons responding to an attack will need flexibility in the statutes and regulations that govern disaster emergencies. Rapidly amending statutes and regulations by the usual legislative and administrative processes is not feasible in an emergency; “flexibility” in this sense means the legal authority manifest in statutes and regulations must necessarily be nonspecific and allow for quick action by

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elected officials and public health leaders. Emergency or executive orders issued by the president, governor, or mayor are the most straightforward legal method for directing response activities customized to the details of a given attack.

In the Colorado bioterrorism statute enacted in 2000, no new powers were authorized for either the health department director or the governor because existing authority to manage emergencies, disasters, and epidemics, though long-standing, was determined to have sufficient latitude to deal with the new threat of bioterrorism. The overall purpose of the introduced bill was to remove legal barriers that discouraged institutions and persons from working together for the overall good of the community. Because the introduced bill did not seek new powers, it was more acceptable to state legislators.

Obtaining Expert Advice

My experience in state government1 has been that after natural or manmade point-source disasters, a governor reflexively turns to the office of emergency management, the department of public safety (or homeland security), or the National Guard for advice and counsel. The staffs of these agencies, however, have neither the expertise necessary to guide the response to an epidemic nor an established, ongoing communications and surveillance system with hospitals, laboratories, and medical providers. It is essential, therefore, to establish a formal process that allows public health and medical experts to assist elected officials in analyzing and interpreting information about the outbreak and in coordinating the public health response to the outbreak. Guidance for fiscal year 2002 supplemental funds for public health preparedness and response for bioterrorism issued by the Centers for Disease Control and Prevention (CDC) requires that a state establish an advisory committee that includes representatives from health departments, first responders, hospitals, and voluntary organizations such as the Red Cross (11).

In Colorado this advisory committee includes not only the nine groups listed in the CDC announcement but also the presidents of the state board of health, state medical society, and state hospital association; the state veterinarian; a wildlife disease specialist; a medical examiner; a specialist in posttraumatic stress management; a pharmacist member of the Board of Pharmacy; the Attorney General; the chief public information officer for the state health department; and, as an ex-officio member, the chief of the Colorado National Guard (3). These persons were named to the committee because they possess useful expertise or connections to the community. The statute authorizing the formation of the committee provided legal immunity to members for their advice (4), and the members pledged that they would attend the committee meetings during a bioterrorist attack rather than report to their regular jobs. By meeting regularly the committee members learn about each other’s skills, experience, and roles and develop a working relationship that, by itself, can be extremely valuable during a crisis.

One notable absence in the composition of the advisory committee is representation from federal agencies, such as CDC, the Federal Emergency Management Agency, the Environmental Protection Agency, and the Federal Bureau of Investigation. Although these agencies cannot, as a practical matter, attend meetings in every state and large municipality, during a crisis they will have an integral role, and disputes are more likely if the leaders are meeting for the first time in a highly stressful situation. For example, local-state-federal disagreements occurred in the management of the pneumonic plague epidemic in Los Angeles in 1924, the last instance of person-to-person transmission of plague in the United States, as well as during the anthrax outbreak in 2001 (12,13).

Removing Legal Barriers

Some existing state regulations, which in normal times are intended to ensure quality medical care, could hinder community efforts during a bioterrorist attack. For example, consideration should be given to modifying, for a limited period through executive orders, the regulations that control the prescription and dispensing of medicine, licensing of physicians and nurses, and transfer of patients between hospitals. Providing antibiotics or vaccinations in mass clinics and obtaining the services of retired or out-of-state physicians and nurses may be necessary.

In Colorado, executive orders that address these concerns have been drafted by the governor’s technical advisory committee. The orders would permit a) health-care providers other than pharmacists and physicians, such as nurses and emergency management technicians, to dispense medications, b) medicines to be distributed without an identified patient’s name on the packet or bottle, c) practice of medicine and nursing by professionals who are not currently licensed in Colorado, provided the practice is restricted to caring for epidemic-associated illnesses and the persons are working under the supervision of a licensed practitioner (who is given legal immunity for the supervisee’s work), and d) persons seeking medical care at one facility to be redirected to another facility without initial assessment or stabilization attempt if the initial hospital is unable to care for any more persons or if a specific facility (established or temporary) has been directed to receive epidemic patients, e.g., those with smallpox. These draft orders must still be tailored to the actual emergency and signed by the governor, but the background legal work can be completed ahead of time.

Two additional features of the Colorado bioterrorism statute exist; these features were designed to encourage volunteers and remove legal barriers to cooperation among institutions

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1My perspective is based on experience serving as the state epidemiologist for Colorado from 1987 to 2001; as chief medical officer from 1998 to 2001; as a participant in Operation Topoff (a full-scale, federal-state-county 4-day bioterrorism exercise conducted in May 2000) (2); and as the principal author of Colorado’s bioterrorism response statute (enacted March 2000) (3–8), its bioterrorism preparedness regulations (adopted May 2001) (9), and its bioterrorism reporting regulations (adopted 1999) (10).
and agencies. First, the statutory definition of “civil defense worker” was modified to include a “physician, health care provider, public health worker, or emergency medical service provider who is ordered by the governor…to provide specific medical or public health services during and related to an emergency epidemic and who complies with this order without pay or other consideration” (7). With this amendment, civil defense workers may receive compensation for injury, including illness caused by bioterrorism, which is suffered as a result of civil defense service. Second, the statute provides that “persons and entities [including hospitals] that in good faith comply completely with board of health rules regarding the emergency epidemic and executive orders…shall be immune from civil or criminal liability for any action taken to comply with the executive order or rule” and that the state shall provide “compensation for property...if the property was commandeered or otherwise used in coping with an emergency epidemic…” (4).

Requiring Plans for Bioterrorist Events
To ensure that a sufficient number of health-care providers, laboratory technicians, public health epidemiologists, and administrative support workers show up for work during a bioterrorist attack, appropriate personal protection (e.g., respiratory protection, vaccination, or chemoprophylaxis) for the worker and, probably, for household members of the worker are essential. When performing nonstandard work, the worker may also need legal protection, as discussed above. Plans for a bioterrorist attack should include these factors and be written by the employer who knows how the agency operates and is staffed because people work for an agency, hospital, or institution, not a region. Nonetheless, it makes sense to develop mutual aid agreements with neighboring jurisdictions and integrate single institution or agency plans into community, regional, or statewide plans.

In the 2000 Colorado bioterrorism statute, the state board of health was given the new authority to promulgate rules requiring each state and local health department, general or critical access hospital, and managed-care organization to write a plan for responding to bioterrorism (7). Such rules were adopted in May 2001 (8). While hospitals and health departments may have previously written plans for managing mass casualties resulting from aircraft, bus, or train crashes or natural disasters, such plans need to be modified to include consideration of the special circumstances of bioterrorism (e.g., chemoprophylaxis and personal protective equipment for workers, infection control, and handling of laboratory specimens). Because pandemic influenza may pose challenges to the medical and public health systems similar to those of bioterrorism, a single plan for both types of epidemics should be drafted.

Ensuring Good Communications
During “typical” outbreaks of communicable diseases, clear and timely communication by the state health department with multiple local health departments and hospitals can be a challenge. In a bioterrorist attack, the communications challenge will likely be greater because many more persons and agencies will be involved. The telephone system may not have sufficient capacity for the increased demand or it may be damaged and disorganized, as happened during the response to the attacks on the World Trade Centers in New York City in September 2001 (14). Furthermore, a large, sometimes overwhelming, number of inquiries made by members of the public to the public health agency usually occur during public health crises, and therefore, administrative plans for a bioterrorist event should include consideration of this workload.

Legal and administrative strategies should be developed in anticipation of communication challenges. Rather than relying on hospital personnel, public health agencies may find it advantageous to station their own personnel with mobile telephone or radio communications equipment in individual hospitals to assure that public health agencies get the information they need as rapidly as possible. Accomplishing this may require an executive order of the governor that commandeers two-way radios. In Colorado, board of health regulations require the state and local health departments to include assignment of employees to hospitals in the agency’s emergency plan (8).

Disease Reporting and Surveillance
Disease reporting requires specification of what to report in what manner and timeframe to which parties. A first legal step in this process is to require immediate reporting of any suspected or confirmed illness, syndrome, or outbreak caused by any potential bioterrorist agent. For example, Colorado regulations were modified in 1999 so that cases of plague, which had been required to be reported within 24 hours of diagnosis by telephone, fax, or through a Web-based system, were to be reported immediately only by telephone to an on-call person if the physician or hospital suspected the case was related to a bioterrorist event (9).

Disease surveillance systems are critical not only for the initial detection of an outbreak but also for monitoring the extent and spread of the outbreak and for determining when it is over. Managing a large outbreak would require gathering information from contact tracing and source-of-exposure investigations as well as information about the availability of critical medicine, medical equipment, and the handling of corpses. These information needs are much different than those needed for early detection of an attack. Therefore, legal authority for surveillance should be modified as necessary to ensure collection of all information that could be needed by the public health agency to fulfill its duties throughout the epidemic. This legal authority may include requirements for groups that do not commonly report information, such as pharmacists, to provide it.

Restrictive Measures, Isolation, and Quarantine
Administrative public health orders restricting personal behavior of persons with certain diseases, such as tuberculosis,
are relatively common in this country (15). Such orders are usually hand-delivered to a specific person(s), and the restrictions are removed after a specified period, such as after one incubation period or when an ill person is no longer infectious. Another type of public health order might involve work restriction, e.g., health-care providers who cannot demonstrate evidence of immunity to a vaccine-preventable disease are not permitted to work during an outbreak of such disease.

Few, if any states, however, have experience issuing and enforcing large-scale quarantine orders that last more than 1–2 days. Orders restricting large numbers of contacts of cases of plague to home were issued in Florence, Italy, in 1630 and described in the 1999 book, Galileo’s Daughter (16). The enforcement of orders restricting the movement of residents of an entire town in which there was an outbreak of viral hemorrhagic fever was depicted in the 1996 movie, Outbreak. The images of severe disease and enforced quarantine are similar in the book and movie and are plausible and disturbing to lay audiences. A more recent, well-documented example of a large-scale movement restriction was the British epidemic of foot-and-mouth disease of 2001, which affected many farms and businesses and led to the quarantine and slaughter of 4 million sheep, cattle, and pigs for disease control purposes (17). In all three examples, a decentralized quarantine was imposed. In general, the advantage of a decentralized strategy (e.g., persons are restricted to home) is that it may reduce the risk for transmission of disease because fewer persons congregate. However, a decentralized strategy may require more community resources to implement and enforce. Alternatively, the centralized strategy (e.g., restricted persons are taken to a sports arena, auditorium, theater, school, or hospital) is seemingly easier for the government to care for restricted persons and to enforce the order but could allow contagious and non-contagious persons to come into contact with each other.

Another example of large-scale quarantine occurred in Los Angeles in 1924 during the last epidemic of pneumonic plague in this country (12). Three days after the first 15 cases in this outbreak became known to public health officials, eight city blocks that housed approximately 2,500 Mexicans were placed in quarantine. Public health nurses were sent to the area to make house-to-house inspections to identify new cases, and all patients with suspected cases in the area were examined by physicians at the patient’s home and then sent to the county hospital. The Los Angeles County Board of Charities provided 7-day rations to each household. All persons who lived at addresses where cases had occurred were quarantined in the county general hospital, and a Spanish-speaking priest and social workers were placed in the area to reassure and calm the residents. The quarantine actions taken in this outbreak were a combination of centralized and decentralized strategies.

As has been discussed by Barbera et al. (18), numerous concerns regarding large-scale quarantine exist. All states currently have in place varying degrees of legal authority enabling isolation, quarantine, or travel restrictions if needed to maintain the welfare and safety of the public. Drafting restrictive orders in advance is less helpful than with the other types of orders discussed above because restrictive orders require more tailoring to the specific circumstances and parameters of an outbreak. Factors such as duration and location of restriction are dependent on what the bioterrorist agent is, how it is transmitted, how widely the agent has been disseminated, whether exposed persons can be personally identified, and what resources are available to care for restricted persons. Not drafting such orders in advance, however, means that they may be written during the turmoil of multiple agencies trying to control an outbreak. Authorities should never hesitate to revise the orders on the basis of updated information.

At the end of the Operation Topoff exercise, for instance, when the governor had issued a travel restriction order for all of metropolitan Denver and CDC had quarantined the entire state of Colorado, such orders created many unforeseen problems, including how to enforce the orders, maintain essential community services, and distribute foods and prescription medicines. The exercise ended before any of these problems were addressed and resolved.

Conclusions

Accurate and substantive information given to the public by credible public health and medical experts can do much to allay the fears of the public and encourage their cooperation and participation in constructive, organized community response efforts (19,20). The foundation for this is thoughtful, detailed preparations. In this article, I have discussed a number of ideas about legal and administrative preparations for a bioterrorist attack, but more work can be done, including development of strategies addressing issues related to mental health, disposal of corpses, performing forensic autopsies, signing death certificates, and managing potential animal vectors of disease.

I have not discussed the sharing of medical and epidemiologic information between public health agencies and law enforcement agencies, such as the Federal Bureau of Investigation. Under normal circumstances, public health officials typically argue that release of disease surveillance information to the criminal justice system will discourage persons with reportable conditions from disclosing to public health officials where they have been and with whom they have had contact. However, a bioterrorist attack is not a routine event, and I recommend that state and local public health agencies review the laws and regulations governing the confidentiality of disease surveillance records and develop a legal and administrative protocol for sharing pertinent and relevant information with law enforcement agencies during a bioterrorist attack (21).

Finally, I have not discussed the protection of civil liberties and due process for persons affected by executive orders of the governor and public health officials. This is an important and difficult issue, especially when well persons are quarantined solely on the basis of their having visited, worked, or resided in a particular location at a particular time, as opposed to having had face-to-face contact with a known contagious person.
Public health officials and attorneys general should review existing safeguards for the protection of civil liberties and determine whether modifications need to be made for the special circumstances created by a bioterrorist attack.

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B-Virus (Cercopithecine herpesvirus 1)
Infection in Humans and Macaques:
Potential for Zoonotic Disease

Jennifer L. Huff* and Peter A. Barry*

Nonhuman primates are widely used in biomedical research because of their genetic, anatomic, and physiologic similarities to humans. In this setting, human contact directly with macaques or with their tissues and fluids sometimes occurs. Cercopithecine herpesvirus 1 (B virus), an alphaherpesvirus endemic in Asian macaques, is closely related to herpes simplex virus (HSV). Most macaques carry B virus without overt signs of disease. However, zoonotic infection with B virus in humans usually results in fatal encephalomyelitis or severe neurologic impairment. Although the incidence of human infection with B virus is low, a death rate of >70% before the availability of antiviral therapy makes this virus a serious zoonotic threat. Knowledge of the clinical signs and risk factors for human B-virus disease allows early initiation of antiviral therapy and prevents severe disease or death.

Structure and Life Cycle
B virus is a large, double-stranded DNA virus with numerous open reading frames, some of which share approximately 79% amino acid sequence identity with HSV-1 and HSV-2 (4). The viral genome is G+C rich (75% G+C), the highest of any known herpesvirus (4). The B-virus genome is only partially sequenced, but thus far, is colinear with that of HSV (5). Electron micrograph studies of B virus show a typical herpesvirus structure (6), including an electron dense core with viral DNA inside an icosapentahedral capsid surrounded by an amorphous tegument protein layer and a lipid envelope studded with viral glycoproteins. Glycoproteins on the viral envelope mediate attachment to and entry into the host cell. For HSV, 11 glycoproteins are known (gB–gM), and another is predicted (gN). Of these, nine have been identified in B virus (5).

In general, alphaherpesviruses infect mucosal epithelia followed by one or more rounds of replication in epithelial cells. B virus likely replicates with three consecutive rounds of transcription (the α, β, and γ genes), as has been established for HSV. The infected cells are lysed, releasing virus to spread to other cells and sensory nerve endings, although direct entry into neurons without replication can occur (3). Virus can also spread from cell to cell without contacting the extracellular environment. Spread of the virus to and from the nerve ganglia occurs by axonal transport, which has been demonstrated for B virus in experimentally infected mice (7). The virus establishes latency in the nerve ganglia. Latency is characterized by a lack of viral replication and limited viral transcription. Periodic reactivation from latency delivers the virus to mucosal epithelial cells, where it replicates; infectious virus is released.

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from the mucosal epithelium. A heavy viral load in the ganglia may increase the frequency of reactivation and shedding (8). Recent findings from the study of primary and recurrent HSV-2 infection indicate that most episodes of recurrent viral shedding are asymptomatic. B-virus shedding in macaques also appears to be primarily asymptomatic (9–11).

**B-Virus Infection in Macaques**

B-virus infection has been reported most commonly in the rhesus and cynomolgus macaque (*M. fuscata*), two species used extensively in biomedical research. B virus has also been isolated from the stump-tailed (*M. arctoides*), pig-tailed (*M. fuscata*), bonnet (*M. radiata*), and Taiwanese (*M. cyclopis*) macaque (12). Sequence comparisons and restriction fragment length polymorphism analysis of viral genomes have demonstrated strain differences between B-virus isolates from different macaque species (13). Research suggests that B virus from rhesus macaques may be more pathogenic for humans than B virus from other macaque species (13). Where the species of macaque is noted, cases of human B-virus infection have all been associated with direct or indirect exposure specifically to rhesus macaques (14–19). The ability of nonrhescus strains of B virus to infect humans is not well understood.

Little is known about the biology of B virus in its natural host. Infection is usually acquired at sexual maturity (2–4 years of age for rhesus macaques). As seen in humans with HSV, B-virus seropositivity increases with population age; seropositivity rates of 80% to 100% occur among most adult captive macaque populations (10,20,21). Oral herpetic lesions such as gingivostomatitis, oral and lingual ulcers, and conjunctivitis have been described, but are usually associated with immunosuppression or stress attributable to recent importation or crowded housing conditions (12,22,23). Genital lesions have not been observed in macaques, although genital infection has been demonstrated by polymerase chain reaction (PCR) (9), virus isolation from the genital mucosa (10,11), and culture of the sacral ganglia (11). In general, macaques remain asymptomatic, and identification of oral herpetic lesions is sufficient grounds for euthanasia of the affected animal. The infrequent cases of disseminated B-virus disease in macaques are most often associated with immunosuppression, caused by either chemotherapy or concurrent infection as with simian type D virus (22). Although severe HSV disease is commonly observed in humans co-infected with HIV, no cases of B-virus disease associated with simian immunodeficiency virus infection have been reported (24).

Relatively few studies have surveyed macaques for B-virus shedding, and detection of B virus by culture is rare. Most cases of B-virus detection in asymptomatic macaques by culture or PCR are associated with breeding season stress (9,10), immunosuppression (25), or primary infection (10,11). The true frequency of B-virus shedding in macaque populations is not known but is likely to be low.

Most cases of human B-virus infection have been associated with apparently healthy macaques (i.e., no obvious herpetic lesions), which indicates asymptomatic shedding of the virus. Lack of clinical signs of recurrent infection makes identification of shedding animals difficult. People working with these animals should consider every animal a potential source of B virus and use proper protective equipment and care when handling them (21,26–28).

**Human B Virus Infection**

Most cases of human B-virus infection have involved direct contact with macaques, such as a bite, scratch, or mucosal contact with body fluid or tissue (12,14–16,19,27,28). Indirect contact, such as injury from a contaminated fomite (e.g., needle puncture or cage scratch), has also resulted in human infection. Human-to-human transmission has been documented in one case (15); however, further investigation has indicated that the risk for secondary transmission is low (18).

Human B-virus disease generally occurs within 1 month of exposure (21), commonly with an incubation period of a few days to a week. The development and progression of disease depend on the site of exposure and the amount of virus inoculated. Vesicular lesions have not been consistently found at the site of exposure (12,14–19). Disease often starts with general influenzalike symptoms of fever, muscle aches, fatigue, and headache (12,14). Other variable symptoms include lymphadenitis and lymphangitis, nausea and vomiting, abdominal pain, and hiccups (12,14). Neurologic signs develop when the virus spreads to the central nervous system and vary with the part of the brain or spinal cord affected. Hyperesthesias, ataxia, diplopia, agitation, and ascending flaccid paralysis have been described after virus spread to the brain (12,14–19). Virus spread to the central nervous system is an ominous sign; even with antiviral therapy and supportive care, most patients die, and those who survive often have serious neurologic sequelae. Deaths are often attributed to respiratory failure associated with ascending paralysis.

The possibility of asymptomatic or mild B-virus infection in humans has been suggested (2,29). A carefully controlled study of B-virus antibodies in persons with macaque contact and controls without contact showed no evidence of asymptomatic human infection or a carrier state for B virus (29). Although HSV antibodies can neutralize the virus in vitro, antibody titers to HSV are not protective in human cases of B-virus exposure or infection (21,29) and can confound diagnostic testing because of cross-reactivity. Asymptomatic human infection with B virus appears exceedingly rare if it occurs at all.

With the discovery of simian immunodeficiency virus and its identification as a model for HIV infection, the number of macaques used in research has increased, as has the number of human B-virus cases. Guidelines for reducing and controlling exposure were first published in 1987 (26) by a group of veterinarians, physicians, and research scientists called the B Virus Working Group. Guidelines were again published in 1995 (21).
by a new B Virus Working Group to include new information and provide protocols for handling exposures. Further recommendations were made in 1998 to emphasize the need for limiting mucosal exposure to potential sources of B virus (19,28). New guidelines by another B Virus Working Group have recently been published (30).

Treatment of B-Virus Infection in Humans

The 2002 B Virus Working Group guidelines address issues to be considered in cases of possible exposure to or infection with the virus (30) and reflect consensus of opinion at the time the guidelines were written. In cases of exposure, an established and frequently updated protocol should be used based on these guidelines and on current literature regarding human cases of B-virus infection. Additional information and contacts are available from: URL: http://www.cdc.gov/niosh/hid5.html, http://www.haz-map.com/Macaque.htm.

According to the guidelines, the most important action in a case of potential exposure to B virus is to rapidly and thoroughly cleanse the wound or exposure site. HSV can enter sensory nerve endings within 5 minutes of exposure, and B virus is likely to infect just as rapidly. Bite wounds, scratches, or puncture wounds of nonmucosal surfaces should be cleansed with soap or detergent for at least 15 min (30). The time spent mechanically cleansing the area is more important than the type of cleansing solution used. Mucosal surfaces should be rinsed with sterile saline or running water for 15 min. Immediate cleansing or rinsing can inactivate and wash away virus present in the exposure site. After immediately cleansing the wound or exposure area, the person should seek medical attention, specifically from a physician identified in the facility’s protocol as someone familiar with treating these B-virus exposure cases.

A physician with a patient who has potentially been exposed to B virus faces a conundrum. Before onset of neurologic symptoms, antiviral therapy is successful. However, few cases of potential exposure lead to infection. Prophylactic treatment is unnecessary in most cases of potential exposure because treatment can confound diagnostic testing by interfering with the humoral immune response (21). However, the 2002 B Virus Working Group viewed prophylactic treatment more favorably in light of the efficacy of postexposure prophylaxis for nosocomial HIV exposure and the availability of new antiviral therapies, such as valacyclovir, that achieve higher serum levels with a more reasonable dosing schedule (30). Although severity of injury may prompt use of antiviral therapy, the amount of inoculated virus determines if infection is likely to occur. In some cases, minor scratches or needle-sticks have transmitted B virus, while bites with severe tissue laceration have healed without infection. The primary factor to consider is whether cleansing (or rinsing, if it is a mucosal surface) was initiated immediately and performed for the recommended 15 min (21). Inadequate cleansing of the wound or exposed area in a timely manner could warrant prophylactic antiviral therapy. Other indications for immediate initiation of antiviral treatment include the identification of herpetic lesions in the source animal, injuries involving the head or neck, and mucosal exposure to macaque fluid. Because of the prevalence of asymptomatic B-virus shedding in macaques, the clinical appearance of the monkey involved (if the animal is identified) may not be helpful in evaluating the possibility of transmission.

In addition to working closely with a physician trained to handle cases of B-virus exposure and infection, taking samples from the exposed person and the source animal is important for virus culture and serologic testing. A list of recommended swabs for virus culture and serum samples is available from: URL: http://www.gsu.edu/~wwwvir/index.html.

Detection of B Virus

Early suspicion and rapid diagnosis of B-virus infection are critical to the control of human infection. The extreme cross-reactivity of primate alphaherpesviruses has required the development of diagnostic methods that can differentiate between HSV and B-virus infection. Despite the inherent risk for exposure, direct culture of B virus has been the standard for diagnosis of infection. Culture of B virus requires a special containment facility since the virus is a biosafety level 4 pathogen (31). Serologic methods for the detection of B-virus infection have also involved propagation of the virus in tissue culture to produce antigen. However, the substitution of related antigens appears to work well for serologic tests. The most promising of these antigens is herpesvirus papio 2, an alphaherpesvirus of baboons that is as closely related to B virus as HSV-1 and HSV-2 are to each other (32,33). Serologic methods are useful only for retrospective analysis, not for therapeutic decisions, which need to be made rapidly in cases of potential human infection.

More recently, PCR methods have allowed direct demonstration of B-virus infection without the risk of working with virus cultures (9,34,35). PCR methods have been hampered by the close genetic relationship between primate alphaherpesviruses; many require post-PCR techniques to definitively differentiate between HSV and B virus. To specifically detect B virus, we developed a method using quantitative real-time PCR, whose potential application for human clinical samples in cases of exposure warrants further study (9). Samples to be tested by PCR may contain B virus and must be handled accordingly (31).

B Virus Outside the Research Setting

The cases of human B-virus infection that have been described have all occurred in relation to contact with macaques in a biomedical research setting. However, this setting is not the only one in which humans have contact with macaques. The Woburn Safari Park in the U.K. recently culled all B-virus–positive macaques from its facility (36). No cases of human infection have been documented despite contact between macaques and humans driving through the park, but the risk perceived by this situation warranted the action. B virus is also prevalent in free-ranging macaques native to
Southwest Asia (12,37). A recent survey of workers at a Balinese Hindu temple that is a refuge for free-ranging macaques and a tourist attraction showed that contact between humans and macaques sufficient to transmit B virus commonly occurred. A serosurvey of 38 macaques in the area showed that 31 (81.6%) were B-virus seropositive. No cases consistent with B-virus disease in humans have been described in this area of Bali or in other areas of Southeast Asia where humans are in contact with free-ranging macaques. However, in cases of encephalitis, B virus may not be considered.

In other situations, particularly when potentially seropositive macaques have been domesticated as pets, opportunities for exposure to B virus are frequent. One report documented many instances of potential exposures from bites, scratches, food sharing, close physical contact, and even shared chewing gum (38). This study also found that children were three times more likely than adults to be bitten by pet macaques. Although the number of macaques kept as pets is probably small, the risk of B-virus infection is increased because of the lack of precautions and the extent of contact between monkey and owner. The risk of B-virus infection is low, but the risk for death is high.

**Specific Pathogen-Free Colony Development**

In 1989, the National Institutes of Health’s National Center for Research Resources started funding specific pathogen-free macaque colony development. The timing and local nature of B-virus reactivation and shedding make detecting infection in an animal difficult. Therefore, serologic methods are used to screen and monitor animals for consideration as pathogen-free. Numerous negative serologic results are necessary to determine a macaque’s B-virus status. Although specific pathogen-free status reduces the likelihood of infection, this status does not eliminate the risk for infection entirely. Full protective equipment should be used for working with all macaques regardless of their pathogen status. While population numbers in these specific pathogen-free colonies are increasing, the demand for pathogen-free animals will continue to exceed the supply for some time (24).

**B-Virus Vaccine Development**

While antiviral therapy has substantially improved the survival rate for human B-virus infection, fatal cases still occur (19,28). The ability of the virus to modulate and evade the immune response has stymied vaccine development for most herpesvirus infections. A vaccine for use in rhesus macaques would reduce transmission of the virus and, over time, reduce the prevalence of infection in captive macaque populations. Given the lack of an effective vaccine for HSV after years of research effort and clinical trials, development of a B-virus vaccine presents a challenge.

A formalin-inactivated B-virus vaccine was developed and tested in the 1960s (39). Although this vaccine did induce an antibody response, antibody titers were low, and frequent boosters (every 3 months) were required (39). Recently, successful use of a vaccinia vector to deliver the gD gene of B virus was demonstrated in rabbits with protection of 10 (91%) of 11 animals from B-virus challenge (40). A DNA vaccine against B virus has also recently been described (41). Glycoprotein B of B virus delivered in a plasmid vector induced a humoral response in both mice and rhesus macaques. Although no challenge experiments were performed in monkeys, an anamnestic-like response upon boosting was noted. While the ability of a B-virus antibody response to protect from infection is not known, studies of HSV suggest that an antibody response alone is not protective. Both the vaccinia and DNA vaccine approaches described above are likely to induce cellular immunity to B virus, although the cellular response was not studied by either group (40,41). As clinical trials of candidate HSV vaccines progress, the development of a B-virus vaccine for use in macaques or humans at risk for exposure should be considered.

**Conclusion**

The potential for fatal human infection with B virus is a constant concern because frequent exposures occur to humans in the course of caring for and using macaques in a research setting. Personal protective equipment and safe handling procedures have limited the incidence of human disease. However, little is known about the biology of B virus in the natural macaque host. A clear understanding of the real risk for B-virus shedding in its natural host will help identify opportunities to prevent or limit zoonotic B-virus disease.

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Dr. Huff is a postdoctoral fellow at the University of California, Davis. This review stems from her dissertation work performed with Peter A. Barry at the Center for Comparative Medicine. Dr. Huff studies infectious diseases in the rhesus macaque model system.

Dr. Barry is an associate professor in the Center for Comparative Medicine and Department of Medical Pathology at the University of California, Davis, and a staff scientist at the California National Primate Research Center (CNPRC). Dr. Barry’s primary research addresses the mechanisms of rhesus cytomegalovirus persistence and pathogenesis. He is also working to improve B-virus diagnostics and understand B-virus pathogenesis in the CNPRC macaque colony.

**References**

SYNOPSIS


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**Photorhabdus Species: Bioluminescent Bacteria as Emerging Human Pathogens?**

John G. Gerrard,* Samantha McNevin,† David Alfredson,* Ross Forgan-Smith,† and Neil Fraser‡

We report two Australian patients with soft tissue infections due to *Photorhabdus* species. Recognized as important insect pathogens, *Photorhabdus* spp. are bioluminescent gram-negative bacilli. Bacteria belonging to the genus are emerging as a cause of both localized soft tissue and disseminated infections in humans in the United States and Australia. The source of infection in humans remains unknown.

Bioluminescence is the production of visible light by a chemical reaction in a living organism. The property is rarely reported in the clinical bacteriology laboratory because bacterial bioluminescence is seen primarily in marine species. *Photorhabdus* spp (family: Enterobacteriaceae) are the only terrestrial bacteria known to exhibit this property (1). The classification within the genus is complex with three currently recognized species: *P. luminescens*, *P. temperata*, and *P. asymbiotica* (2). Several subspecies are recognized.

*Photorhabdus* spp. have been the subject of intensive study by agricultural scientists because of the role these bacteria play in controlling insects. Insects, like humans, are subject to infestation by nematodes (3). *Photorhabdus* spp. inhabit the gut of some insect-pathogenic nematodes (*Heterorhabditis* spp.), where they form a symbiotic relationship. Nematode species of this type are able to invade the larvae of susceptible insects and release *Photorhabdus* spp. The bacteria proliferate and promote nematode reproduction by killing the insect larvae.

Insect-pathogenic nematodes harboring *Photorhabdus* spp are used as biopesticides in a number of countries, including the United States and Australia. Agricultural scientists are also attempting to develop insect-resistant transgenic crops by using insecticidal toxin genes derived from *Photorhabdus* spp. (4).

Genes encoding homologues of insecticidal toxins from *Photorhabdus* spp. occur naturally within the genome of *Yersinia pestis*, the cause of plague. Lateral transfer of genetic material between *Photorhabdus* and *Yersinia* species is thought to have resulted from their common association with insects as bacterial pathogens (5).

Human infection with *Photorhabdus* spp. has been described in two previous publications—six cases from the United States (6) and four cases from South Eastern Australia (Victoria and New South Wales) (1). We report two additional recent human cases of *Photorhabdus* infection from the Australian state of Queensland.

**The Study**

**Patient 1**

A 39-year-old male pest controller from Gladstone on a routine visit to his general practitioner in April 2001 inquired about the recent appearance of a red macule, 8 mm in diameter, on the medial aspect of his right ankle. No specific treatment was given. When he was seen again 18 days later, a painful, necrotic ulcer, about 12 mm in diameter, had developed at the original site of the red spot. A gram-negative organism later identified as *Photorhabdus* sp. was isolated in pure growth from the exudate. The patient began a 10-day course of oral cephalixin. When he was observed again 11 days later, he exhibited a persistent discharge with surrounding cellulitis. He was therefore prescribed a 10-day course of oral amoxicillin-clavulanate. Three weeks later, the ulcer appeared to be healing; after another 6 weeks, signs of infection had again developed. A gram-negative organism was isolated from the exudate but was not formally identified.

The patient was prescribed an additional 7-day course of oral cephalixin. When he was observed 3 months later, the infection had resolved. In his recent work as a pest controller, he had been spraying chemical insecticides under houses and in foreign cargo ships. He had never used insect pathogenic nematodes as a biopesticide.

**Patient 2**

A 78-year-old man from the Queensland Gold Coast sought treatment in January 1999 with a 3-day history of a painful, swollen right foot. The patient had a history of polymyalgia rheumatica for which he was taking prednisone, 8 mg daily. In January 1999, after working barefoot in the garden, the man noted intense pain in his right forefoot and a very small amount of bloody discharge from the web space between his fourth and fifth toes.

The next day he was seen by his general practitioner who treated him with oral dicloxacillin. Two days later he was admitted to the hospital with increasingly severe pain with extensive redness and swelling extending to his right knee. He was noted to be afebrile with a mild neutrophil leukocytosis. He was started on a regimen of intravenous gentamicin.

Surgical debridement of the right foot was required on three occasions during the first 8 days of his admission. Pus was collected for culture on two of these occasions, and tissue was obtained during the third. An organism identified as
Photorhabdus sp. was isolated in pure culture from each of these operative specimens. The same organism was also isolated, together with Staphylococcus aureus, from a superficial swab collected in the emergency department on presentation. No bacterial growth was obtained from blood cultures collected on admission.

The patient was treated with intravenous gentamicin for 2 weeks and ceftazidime for 1 week. He was discharged on a 6-week course of oral ciprofloxacin. The foot remained healed on follow-up 3 months later.

Photorhabdus spp. can be isolated and identified to genus level by using techniques available in most clinical bacteriology laboratories. A total of five isolates from the two patients described in the current report were examined in our laboratories with standard techniques (one from patient 1 and four from patient 2). The phenotypic characteristics that the isolates displayed were typical of the genus.

Colonies were formed after 24–48 hours on tryptic soy agar containing either 5% sheep or horse blood (bioMérieux, Baulkham Hills, Australia) at both 35°C and at room temperature, with a tendency to “swarm” (Figure 1). The isolates also grew on MacConkey agar. On sheep and horse blood agar, a thin line of annular hemolysis was observed 4–12 mm from the colony edge. The hemolysis was more apparent when the isolates were incubated at room temperature (Figure 2). The organisms were motile, gram-negative, rod-shaped bacteria. They were facultatively anaerobic, oxidase negative, and strongly catalase positive. Other biochemical reactions were as described previously (1).

The defining characteristic was the presence of faint luminescence, which could be clearly seen with the naked eye when the colonies were examined under conditions of total darkness. It was critical to this examination that the observer’s eyes be allowed to adjust to the darkness for 10 minutes.

Two commercially available automated bacterial identification systems were used in our laboratories: MicroScan Walkaway (Dade Behring Inc., MicroScan Division, West Sacramento, CA) and bioMérieux Vitek (bioMérieux; Hazelwood, MO). Photorhabdus spp. do not currently appear on the databases of either of these systems, which leads to misidentification (Table 1).

Photorhabdus spp. have been shown to form a heterogeneous group based on DNA-DNA hybridization studies, 16S rDNA sequencing and polymerase chain reaction ribotyping (2). A polyphasic approach is now applied to classifying isolates within the genus, dividing it into three species and several subspecies. The American clinical isolates described by Farmer et al. (6) belong to a new species, Photorhabdus asymbiotica (2). A specific epithet has not yet been assigned to the Australian clinical isolates but they also may form a new species within the genus (7).

Antimicrobial sensitivity was assessed by using broth microdilution. The isolates were sensitive to a broad range of antimicrobial agents with activity against gram-negative bacteria including ciprofloxacin, gentamicin, tetracycline, ceftriaxone, and amoxycillin-clavulanate. Isolates from both patients were resistant to cephalothin and ampicillin.

**Conclusions**

Publication of information about these two cases brings to a total of 12 the number of human infections with Photorhabdus spp. documented in the medical literature (Table 2 and Figure 3). The clinical picture described in the 12 cases has generally been one of localized or more commonly multifocal skin/soft tissue infection. Such infection has had a tendency to relapse. The disseminated distribution of skin/soft tissue infection in several cases suggests hematogenous spread. Bacteremia was documented in 4/12 case-patients. Cough was
documented in two of the bacteremic case-patients. In one of these, isolates of a *Photorhabdus* sp. were obtained from sputum as well as from blood and skin/soft tissue.

Given the very limited clinical experience, making definitive recommendations about treatment is not possible. Antimicrobial therapy should be guided by in vitro sensitivities. The tendency for *Photorhabdus* infection to relapse suggests that prolonged therapy for a period of weeks would be prudent, perhaps with an oral fluoroquinolone.

*Photorhabdus* spp. are not human commensals. The patients apparently acquired the pathogen from an unidentified source in the terrestrial environment. This hypothesis is supported by the observations that at least 4/6 of the Australian patients were engaged in outdoor activities around the time of acquisition and that the initial site of infection was on the lower limbs in more than half of Australian and American case-patients.

*Photorhabdus* spp. have never been shown to live freely in soil, although they will survive in soil under laboratory conditions (8). *Photorhabdus* spp. have only been isolated naturally from two nonclinical sources: insect-pathogenic nematodes (*Heterorhabditis* spp) and the insects they parasitize (beetles, moths, and the like). It seems likely therefore that *Photorhabdus* spp are transmitted to humans by a terrestrial invertebrate (nematode or arthropod), but that vector has not yet been identified.

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**Table 1. Misidentification of *Photorhabdus* isolates from patients 1 and 2 by commercially available bacterial identification systems**

<table>
<thead>
<tr>
<th>System</th>
<th>Misidentification</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>MicroScan Walkaway Rapid Neg BP Combo Panel Type 4</td>
<td><em>Shewanella putrefaciens</em></td>
<td>99.97%</td>
</tr>
<tr>
<td>MicroScan Walkaway Neg BP Combo Panel Type 11</td>
<td><em>Pseudomonas oryzihabitans</em></td>
<td>85.46%</td>
</tr>
<tr>
<td>BioMérieux Vitek GNI+ V1316</td>
<td><em>Providencia stuartii</em></td>
<td>99%</td>
</tr>
</tbody>
</table>

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**Table 2. Published human cases of *Photorhabdus* infection**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Year</th>
<th>Country</th>
<th>Location</th>
<th>Age/sex</th>
<th>Clinical</th>
<th>Alleged vector</th>
<th>Source of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2001</td>
<td>Australia</td>
<td>Gladstone, Queensland</td>
<td>39M</td>
<td>Soft tissue infection right ankle (professional pest controller)</td>
<td>Pus from ankle ulcer</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1999</td>
<td>Australia</td>
<td>Gold Coast, Queensland</td>
<td>78M</td>
<td>Soft tissue infection right foot</td>
<td>Pus and tissue from right foot</td>
<td></td>
</tr>
<tr>
<td>3 (1)</td>
<td>1998</td>
<td>Australia</td>
<td>Murwil-lumbah, New South Wales</td>
<td>55M</td>
<td>Multifocal soft tissue infections (upper and lower limbs, abdomen), pneumonia</td>
<td>Blood, sputum, pus and tissue</td>
<td></td>
</tr>
<tr>
<td>4 (1)</td>
<td>1998</td>
<td>Australia</td>
<td>Wangaratta, Victoria</td>
<td>50M</td>
<td>Multifocal soft tissue infections (upper and lower limbs)</td>
<td>Spider</td>
<td>Pus from soft tissue abscesses</td>
</tr>
<tr>
<td>5 (1)</td>
<td>1998</td>
<td>Australia</td>
<td>Melbourne, Victoria</td>
<td>90M</td>
<td>Cough and fever</td>
<td>Blood</td>
<td></td>
</tr>
<tr>
<td>6 (1)</td>
<td>1994</td>
<td>Australia</td>
<td>Melbourne, Victoria</td>
<td>11F</td>
<td>Multifocal soft tissue infections (lower limbs and chest)</td>
<td>Pus and soft tissue biopsies</td>
<td></td>
</tr>
<tr>
<td>7 (6)</td>
<td>1989</td>
<td>USA</td>
<td>San Antonio, Texas</td>
<td></td>
<td>Groin infection</td>
<td>Groin</td>
<td></td>
</tr>
<tr>
<td>8 (6)</td>
<td>1987</td>
<td>USA</td>
<td>San Antonio, Texas</td>
<td>45M</td>
<td>Multifocal soft tissue infection, left lower limb</td>
<td>Spider</td>
<td>Pus from lower limb abscess</td>
</tr>
<tr>
<td>9 (6)</td>
<td>1986</td>
<td>USA</td>
<td>San Antonio, Texas</td>
<td>78M</td>
<td>Multifocal soft tissue infection left lower limb</td>
<td>Pus from lower limb abscess and ulcer</td>
<td></td>
</tr>
<tr>
<td>10 (6)</td>
<td>1984</td>
<td>USA</td>
<td>San Antonio, Texas</td>
<td>36F</td>
<td>Disseminated bacterial infection</td>
<td>Submandible, abdomen</td>
<td></td>
</tr>
<tr>
<td>11 (6)</td>
<td>1979</td>
<td>USA</td>
<td>Pennsylvania</td>
<td>72F</td>
<td></td>
<td>Blood, skin</td>
<td></td>
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<tr>
<td>12 (6)</td>
<td>1977</td>
<td>USA</td>
<td>Maryland</td>
<td>80F</td>
<td>Endocarditis</td>
<td>Blood</td>
<td></td>
</tr>
</tbody>
</table>
Dr. Gerrard is Director of Infectious Diseases at the Gold Coast Hospital and a clinical senior lecturer at the University of Queensland, Australia. His research interests include clinical and laboratory aspects of emerging bacterial pathogens.

References


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Life-Threatening Infantile Diarrhea from Fluoroquinolone-Resistant Salmonella enterica Typhimurium with Mutations in Both gyrA and parC

Hideo Nakaya,* Akihiro Yasuhara,* Ken Yoshimura,* Yukio Oshioi,* Hidemasa Izumiya,† and Haruo Watanabe†

Salmonella Typhimurium DT12, isolated from a 35-day-old infant with diarrhea, was highly resistant to ampicillin, tetracycline, chloramphenicol, streptomycin, gentamycin, sulfamethoxazole/trimethoprim, nalidixic acid, and fluoroquinolones. The patient responded to antibiotic therapy with fosfomycin. Multidrug-resistance may become prevalent in Salmonella infections in Japan, as shown in this first case of a patient infected with fluoroquinolone-resistant Salmonella.

Salmonella enterica serovar Typhimurium (S. Typhimurium) is one of the most important causative agents of acute human Salmonella gastroenteritis. In particular, S. Typhimurium definitive phage type104 (DT104), which has developed multidrug resistance to ampicillin, tetracycline, chloramphenicol, streptomycin, sulfa drugs, and other antibiotics, has quickly become widespread in developed countries and drawn much attention worldwide (1–6). In European countries, other types of Salmonella resistant to fluoroquinolones, including DT104, have been detected, and adequate treatment of infected patients is now a serious issue. We isolated S. Typhimurium DT12, highly resistant to fluoroquinolones, from diarrhetic stools from an infant and reported the first clinical infection in Japan (7). We describe our analysis of this isolate’s antibiotic susceptibility and drug resistance genes: three point mutations in the region determining quinolone resistance were identified in gyrA and parC.

Case Report

The patient was a 35-day-old infant boy with fever, diarrhea, and vomiting. He was born at 38 weeks, weighing 3,296 g. Hyperbilirubinemia developed at 1 week of age. He was fed by both breast milk and formula. The family history was unremarkable. The baby vomited on the night of September 4, a.m. the next day. His parents consulted the maternity office where he was born, and the obstetrician prescribed oral fosfomycin. When his fever did not subside, the obstetrician referred him to the outpatient department of pediatrics, Kansai Medical University Kohri Hospital, Osaka, Japan. Acute enteritis was diagnosed, and the patient was admitted to the hospital on September 5.

On admission, the infant was 55.0 cm long and weighed 4,536 g. His temperature was 38.6°C, heart rate 162 beats/min, respiratory rate 52/min, and blood pressure 102/palpable mmHg. He was pale and lethargic with cold extremities and cyanosis around the nose and mouth. His anterior fontanelle was 1 cm in diameter without swelling. Small eruptions were observed on his face and neck.

Laboratory evaluation was remarkable for the following: total protein 4.9 g/dL (normal range: 5.0–6.5 g/dL), albumin 2.8 g/dL (normal range: 3.3–4.2 g/dL), C-reactive protein 2.5 mg/dL (normal: <0.3 mg/dL), leukocyte 3,730/mm³ (normal range 5,000–19,500/mm³), neutrophils 59.4%, lymphocytes 33.0%, sodium ion 135 mEq/L (normal range: 135–147 mEq/L), potassium ion 4.5 mEq/L (normal range: 3.6–5.0 mEq/L), chloride ion 108 mEq/L (normal range: 98–108 mEq/L), calcium 5.0 mEq/L (normal range 4.2–5.7 mEq/L), blood urea nitrogen 9.8 mg/dL (normal range: 4–15.4 mg/dL), creatinine 0.27 mg/dL (normal range: 0.23–0.6 mg/dL), uric acid 3.6 mg/dL (normal range: 1.4–3.5 mg/dL), and blood sugar 93 mg/dL (normal range: 60–100 mg/dL). S. Typhimurium DT12, named KKH712, was isolated from his stool. Liver panel and chemistries were otherwise normal.

Upon admission, fosfomycin by injection was administered for bacterial enteritis. Frequent diarrhea and vomiting decreased. The baby’s body temperature and C-reactive protein level normalized, and his general condition improved. He was discharged on day 14. Outpatient follow-up showed that, with fosfomycin, his stool culture eventually tested negative for Salmonella. Stool cultures from his family members (father, mother, sister, and brother) did not show the causative Salmonella isolate, and no member of family had diarrhea or took antimicrobial drugs. The family did not have a pet and had not traveled overseas recently. We did not find a route of infection.

Conclusions

We examined the S. Typhimurium DT12 (KKH712) isolated from the patient for drug susceptibility. We determined MICs of 18 antibiotics (ampicillin, cefaclor, cefazolin, cefazidime, ceftriaxone, imipenem, streptomycin, kanamycin, gentamycin, amikacin, tetracycline, chloramphenicol, sulfamethoxazole/trimethoprim, fosfomycin, nalidixic acid, levofloxicin, ciprofloxacin, and norflaxacin) against the strain by the agar plate dilution method provided by the National Committee for Clinical Laboratory Standards (8). The strain, S. Typhimurium ATCC13311, was used as a sensitive strain for the comparison, and Escherichia coli ATCC25922 was used as the quality control reference strain. Susceptibilities of S. Typhimurium DT12
Sequence analysis of the gyrA and parC genes was performed by the method described by Giraud et al. (9). In brief, DNA fragments of each gene were amplified in 50-µL reaction mixture by using boiled bacterial suspension with 200 µM of deoxynucleotide triphosphate, 1 µM of the primer pairs, Taq buffer (QIAGEN GmbH, Hilden, Germany), and 2.5 U of Taq DNA polymerase (QIAGEN GmbH). Polymerase chain reaction was run at 93°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min for 35 cycles. The DNA fragments were purified by using MicroSpin Column S-300HR (Amersham Pharmacia Biotech, Piscataway, NJ). Sequence was determined by the method described by Sanger et al. (10) in an automatic DNA sequencer (Applied Biosystem 310, Perkin-Elmer Inc., Foster City, CA) by using primer STGYRA1 or STPARC1 for gyrA or parC fragments, respectively (Table 2). Nucleotide sequences of its gyrA and parC genes were determined and point mutations were detected: Ser83Phe (TCC→TTC) and Asp87Asn (GAC→GAC) in the quinolone resistance-determining region (QRDR) of gyrA and Ser80Arg (AGC→CGC) in the QRDR of parC.

The World Health Organization has determined that Salmonella is reemerging as one of the most important infectious diseases in the world. Drug-resistant Salmonella strains, for which infections are increasing worldwide, are of special concern (1–6). In Japan, quinolone-resistant S. Typhimurium strains from domestic cases have been emerging since 1995 (11). The phage type of the multidrug-resistant S. Typhimurium that we isolated was DT12, not DT104, the prevalent type in developed countries (12), which suggests that the strain in this study may differ from the prevalent ones. BlnI-digested pulsed-field gel electrophoresis patterns were different between S. Typhimurium DT12 KKH712 and typical Japanese isolates of S. Typhimurium DT104 (12, data not shown), which also supports the idea that this strain is different. Some multiple drug-resistant S. Typhimurium DT12 strains have been reported in Japan, but the frequency of this strain is not as high as that of DT104 (12). Recently, other S. Typhimurium DT12 strains with high fluoroquinolone resistance in humans were isolated in Japan (pers. commun., H. Izumiya). However, scant data are available on fluoroquinolone resistance of S. Typhimurium DT12 originating from cattle in Japan.

The main mechanism of fluoroquinolone resistance by Enterobacteriaceae, including Escherichia coli, is reported to be several point mutations in the QRDR in the structural gene of DNA gyrase or DNA topoisomerase IV. Analysis of the quinolone resistant gene in the strain obtained from our patient showed three point mutations in QRDR: Ser83Phe (TCC→TTC) and Asp87Asn (GAC→GAC) in QRDR of gyrA and Ser80Arg (AGC→CGC) in QRDR of parC. These same three mutations have been reported previously in fluoroquinolone-resistant bacteria (9,13–15). However, to the best of our knowledge, this report is the first of a Salmonella isolate highly resistant to fluoroquinolones from a clinical case with three point mutations in the QRDR (16).

Ampicillin, chloramphenicol, sulfon drugs, and fluoroquinolone have been established as standard first-line therapy for Salmonella infections. If the Salmonella is a multidrug-resistant strain as in this case, however, all of these antibiotics will be ineffective, and treatment will be difficult. In fact, a previous report describes a patient death after a nosocomial Salmonella outbreak in a U.S. hospital (17).

Fosfomycin, administered to our patient, has been used to treat various infectious diseases in Japan. This drug is one of the most commonly used antibiotics in Japan because it produces relatively few side effects. In our case, fosfomycin was quite effective against the multidrug- and fluoroquinolone-resistant Salmonella Typhimurium DT12 isolate KKH712.

Table 1. MICs (µg/mL) of 18 antibiotics for Salmonella Typhimurium strains (ATCC13311 and KKH712)

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>ATCC13311</th>
<th>KKH712</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>≤0.5</td>
<td>512</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ceftriaxime</td>
<td>0.13</td>
<td>0.25</td>
</tr>
<tr>
<td>Ceftriazone</td>
<td>≤0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>8</td>
<td>512</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Gentamicin</td>
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<td>32</td>
</tr>
<tr>
<td>Amikacin</td>
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<td>1</td>
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<tr>
<td>Tetracycline</td>
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<td>128</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>4</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Sulfamethoxazole/trimethoprim</td>
<td>1</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>4</td>
<td>&gt;512</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>≤0.03</td>
<td>8</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0.06</td>
<td>16</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≤0.03</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2. Primers for sequence analysis of gyrA and parC

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>STGYRA1</td>
<td>5’-TGTCCGAGATGGCCTGAAGC-3’</td>
</tr>
<tr>
<td>STGYRA2</td>
<td>5’-CGTTGATGCTTCCCGTGAGC-3’</td>
</tr>
<tr>
<td>STPARC1</td>
<td>5’-ATGAGCGATATGGCAGAGCG-3’</td>
</tr>
<tr>
<td>STPARC2</td>
<td>5’-TGACCGAGTTCGCTTAACAG-3’</td>
</tr>
</tbody>
</table>
resistant Salmonella; our patient recovered after taking this antibiotic, which is considered relatively safe. Fosfomycin is often administered to babies and children and expected to be effective; however, fosfomycin-resistant Salmonella has been reported in Japan (18). In Japan, fosfomycin was approved for use in animals in 1986. Thus far, fosfomycin-resistant Salmonella has not increased. We must be very careful not to overuse this antibiotic and thereby introduce Salmonella strains resistant to fosfomycin as has happened with the fluoroquinolones.

The emergence of fluoroquinolone-resistant Salmonella in European countries is attributed to the use of fluoroquinolones in livestock and the accompanying natural selection of the resistant strain. In Japan, fluoroquinolones were approved for use in animals in 1991. Fluoroquinolones tend to be used more frequently in Japan than in Europe. Persons in Japan, then, are at risk of having more infectious diseases caused by fluoroquinolone-resistant Salmonella and other bacteria. Surveillance for antimicrobial resistance of Salmonella should be continued, particularly to monitor the emergence of strains with high fluoroquinolone resistance from humans and livestock.

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References

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Invasive Type e Haemophilus influenzae Disease in Italy

Marina Cerquetti,* Marta Luisa Ciofi degli Atti,* Rita Cardines,* Stefania Salmaso,* Giovanna Renna,* Paola Mastrantonio,* and the Hi Study Group

We describe the first reported cases of invasive type e Haemophilus influenzae disease in Italy. All five cases occurred in adults. The isolates were susceptible to ampicillin and eight other antimicrobial agents. Molecular analysis showed two distinct type e strains circulating in Italy, both containing a single copy of the capsulation locus.

In Italy, the vaccine against Haemophilus influenzae type b (Hib) was licensed in 1995, but vaccination is voluntary. Vaccination coverage by 24 months of age was estimated in 1998 at 19.8% for the 1996 birth cohort (1). Coverage increased in 2000 to 53.1% for the 1998 birth cohort (2). Since 1994, surveillance of H. influenzae meningitis has been conducted within the National Surveillance of Bacterial Meningitis (3). A laboratory-based active surveillance of invasive H. influenzae disease was implemented in a sample of Italian regions in 1997 (4). In 1997–1998, the incidence of invasive Hib disease estimated by this system was lower than that reported in northern and central Europe and in the United States before mass vaccination was introduced, yet was comparable with the incidence reported in other Mediterranean countries with similar vaccination coverage (4).

In 1998–2001, laboratory-based active surveillance was conducted in seven Italian regions, including a population of approximately 24 million persons (33% of the Italian population). Participating regions were located throughout the country and included rural, urban, and large metropolitan areas. Active H. influenzae case finding was conducted by contacting, monthly, microbiologists from the regional laboratories of hospitals with infectious disease or pediatric wards. A patient with invasive disease was defined as a patient with a compatible illness, accompanied by isolation of H. influenzae from a normally sterile site or detection of Hib antigen in cerebrospinal fluid. Hospital microbiologists were asked to send H. influenzae isolates to the national reference laboratory at Istituto Superiore di Sanità, where all strains were assayed by polymerase chain reaction (PCR) capsular genotyping. Serotyping by slide agglutination was performed at the regional level, when possible, and at the national reference laboratory.

From 1998 to 2001, a total of 219 cases of invasive H. influenzae disease were reported; 165 were diagnosed by isolation of H. influenzae from a normally sterile site, and 54 were diagnosed by detection of Hib antigen in cerebrospinal fluid. Of the 165 isolates, 97 (58.8%) were sent to the national reference laboratory; the percentage of isolates sent to the reference laboratory remained relatively stable over the years (from 62.3% in 1998 to 58.3% in 2001). Analysis of incidence data by serotype showed that the annual number of cases of invasive Hib disease decreased from 69 in 1998 to 17 in 2001, while the number of nontypable H. influenzae remained constant (mean 8 cases/year; range 7–11) (Table 1). No cases attributable to capsulated H. influenzae other than type b were detected in years 1998–1999; in the next 2 years, a total of seven cases were traced; five were due to type e strain.

We describe these five cases of invasive disease caused by H. influenzae type e (Hie). Genetic relationship among the five Hie isolates was assessed by pulsed-field gel electrophoresis (PFGE). Susceptibility to nine antimicrobial agents, including ampicillin, was also determined. Because amplification of capsulation (cap) locus is assumed to contribute to strain virulence, the copy number of cap e locus in each isolate was also identified.

The Study

Five Hie isolates were detected through surveillance from January 2000 to December 2001. Characteristics of patients were obtained by reviewing clinical records (Table 2). Briefly, two cases, both with meningitis, occurred in young adults, who recovered. The remaining three cases, two with bacteremic pneumonia and one with septicemia, were in elderly patients, who died. All but one (no. 2) of the patients were from neighboring regions in northeastern Italy. However, the towns they lived in were quite distant, and the patients were admitted to different hospitals in different periods. Serotyping of the isolates by slide agglutination was performed by using polyvalent and monovalent antisera to capsular serotypes a through f (Difco Laboratories, Detroit, MI). Capsular genotype was identified by PCR (5). Briefly, in a first round of PCR, primers to the ompP2 gene were used to confirm the H. influenzae species, while primers directed to the hex region confirmed capsulation. A second round of PCR with primers directed to the cap e–specific region (6) generated an expected product of 1,350 bp. Further characterization of the isolates was performed by phenotypic and genotypic methods. Bio- types were assigned by determining indole production, urease


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level, and ornithine decarboxylase activities. MICs of ampicil-
in were determined by E-test (AB Biodisk, Solna, Sweden).
Hib strain ATCC 10211 was used as control. Susceptibilities to
trimethoprim/sulphamethoxazole, ceftazidime, chlorampheni-
col, azithromycin, aztreonam, ciprofloxacin, imipenem, and
tetracycline were tested by disk diffusion assay. Both the E-
test and the disk diffusion assay were performed by using 
Hae-
mophilus test medium, as recommended by the National Com-
mittee for Clinical Laboratory Standards (NCCLS) (7).
Interpretative breakpoints and zone diameters were also based
on NCCLS criteria. Production of β-lactamase was detected by
the cefinase disk test (BBL, Becton-Dickinson, Sparks, MD).
Restriction fragment length polymorphism analysis by PFGE
was conducted as described (5), except for digestion of
genomic DNA, performed by using Smal (20 U) or Apal
(20 U) restriction enzymes (New England BioLabs, Wilbury
Way Hitchin, U.K.). The copy number of cap e locus was
determined by Southern blot analysis, using as probe the
1,350-bp amplicon obtained by PCR of a prototype type e
strain. Since the KpnI and Smal sites flank the cap locus of
capsulated H. influenzae strains, the copy number of the
 locus can be estimated by the size of the restriction fragment
obtained after digestion of the chromosome with these
enzymes (8). Restriction fragments were separated by PFGE
as described (5), transferred to nylon membranes, and hybrid-
ized with the probe. Labeling of the probe and hybridization
reactions were obtained by using the ECL kit (Amersham
Pharmacia Biotech, Little Chalfont, U.K.). Hybridizing bands
were visualized by autoradiographs. Although no data are
available on the size of cap e locus, on the basis of results
obtained on Hib strains, the DNA fragment for a single-copy
strain was expected to be approximately 27–28 kb. In fact, the
KpnI/Smal fragment includes the cap locus, whose size is 18
kb in Hib strains, plus additional segments (about 10 kb)
upstream and downstream of the cap region (8). Strains with
two or more copies of the cap b locus featured fragments of
increased size (45 kb, 63 kb, 81 kb, 99 kb) (8).

By PCR, all isolates exhibited the type e capsular geno-
type. By slide agglutination method, performed at the national
reference laboratory, four isolates were detected as type e, and
one was misidentified as nontypable. At the regional level,
only one of the five PCR-positive strains had been recognized
as type e, two had been identified as nontypable, and two had
not been typed. By biotyping, four isolates were classified as
biotype IV, and one as biotype I (patient no. 5) (Table 2). MICs
of ampicillin ranged from 0.125 µg/mL to 0.25 µg/mL, indi-
cating that all isolates were susceptible to this antibiotic; none
produced β-lactamase. As assessed by disk diffusion assay, all
isolates were also susceptible to trimethoprim/sulphamethox-
azole, ceftazidime, chloramphenicol, azithromycin, aztre-
onam, ciprofloxacin, imipenem, and tetracycline. PFGE with
SmaI restriction enzyme digestion generated not well-resolved
profiles, as several very close fragments of 194–145 kb were
obtained (data not shown). Following Apal digestion, profiles
were easier to compare (Figure): three isolates (patients no. 3,
4, and 5) shared an indistinguishable pattern (pattern 1); one
isolate (patient no. 1) showed a profile closely related to pat-
ttern 1 but with two band differences (pattern 1a). The isolate
from patient no. 2 appeared clearly different from the others
(pattern 2), according to criteria reported by Tenover et al. (10).

Table 1. Incidence per 100,000 persons and cases of invasive Haemophilus influenzae disease, by serotype, Italy, 1998–2001

<table>
<thead>
<tr>
<th>H. influenzae</th>
<th>1998</th>
<th>1999</th>
<th>2000</th>
<th>2001</th>
</tr>
</thead>
<tbody>
<tr>
<td>All capsular types plus nontypablea</td>
<td>0.35 (84)</td>
<td>0.26 (64)</td>
<td>0.16 (40)</td>
<td>0.13 (31)</td>
</tr>
<tr>
<td>Type b</td>
<td>0.28 (69)</td>
<td>0.18 (44)</td>
<td>0.09 (21)</td>
<td>0.07 (17)</td>
</tr>
<tr>
<td>Nontypable</td>
<td>0.05 (11)</td>
<td>0.03 (7)</td>
<td>0.03 (7)</td>
<td>0.03 (7)</td>
</tr>
<tr>
<td>Capsulated other than b</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.02 (4)</td>
<td>0.01 (3)</td>
</tr>
</tbody>
</table>

*aIncludes isolates that were not serotyped.

Table 2. Clinical data of patients with Hie invasive disease and characterization of isolates

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Mo/yr of</th>
<th>Age</th>
<th>Gender</th>
<th>Signs and symptoms</th>
<th>Underlying condition</th>
<th>Outcome</th>
<th>Site of isolation</th>
<th>Biotype</th>
<th>PFGE pattern</th>
<th>Copy no. of cap e locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>01/2000</td>
<td>75</td>
<td>M</td>
<td>Bacteremic pneumonia</td>
<td>Chronic lymphocytic leukemia</td>
<td>Died</td>
<td>Blood</td>
<td>IV</td>
<td>1a</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>05/2000</td>
<td>35</td>
<td>M</td>
<td>Meningitis</td>
<td>Head trauma from car accident</td>
<td>Survived</td>
<td>CSF</td>
<td>IV</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>10/2000</td>
<td>65</td>
<td>M</td>
<td>Septicemia</td>
<td>Retro-peritoneal sarcoma</td>
<td>Died</td>
<td>Blood</td>
<td>IV</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>02/2001</td>
<td>98</td>
<td>F</td>
<td>Bacteremic pneumonia</td>
<td>Chronic heart disease</td>
<td>Died</td>
<td>Blood</td>
<td>IV</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>12/2001</td>
<td>33</td>
<td>F</td>
<td>Meningitis</td>
<td>None</td>
<td>Survived</td>
<td>CSF</td>
<td>I</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Hie, Haemophilus influenzae type e; CSF, cerebrospinal fluid; PFGE, pulsed-field gel electrophoresis; M, male; F, female.
Conclusions

Although studies on nasopharyngeal carriage of *H. influenzae* have shown the presence of serotype *e* as colonizer (11–13), few studies have described serious infections attributable to this capsular type (14–17). Our results suggest that *Hie* may cause either fatal infections in elderly patients with underlying disease or meningitis in adults whether or not underlying conditions are present. In Italy, these cases of *Hie* infection are the first reported. Despite limited data on the frequency of non-type *b* invasive disease before 1997, our results are based on 4 years of laboratory-based active surveillance on a large population sample. Since no type *e* strains were detected in the first 2 years of this surveillance, this clustering is likely due to the emergence of invasive *Hie* disease. Alternative explanations might include improved laboratory confirmation methods (however, the proportion of meningitis cases without an identified etiologic agent did not decrease from 1998 to 2001) or improved reporting in the last 2 years of surveillance (however, no changes to the system were made, and quality indicators, such as proportion of isolates sent to the reference laboratory, remained stable throughout the 4 years).

An unequivocal method of assigning capsular type is required to monitor infection attributable to uncommon serotypes, especially since, as in some *Hie* isolates, expression of capsular polysaccharide is not sufficient to be detected by slide agglutination (18). In our study, PCR capsular typing allowed the identification of an additional *Hie* strain that had been misidentified by slide agglutination.

The prevalence of ampicillin resistance in both β-lactamase–positive and –negative *H. influenzae* isolates has increased worldwide. Neither β-lactamase production nor intrinsic resistance was detected among our *Hie* isolates, confirming the low incidence of ampicillin-resistant *H. influenzae* strains from invasive disease in Italy (5,9).

PFGE has been successfully used to type *H. influenzae* isolates. Our results suggest that two distinct *Hie* strains circulated in Italy in 2000–2001. Four of the five isolates, found in two neighboring regions, appeared to represent a unique clonal group with two subtypes. This clustering of most strains in one PFGE pattern might be explained with the clonal population structure of encapsulated *H. influenzae* previously observed in Italy (5,9), but further studies are needed to clarify this point. Moreover, since the type *e* isolates analyzed in this study had no close genetic relationship to the invasive *Hib* strain circulating in Italy, their derivation from type *b* isolates by capsular switch is unlikely. *H. influenzae* PFGE typing has a stronger discriminatory power than biotyping (19). In our study, however, in the isolates assigned to the same clonal group by PFGE, one was classified as biotype I, and the others were biotype IV. These findings suggest that biotyping could be a useful adjunct to PFGE when typing *Hie* isolates.

In our study, all five *Hie* isolates contained a single copy of the *cap e* locus, suggesting that they did not possess unusual virulent traits related to the capsule. Since the size of the restriction fragment obtained was smaller than expected on the basis of *cap b* locus size, a possible explanation may be that the size of region 2 of *cap e* locus is smaller than that found in *Hib* strains. Alternatively, the DNA flanking the *cap e* locus may differ from that found in *Hib* strains; therefore, the segments upstream and downstream from the *cap e* region would be smaller.

Both the laboratory-based active surveillance of invasive *H. influenzae* disease and PCR capsular genotyping could have improved the capability to detect cases attributable to serotypes other than *b*. Nevertheless, our data suggest the
emergence of invasive Hie disease among the adult population in Italy and underline the need to closely monitor infection caused by non–type b strains.

Acknowledgments

We are grateful to Dr. Tonino Sofia for editorial assistance and M.P.E. Slack for providing a prototype type e strain.

Dr. Cerquetti is a scientist at the Laboratory of Bacteriology and Medical Mycology of Istituto Superiore di Sanità. She has been reference microbiologist for Italy in the European Union Biomedicine and Health II Haemophilus influenzae type b surveillance project (1996–1998) and in the European Union Health and Consumer Protection Directorate-General surveillance network project for invasive H. influenzae disease (2000–2001). Her research interests include investigations on mechanisms of pathogenesis in H. influenzae and Clostridium difficile.

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Public Health Surveillance for Australian Bat Lyssavirus, in Queensland, Australia, 2000–2001

David Warrilow,* Bruce Harrower,* Ina L. Smith,* Hume Field,† Roscoe Taylor,‡ Craig Walker,§ and Greg A. Smith*

From February 1, 2000, to December 4, 2001, a total of 119 bats (85 Megachiroptera and 34 Microchiroptera) were tested for Australian bat lyssavirus (ABLV) infection. Eight Megachiroptera were positive by immunofluorescence assay that used cross-reactive antibodies to rabies nucleocapsid protein. A case study of cross-species transmission of ABLV supports the conclusion that a bat reservoir exists for ABLV in which the virus circulates across Megachiroptera species within mixed communities.

Since the identification of Australian bat lyssavirus (ABLV) in bat species throughout Australia (1), public health units have been forced to confront its implications for human health. Fortunately, because of the close genetic and serologic relationship between rabies and ABLV, rabies immune sera and vaccines offer postexposure protection from infection (1,2). A bite or scratch from a bat in Australia constitutes a potential exposure to ABLV, and persons affected should be offered postexposure prophylaxis unless the bat can be shown to be uninfected with ABLV. However, such prophylaxis is costly and uncomfortable, and immune sera are in short supply worldwide. As many bats involved in such incidents are uninfected, and a negative result obviates the need for postexposure prophylaxis, determining each bat’s ABLV infection status is preferable. Under instruction from the public health units, Queensland Health Scientific Services Public Health Virology Laboratory has tested bats for ABLV since July 1998.

Two strains of ABLV are known to be circulating. One strain was isolated from a species of insectivorous Microchiroptera, Saccolaimus flaviventris (1). A second strain has been shown to infect the four species of Megachiroptera in the genus Pteropus that occur in mainland Australia (1,3). Isolates from the four pterloid species show minimal sequence variation with geographic origin and species and are essentially identical (I.L. Smith, unpub. data). Pteropid bats are nomadic nocturnal mammals that roost in trees during the day in colonies that frequently number in the thousands. Colonies may contain one or more species, and fluctuate in size, depending on available food resource (4). This dynamic social structure has been evoked to explain the circulation of a single strain of ABLV in pteropids (1).

In this article, we report on surveillance of bats brought in for testing to our laboratory. A case is described in which circumstantial evidence exists for bat-to-bat cross-species transmission of ABLV. This finding is consistent with a model in which the social structure of pteropid camps results in a single strain of circulating virus.

The Study

Bats involved in incidents involving public health were tested for ABLV infection by immunofluorescence assay (IFA) on brain impression smears by using a fluorescein isothiocyanate-conjugated monoclonal (Fujirebio Diagnostics, Malvern, PA) or polyclonal (Biorad, Hercules, CA) antibodies to the nucleocapsid protein. The IFA was performed in parallel with a fluorescent real-time polymerase chain reaction (PCR) assay (5) for pteropid samples, or a heminested reverse transcriptase (RT)-PCR for all others (6). When it was possible, the bat was identified to the species level (7). For molecular analyses, the region encoding the carboxy-terminal of the glycoprotein and its long 3′ untranslated region were amplified by RT-PCR, and the products were directly sequenced by using Big-Dye chemistry (Applied Biosystems, Foster City, CA). Primers and reaction details are available on request.

During February 1, 2000, to December 4, 2001, a total of 119 bats, including 85 Megachiroptera and 34 Microchiroptera, were submitted for testing to the Public Health Virology Laboratory, Queensland Health Scientific Services. Bats submitted for testing had either bitten or scratched a person, or testing was considered to be in the interests of public health. Eight bats tested positive for ABLV infection by IFA (Table 1). Six of the bats positive for ABLV were P. alecto (75%); one bat positive for ABLV was P. poliocephalus (12.5%), and another bat that tested positive was an unidentified member of the genus Pteropus (12.5%). No positive Microchiroptera were obtained during the study period. Positive bats were from the Rockhampton area (37.5%), south of the Brisbane South metropolitan area (25%), Townsville area (12.5%), Sunshine Coast area (12.5%), and Brisbane South Coast area (12.5%). Confirmatory real-time or heminested RT-PCR results were concordant with IFA in all cases. Controls for contamination were negative for ABLV.

In October 2000, a wild Black Flying Fox (bat 1, P. alecto) that was acting aggressively was removed from the top of a dome-shaped wire-mesh enclosure for viewing bats at a zoo in Rockhampton. Inside this enclosure were housed 23 flying foxes, all previously well. No new bats had been added to the

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cage for 12 months. The animal was euthanized and sent for testing to Queensland Health Scientific Services where it tested positive for ABLV by IFA on brain impression smear. One month later a captive Gray-headed Flying Fox (bat 2, P. scapulatus) from within the enclosure was observed behaving abnormally. Normally a highly social animal, the bat was not moving freely and was licking its vulva profusely. The animal was relocated to an isolation cage where, during the next 20 hours, it exhibited a progressive neurologic syndrome. The animal was relocated to an isolation cage where, during the next 3 months, and then for a further 3 months after they returned to their original enclosure. No attributable clinical disease, seroconversion, or behavioral change was observed during this time. The bat enclosure was modified to incorporate an extra outer mesh layer to prevent future direct contact with free-living wild bats outside the enclosure. Existing double-fencing had already prevented the public from having direct contact with flying foxes in the enclosure.

**Conclusions**

The infection prevalence of 9.4% in submitted flying foxes in this study is not statistically significantly higher than that previously observed (6%) in sick, injured, and orphan flying fox submissions (3; H.E. Field, unpub. data); the wide 95% confidence interval (CI) (4% to 18%) reflects the limited sample size in this study. The prevalence is, however, statistically significantly higher than that observed in wild-caught flying foxes (3; H.E. Field, unpub. data), reinforcing the contention that the subpopulation of sick and injured flying foxes poses a higher risk for exposed humans. None of the submitted Microchiroptera was positive for ABLV. The small sample size in this study limits meaningful interpretation of this observation (the 95% CI for 0% prevalence with a sample size of 34 is 0% to 10%). Although this finding could indicate a lower incidence of ABLV in communities of Microchiroptera, downplaying the risk for human exposure posed by Microchiroptera in Australia would be premature.

The incident showing transmission from an ABLV-infected wild flying fox to a captive flying fox is interesting in two respects. First, an incubation period for the virus can be estimated. Assuming the scratch/bite occurred close to the time when the wild bat was discovered, the captive bat was observed to display symptoms after 29 days. This period compares with the only reported incubation time for an ABLV infection in a Black Flying Fox (P. alecto) of 6–9 weeks (9). Incubation times after experimental infection of Vampire Bats with rabies were shorter at 7–26 days for intramuscular injection (10) or 2–4 weeks after subcutaneous or intramuscular injection (11).

Second, this report is the first to describe probable natural cross-species transmission. This finding has implications for

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**Table 1. Bats positive for Australian bat lyssavirus infection**

<table>
<thead>
<tr>
<th>Species</th>
<th>IFA positive&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Megachiroptera</td>
<td></td>
</tr>
<tr>
<td>Pteropodidae</td>
<td></td>
</tr>
<tr>
<td>Pteropus alecto</td>
<td>6/50</td>
</tr>
<tr>
<td>Pteropus scapulatus</td>
<td>0/18</td>
</tr>
<tr>
<td>Pteropus poliocephalus</td>
<td>1/8</td>
</tr>
<tr>
<td>Pteropus conspicillatus</td>
<td>0/4</td>
</tr>
<tr>
<td>Unidentified Pteropus</td>
<td>1/5</td>
</tr>
<tr>
<td>Subtotal</td>
<td>8/85</td>
</tr>
<tr>
<td>Microchiroptera</td>
<td></td>
</tr>
<tr>
<td>Hipposideridae</td>
<td></td>
</tr>
<tr>
<td>Hipposiderosater</td>
<td>0/1</td>
</tr>
<tr>
<td>Molossidae</td>
<td></td>
</tr>
<tr>
<td>Mormopterus beccarii</td>
<td>0/2</td>
</tr>
<tr>
<td>Mormopterus loriae</td>
<td>0/2</td>
</tr>
<tr>
<td>Unidentified Mormopterus</td>
<td>0/2</td>
</tr>
<tr>
<td>Rhinolophidae</td>
<td></td>
</tr>
<tr>
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<td>Miniopterus scotorepens</td>
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<td>Total</td>
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</table>

<sup>a</sup>IFA, immunofluorescence assay; number positive/number tested.
or our understanding of how the virus circulates in bat communities. Evidence of cross-species transmission is consistent with the minimal sequence variation in isolates from sick or injured flying foxes obtained from various species at different sites around Australia (1). The large seasonally nomadic, multispecies colonies in which flying foxes commonly congregate (and interact) provide opportunity for interspecies and interregion transmission of ABLV. Our findings support this model for circulation of ABLV in pteropid colonies.

Acknowledgments
We acknowledge the expertise and dedication of Leanne Law and Lynette Hoskins for their care and daily monitoring of the quarantined colony and for their assistance with blood sample collections.

Dr. Warrilow is a research scientist at the Public Health Virology Laboratory, Queensland Health Scientific Services. His research interests focus on novel approaches to viral antigen production and therapeutic delivery. He is currently engaged in a project to establish a reverse genetics system for Australian bat lyssavirus and is also interested in the development of nucleic acid-based tests to detect viral diseases.

References

<table>
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<td>G</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>A</td>
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<tr>
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<td>G</td>
<td>G</td>
<td>C</td>
<td>G</td>
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</tr>
<tr>
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<td>A</td>
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<td>A</td>
<td>G</td>
<td>T</td>
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<sup>a</sup>Nucleotide position from the Ballina isolate of the pteropid strain of ABLV (8); A, deoxyadenosine; C, deoxycytidine; G, deoxyguanosine; T, thymidine.
<sup>b</sup>Case study, bat 1.
<sup>c</sup>Case study, bat 2.
<sup>d</sup>Queensland Health Scientific Services collection.
<sup>e</sup>From reference 8.

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Infection of Cultured Human and Monkey Cell Lines with Extract of Penaeid Shrimp Infected with Taura Syndrome Virus

Josefina Audelo-del-Valle,* Oliva Clement-Mellado,† Anastasia Magaña-Hernández,† Ana Flisser,‡ Fernando Montiel-Aguirre,‡ and Baltasar Briseño-García†

Taura syndrome virus (TSV) affects shrimp cultured for human consumption. Although TSV is related to the Cricket Paralysis virus, it belongs to the "picornavirus superfamily," the most common cause of viral illnesses. Here we demonstrate that TSV also infects human cell lines, which may suggest that Penaeus is a potential reservoir of this virus.

The Taura syndrome virus (TSV) causes a disease affecting penaeid shrimp, the most important commercial family of crustaceans (1). The causal agent is a single-stranded (+) RNA virus, recently reported to be genomically related to the Cricket Paralysis virus of the Cripavirus genus, family Dicistroviridae of the "picornavirus superfamily" (2–5). This superfamily includes several human pathogens, for example, the common cold viruses and several enteroviruses (e.g., polioviruses). Traditionally, research on the replication of shrimp viruses has been based on the use of cultured fish cellular lines (6). However, because TSV could potentially represent a public health threat, we explored whether this viral agent might be capable of infecting cultured mammalian cells.

The Study

Since Sabin strain LSc 2ab (Sabin 1), the poliovirus used for human vaccination, is usually replicated in monolayer culture cells of human rhabdomyosarcoma (RD), human larynx carcinoma (Hep-2C), or Buffalo green monkey kidney (BGM) (7), we injected these cell lines with a 0.22-µm membrane-filtered whole extract of the hepatopancreas of shrimp (Penaeus stylirostris) affected with TSV. The animals were collected from farms located in northwestern Mexico. Control cell lines were injected with filtered hepatopancreas extracts from healthy shrimp. Cultures were incubated at 37°C and periodically observed under a microscope until any sign of cytopathic effect was detected (usually from 19–23 hours). Cells were then harvested and lysed. Fresh cell lines were inoculated with the lysate, incubated, and processed in a similar way. A third inoculation was again performed with the second lysate (8).

The cytopathic effect in RD cells began with a partial destruction of the cellular layer. Next, small cellular groups and some isolated round cells were observed. The cells showed an apparent increase in size, diffuse cell rounding, and a refringent aspect (Figure 1B). In Hep-2C cells, the cellular monolayer was partially destroyed. Most cells were individualized and clearly rounded; they also presented a refringent aspect. Hep-2C was the most affected of the three lines used (Figure 1D). The cytopathic effect in the BGM cell line began as a partial destruction of the cellular layer, which evolved to a syncytial-like formation of rounded, refringent cells. Some cells remained isolated but with altered morphology (Figure 1F). RD, Hep-2C, and BGM cells injected with an extract similarly processed but from healthy shrimp, showed no cytopathic effects, even after 7 days of culture (Figures 1A, 1C, and 1E). As a positive control, RD cells were injected with Sabin viral extract and showed the characteristic cytopathic effect produced by an enterovirus infection.

To confirm the presence of TSV in the cell culture media, a bioassay was performed by using media from the third passage. For this assay, healthy P. stylirostris shrimp were injected with the infected medium in 10% volume of their corporal mass in the third abdominal segment. Twenty-four hours later, these animals were clearly infected, showing fragile antennas and soft cuticle as well as chromatophore expansion along the whole surface of the body, particularly at the tail fan (telson and uropods). These signs were clinically indistinguishable from those occurring in naturally infected animals and are considered as pathognomonic of the acute phase of infection by TSV (9). Presence of the viral genome in different subcuticular tissues (gills and pleopods) of these animals was confirmed by in situ hybridization by using TSV ShrimProbe (DiagXotics, Inc., Wilton, CT). RNA-DNA hybrids were clearly visible as black spots after the samples were stained with Bismarck

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Figure 1. Image of mammalian cell lines injected with extracts from healthy shrimp: A, human rhabdomyosarcoma (RD) cells; C, human larynx carcinoma (Hep-2C) cells; E, Buffalo green monkey kidney (BGM) cells. Cytopathic effect in cultured cells inoculated with extracts from shrimp affected with Taura syndrome: B, RD cells; D, Hep-2C cells; F, BGM cells.
brown (Figure 2). Shrimp injected with culture media from control cell lines showed no signs of infection after 7 days of observation.

Conclusions
If one takes into consideration the capacity of viruses to modify receptor recognition and host cell tropism and the fact that cell receptors for many of the picornavirus superfamily members seem to be ubiquitous membrane molecules (e.g., decay-accelerating factor, different type of integrins, low-density lipoprotein receptor, sialic acid [10–12]), the potential wide range of host cells for TSV should not come as a surprise. To our knowledge, these cultured human and monkey cell lines are the first reported to be infected with a viral agent isolated from shrimp. Because many members of the picornavirus superfamily are the most common causes of viral illnesses worldwide (including nonspecific febrile illnesses, myocarditis, aseptic meningitis, and sepsis-like disease), such illnesses lead to frequent unnecessary prescription of antibiotics (13). Penaeus could be considered as a reservoir of a virus that could become a potential pathogen to humans and other mammals (11,14).

Acknowledgments
We gratefully acknowledge Dolores Correa and Mirza Romero for helpful and critical discussions.

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References

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Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.
**Fluoroquinolone Resistance in Campylobacter jejuni Isolates in Travelers Returning to Finland: Association of Ciprofloxacin Resistance to Travel Destination**

Antti Hakanen,*† Hannele Jousimies-Somer,‡§1 Anja Siitonen,‡ Pentti Huovinen,* and Pirkko Kottiainen†

Ciprofloxacin resistance was analyzed in 354 *Campylobacter jejuni* isolates collected during two study periods (1995–1997 and 1998–2000) from travelers returning to Finland. The increase in resistance between the two periods was significant among all isolates (40% vs. 60%; p<0.01), as well as among those from Asia alone (45% vs. 72%; p<0.01).

*Campylobacter jejuni* isolates are naturally susceptible to fluoroquinolones (1,2). During the 1990s, however, fluoroquinolone resistance in *Campylobacter* rapidly increased in several countries (3). In Thailand and Spain, for example, up to 80% of *Campylobacter* isolates are fluoroquinolone-resistant (4,5). However, major differences in *Campylobacter* fluoroquinolone resistance levels are known to occur, and in many parts of the world fluoroquinolone resistance levels remain low (3). This study was performed to evaluate the level of fluoroquinolone resistance in *C. jejuni* isolates from travelers returning to Finland and to specify the countries where resistant isolates are acquired.

**The Study**

Our study included 354 clinical human fecal *C. jejuni* isolates collected from travelers returning to Finland from 1995 to 2000. The isolates were collected in two different phases from the laboratory of a large private hospital in Helsinki, Finland. Participants were treated as outpatients, and no data on antimicrobial usage before fecal sampling were available; all participants had a history of traveling abroad within the preceding 2 weeks. From January 1995 to November 1997, we consecutively collected 205 isolates, and from October 1998 to January 2000, 149 isolates. The isolates were cultured and identified by standard microbiologic methods (6). MICs of ciprofloxacin and nalidixic acid for the isolates were determined by the agar plate dilution method, as described (7). *C. jejuni* RH 3583 (a local control strain, originally isolated in Edinburgh, U.K., *C. jejuni* 143483) was used as a control in susceptibility testing and also as a growth control strain. The MIC breakpoint used for the resistance to ciprofloxacin was that recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for non-Enterobacteriaceae (8). To nalidixic acid, the breakpoints were those recommended by NCCLS for Enterobacteriaceae (8).

Data concerning the numbers of travels from Finland to countries of interest (i.e., countries with the largest numbers of all *C. jejuni* isolates or of ciprofloxacin-resistant isolates) during the study months were received from Statistics Finland (available from: URL: www.stat.fi/). The susceptibility data were analyzed by using the WHONET5 computer program (available from: URL: www.who.int/emcWHONET/WHONET.html).

Statistical analysis was made by using the chi-square test and the Fisher exact test. Differences between *C. jejuni* infection rates in travelers returning from various travel destinations were statistically tested with Poisson regression analysis. Differences were quantified with infection rates and 95% confidence intervals; p values of <0.05 were considered significant. Statistical data were analyzed by using the SAS system for Windows, release 8.2/2001 (SAS Institute, Inc., Cary, NC).

Of the 354 *C. jejuni* isolates studied, the country where campylobacteriosis was acquired could be identified for 319 isolates, collected from travelers to 40 different countries. The origin of 22 isolates was traced at least to a continental level; the patients involved had several travel destinations. The origin of 13 isolates remained unknown. The most common countries of origin were Spain with 77 (22%) isolates, Thailand with 50 (14%) isolates, and India with 23 (6%) isolates. During the first study period (1995–1997), 205 isolates were collected from travelers. Of the 34 countries of origin that were identified, the most common were Spain with 40 (20%) isolates, India with 19 (9%) isolates, and Thailand and Turkey, both with 17 (8%) isolates. During the second study period (1998–2000), 149 isolates were collected from travelers; of the 25 countries of origin identified, the most common were Spain with 37 (25%) isolates, Thailand with 33 (22%) isolates, and Portugal and Tunisia, both with 6 (4%) isolates. Of all 354 *C. jejuni* isolates, 172 (49%) were resistant to ciprofloxacin. Of the 205 isolates collected in 1995–1997, 82 (40%) were resistant to ciprofloxacin compared with 90 (60%) of the 149 isolates collected in 1998–2000 (p<0.01). When analyzed by continent, the increase in fluoroquinolone resistance between these two periods was significant among the isolates from Asia alone (45% vs. 72%, p<0.01; Table 1). An

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Dr. Jousimies-Somer is deceased.
increasing tendency for fluoroquinolone resistance was also observed in the isolates from the three additional continents. The numbers and proportions of ciprofloxacin-resistant isolates collected from travelers returning from Spain and Thailand, the two most common countries of origin, were analyzed separately. During the first and second collection periods, the respective numbers and proportions of ciprofloxacin-resistant isolates were 29 (73%) and 26 (70%) in the isolates from Spain, and 13 (77%) and 26 (79%) in the isolates from Thailand. To assess whether the larger proportion of isolates from Thailand during the second period (8% vs. 22%) explained the significant increase in fluoroquinolone resistance in the whole study group, we analyzed the data excluding all isolates from Thailand. The increase in ciprofloxacin resistance, from 37% to 55%, was still significant (p<0.01). A corresponding analysis that excluded all isolates from both Spain and Thailand resulted in an increase in ciprofloxacin resistance from 27% to 48% (p<0.01).

The number of ciprofloxacin-resistant *C. jejuni* isolates collected was compared with the estimated numbers of all trips from Finland during the study period to the five most frequent countries of origin for the ciprofloxacin-resistant isolates. These speculative infection rates were used to calculate rate ratios between these countries. Because the speculative infection rate by fluoroquinolone-resistant *C. jejuni* isolates was highest in travelers returning from Thailand, that country was used as the reference in the rate ratio comparisons. The rate ratios by fluoroquinolone-resistant isolates in travelers returning from Spain and Portugal were 0.11 in both groups; the differences were statistically significant compared with the ratios for the reference country (p<0.01 for both; Table 2). The corresponding rate ratios in travelers returning from India and China were 0.90 and 0.72, respectively; these differences were not significant.

**Conclusions**

We have shown that ciprofloxacin resistance significantly increased (from 40% to 60%; p<0.01) during the study period among all *C. jejuni* isolates from travelers. The increase was also significant in isolates from Asia alone, suggesting a continual presence of selection pressure for the emergence of fluoroquinolone resistance on that continent. Moreover, an

<table>
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<th>Country</th>
<th>Estimated no. of trips from Finland during the study months</th>
<th>No. of all isolates</th>
<th>Speculative infection rate</th>
<th>Rate ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of ciprofloxacin-resistant isolates</th>
<th>Speculative infection rate by ciprofloxacin-resistant isolates</th>
<th>Rate ratio&lt;sup&gt;a&lt;/sup&gt; by ciprofloxacin-resistant isolates</th>
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<td>Spain (incl. Canary Islands)</td>
<td>1,145,872</td>
<td>77</td>
<td>(0.1)</td>
<td>0.12 (0.08 to 0.17)</td>
<td>55</td>
<td>(0.05)</td>
<td>0.11 (0.07 to 0.16)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Thailand</td>
<td>87,842</td>
<td>50</td>
<td>(0.6)</td>
<td>1</td>
<td>39</td>
<td>(0.44)</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>India</td>
<td>27,591</td>
<td>23</td>
<td>(0.8)</td>
<td>1.46 (0.89 to 2.40)</td>
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<td>(0.40)</td>
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<td>China</td>
<td>25,073</td>
<td>12</td>
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<td>0.84 (0.45 to 1.58)</td>
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<td>(0.32)</td>
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<td>Portugal</td>
<td>148,647</td>
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<td>(0.1)</td>
<td>0.13 (0.07 to 0.25)</td>
<td>7</td>
<td>(0.05)</td>
<td>0.11 (0.05 to 0.24)&lt;sup&gt;d&lt;/sup&gt;</td>
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</table>

<sup>a</sup>The five most frequent countries of origin of ciprofloxacin-resistant *C. jejuni* isolates were included in this analysis.
<sup>b</sup>Based on the numbers of Finnish travelers to these countries; data collected from the reports of Statistics Finland.
<sup>c</sup>Infections per 1,000 trips. Since the total number of isolates from Finnish population was not studied, the rate is speculative but could be used to calculate comparable rate ratios.
<sup>d</sup>Thailand as the reference country; 95% confidence intervals in parentheses.
<sup>e</sup>Differences between Thailand and Spain, and Thailand and Portugal, are statistically significant (p<0.01).
increasing tendency in ciprofloxacin resistance was observed in isolates from three additional continents, but either the increase (from 44% to 51% in Europe) or the number of isolates (7 isolates from America and 32 isolates from Africa) was small, and the changes were not statistically significant. Throughout the study, the rates of ciprofloxacin resistance remained on a high level in Spain and Thailand, the two most frequent countries of origin for all foreign isolates, as well as for resistant isolates. The fact that the increase in ciprofloxacin resistance remained significant even after all isolates from Spain and Thailand were excluded from the analysis illustrates that the emergence and spread of fluoroquinolone-resistant *C. jejuni* are not restricted to a few highly Campylobacter-endemic countries. Rather, these findings show that *C. jejuni* fluoroquinolone resistance, which manifested at the beginning of the 1990s, continues to grow rapidly in many parts of the world.

Several studies have focused on the quinolone resistance of *Campylobacter* spp. In Spain, a rapid increase in quinolone resistance was observed after 1988, with up to 50% of *C. jejuni* isolates resistant by 1991 (9,10). In recent years, fluoroquinolone resistance rates among Spanish *C. jejuni* isolates have been reported to reach 80% (5); these findings are in accordance with the 70% to 73% resistance rates we observed in isolates from Spain. Similarly, the 77% to 79% rates of ciprofloxacin resistance among our isolates from Thailand concur with the surveillance data, indicating that fluoroquinolone resistance rates already exceed 80% in Thailand (4).

Our study provides data on *C. jejuni* fluoroquinolone resistance in 40 countries and on four continents, rendering possible the evaluation of the relative risk for a Finnish traveler to acquire an infection by ciprofloxacin-resistant *C. jejuni* in different travel destinations. When assessing the actual infection rate by ciprofloxacin-resistant *C. jejuni* in any destination, the number of ciprofloxacin-resistant *C. jejuni* isolates imported from that destination during the study months should be divided by the number of simultaneous trips from Finland. In our study, the total number of resistant *C. jejuni* isolates imported to Finland was not known, since we examined isolates from one hospital only. Thus, when the numbers of ciprofloxacin-resistant *C. jejuni* isolates identified were divided by the simultaneous numbers of trips from the whole country, the figures (referred to as speculative infection rates in Table 2) did not provide any real data on infection rates by resistant isolates. Nevertheless, these figures could be used to calculate rate ratios between different travel destinations. Despite the high proportions of ciprofloxacin-resistant isolates in Spain (71%) and Portugal (64%), the risk of acquiring fluoroquinolone-resistant campylobacteriosis appeared to be 10 times smaller in those countries than in Thailand. These results are in line with our previous results, which showed that a tourist’s risk of acquiring quinolone-resistant salmonellosis was significantly higher in Thailand and Malaysia than in other travel destinations (11).

In conclusion, we demonstrated a significant increase in ciprofloxacin resistance among all *C. jejuni* travelers’ isolates, as well as among the isolates from Asia alone. The rate of ciprofloxacin resistance remained on a high level throughout the study in Spain and Thailand, the two most frequent countries of origin of the ciprofloxacin-resistant isolates. These data support the concept of continuous selection pressure for the emergence and spread of fluoroquinolone resistance not only in Asia but also in many other parts of the world. Efforts should be made to elucidate and alleviate the factors behind this selection pressure.

Acknowledgments

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References


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Dual Infection by Dengue Virus and Shigella sonnei in Patient Returning from India

To the Editor: Two days after returning from a 4-week trip in India, a 44-year-old woman was admitted to a local French hospital with diarrhea and a fever (40°C). The fever had started 2 days before her return and was associated with myalgia and backache. The patient had not been vaccinated against yellow fever and did not take malaria prophylaxis during her trip. Blood smears were negative for malaria parasites. Biological analyses (complete blood count, liver enzymes, urine culture, stool culture, blood cultures) were ordered. She was sent home with an empirical treatment with ofloxacin (200 mg per day). Biological parameters were within the normal range, except for her platelet count, which was at the lower limit (170 G/L). Microbiologic analyses of stools yielded an isolate of Shigella sonnei serotype 9.

One week later, the patient was admitted to the infectious diseases unit of a university hospital in Marseilles, France, with persistent fever, myalgia, and a 7-kg weight loss; she had no digestive manifestations. Results of viral serology tests were negative, except that immunoglobulin (Ig)M, but not IgG, specific for dengue virus (formal name: Dengue virus; [DENV]) was present. This result was obtained with the Dengue Virus IgM and IgG Rapid Immunochromatographic Card Test (Biotrin, PanBio Pty. Ltd., Brisbane, Australia) and was confirmed by the Dengue Duo IgM-capture and IgG-capture enzyme-linked immunosorbent assay (ELISA) (PanBio) and a previously described in-house IgM antibody capture ELISA tests (1). Forty days later, a second serum specimen was collected and tested positive for DENV IgG with the persistence of IgM by three techniques. In light of these results, the diagnosis of primary dengue infection was established, according to criteria of the Centers for Disease Control and Prevention (2). A literature review did not find any documented case of coinfection by DENV and Shigella.

In India, DENV causes epidemic and sporadic cases year-round, with a peak in frequency from August to November, during the humid season (3). During the patient’s trip, she successively visited Mumbai (Bombay); went north to the Shimla district, Himachal Pradesh, the Agra district, and Uttar Pradesh; and came back to Bombay 3 days before leaving for France. If one assumes a 3- to 6-day incubation period, she likely acquired the dengue infection in Uttar Pradesh; the virus has been reported to circulate in this area (4).

Although severe forms are increasingly reported, most cases of dengue fever consist of a mild illness with nonspecific symptoms such as headache, myalgia, and malaise. Dengue fever may go undiagnosed in travelers returning from DENV-endemic areas. This case underlines the importance of a thorough interview and clinical examination to detect characteristic signs (photophobia, painful ocular mobilization, skin rash) in patients returning from areas endemic for dengue fever, when clinical and biological signs incompletely correlate with the primary diagnosis. Since dengue fever is the second most frequent cause of febrile illness in persons returning from tropical areas, such patients should be routinely screened for the disease.

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St. Louis Encephalitis in Argentina: The First Case Reported in the Last Seventeen Years

To the Editor: St. Louis encephalitis is a mosquito-borne viral disease that affects humans. The causative agent, SLEV (formal name: Saint Louis encephalitis virus), is a member of the Flaviviridae family.Severity of the clinical syndromes increases with age, and persons >60 years old have the highest frequency of encephalitis. The primary transmission cycle involves wild passeriform and columbiform birds, and Culex sp. mosquitoes (1). In Argentina, an urban cycle may involve Cx. quinquefasciatus, which is a source of a viral isolate, and abundant birds (house sparrows, doves, or chickens) (2). The distribution of SLEV in Argentina is wide; seroprevalence ranges from 3% to

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50% of the country’s population (3). Spinsanti et al. reported results of a serologic screening in persons ages 0–87 years who live in the city of Córdoba; antibodies were most frequently found in persons >60 years of age (4). However, cases of St. Louis encephalitis reported in Argentina are very rare. Two cases with serologic diagnosis were reported in 1964 and 1968, respectively (2). In 1971, two more cases were diagnosed on the basis of viral isolation (5). Finally, the last case reported was a patient with meningoencephalitis diagnosed in the province of Buenos Aires by hemagglutination inhibition assay (6). Herein, we report a case of Saint Louis encephalitis that occurred in the province of Córdoba, Argentina.

A 61-year-old man was admitted to the hospital in February 2002, complaining of headache, fever, and diplopia. He had been well until 3 months before admission, when ophthalmic herpes zoster was diagnosed. He underwent therapy with oral acyclovir and had a good clinical outcome. Ten days before admission, he developed unstable gait with misbalance and hand tremors, mainly at his left side. On admission, he had occipital headache, diplopia, and nausea and vomiting associated with high fever and chills. Somnolence appeared a few hours before the consultation.

The patient was a right-handed businessman, a native of Córdoba. He was married and had no risk factors for sexually transmitted diseases. He had not traveled inside or outside the country during the last year. He lived near a river with a high-density population of mosquitoes.

Vital signs on admission showed axillary temperature of 39°C, pulse of 90 beats per minute, respiratory frequency of 20 per minute, and blood pressure of 110/70 mmHg. Physical examination demonstrated a somnolent patient who was easily aroused and oriented. His speech was slurred. Results of a fundoscopic examination appeared normal. Results of a cranial-nerve examination showed horizontal left diplopia with left sixth nerve paresis. A resting, postural, and intentional hand tremor was evident. Motor strength was 5/5 throughout with normal bulk and tone, tendon reflexes, and coordination. Examination of sensitivity showed no abnormalities. A slight neck rigidity was detected.

Routine laboratory analysis was unremarkable, and results of serologic tests for coxsackie virus, echovirus, and HIV were negative. HIV-1 RNA by polymerase chain reaction (PCR) and p24 antigen were also negative. Cerebrospinal fluid study revealed a leukocyte count of 18/mm³ (80% lymphocytes), a glucose level of 48 mg/dL, and a protein level of 87 mg/dL. Cryptococcal antigen, antibodies for syphilis, Human herpesvirus 1 and 2, and PCR for varicella-zoster virus 1 and Human herpesvirus were also negative. Results of an electroencephalogram and a chest radiograph were normal. Therapy with intravenous acyclovir was initiated. A magnetic resonance imaging (MRI) scan of the brain showed a striking signal change on T2 in the substantia nigra of the midbrain, mainly at the right side.

The patient continued febrile, diplopia disappeared, and meningeal signs progressed with frank cervical stiffness, positive Kerning sign, and photophobia. Diffuse tremulousness and axial rigidity appeared. Upper extremities showed rigidity with cogwheel phenomenon. Conversely, lower extremities showed spasticity with bilateral Babinski sign. Tendon reflexes became enhanced. His gait showed retropulsion with wide base sustentation. Dysdiadochokinesia appeared. On the third day, a new lumbar puncture showed worse results: a leukocyte count of 210/mm³ (82% lymphocytes), a glucose level of 51 mg/dL, and a protein level of 106 mg/dL. Another electroencephalographic examination showed unspecific extrapyramidal signs are described in St. Louis encephalitis and Japanese encephalitis (10). The typical MRI finding of patients with St. Louis encephalitis is localized in the substantia nigra (11).

Acute- and convalescent-phase serum samples (taken 10 and 16 days after onset of illness, respectively) were sent to the Arbovirus and Arenavirus Disease Laboratory, Instituto de Virología, Córdoba, SLEV immunoglobulin (Ig) M antibodies were positive by indirect immunofluorescence assay (IFA). Seroconversion for IgG antibodies was demonstrated by IFA (7) and hemagglutination inhibition assay, with titers of 640 and 80 in the first sample and 2,560 and 320 in the second sample. These results were confirmed by neutralization test using the reduction of plates technique in Vero cells culture, as described (8). Eastern equine encephalomyelitis virus and Western equine encephalomyelitis viruses with known circulation in Argentina were included in the assay with negative results (3). An increase in antibodies titers between acute- (320) and convalescent-phase (1,280) samples was found only for SLEV. Among other flaviviruses, dengue, yellow fever, and Ilhéus circulate only in subtropical areas of Argentina (the province of Córdoba is not included in this area); only dengue virus was investigated (by neutralization test) because of a current epidemiologic surveillance program; results were negative. No evidence that West Nile virus is currently circulating or has entered Argentina was found, so we did not perform tests to detect it (2,9). Isolation of SLEV from the cerebrospinal fluid and blood was attempted in newborn mice and Vero cell cultures with negative results.

While the typical clinical manifestations of viral encephalitis (fever, headache, and altered level of consciousness) are indistinguishable from each other, tremor and other extrapyramidal signs are described in St. Louis encephalitis and Japanese encephalitis (10).
In summary, the occurrence of St. Louis encephalitis in a 61-year-old patient, after >10 years of no reports in Argentina, along with specific epidemiology, suggest that further studies are needed to assess the risk for human infection by SLEV in Argentina and the role of several mosquito species in its transmission.

Acknowledgments

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Streptomyces bikiniensis Bacteremia

To the Editor: Carey et al. recently reported in this journal a case of catheter-related bacteremia attributed to Streptomyces in a patient receiving holistic infusions (1). We describe the isolation of Streptomyces bikiniensis from multiple blood cultures in a single patient over the course of 1 week, further illustrating that Streptomyces is pathogenic and a cause of bacteremia even in the absence of overt clinical symptoms and risk factors.

A 14-year-old girl with osteosarcoma of the right proximal tibia came to our hospital 13 months after diagnosis for her final course of chemotheraphy. At the time of diagnosis, a double-lumen central venous catheter was inserted. Her course was complicated by poor response to chemotherapy, and a limb salvage procedure was performed 3 months after diagnosis. The proximal tibia was replaced with a cadaveric bone graft. Several hours after the patient received methotrexate, a fever of 39.2°C developed. No sign of infection was observed on physical examination. Her leukocyte count was 6,300 cells/mm³ with an absolute neutrophil count of 4,914 cells/mm³. She received a single dose of acetaminophen and was without fever for the remainder of her hospitalization. A blood culture obtained from the central venous catheter at the time of fever grew Streptomyces. Repeat blood cultures obtained from both ports of the central venous catheter on day 3 and a peripheral blood culture obtained on day 4 also grew Streptomyces. Treatment with vancomycin and cotrimoxazole was started on day 4 in the hospital. The Streptomyces isolate was susceptible to vancomycin, amikacin, cotrimoxazole, erythromycin, cephalizin, and tetracycline and was resistant to ampicillin, penicillin, oxacillin, and clindamycin. A blood culture drawn from the central venous catheter on day 3 of antibiotic therapy (the 6th day in the hospital) grew Streptomyces after 9 days of incubation. All subsequent blood cultures were without growth. The central venous catheter was removed, and the patient received vancomycin intravenously for 6 weeks, without recurrence of Streptomyces bacteremia.

The bone graft was considered a potential source of infection. As most cases of disease from Streptomyces occur in the tropics, we requested information on whether the donor traveled or resided outside the United States. However, the donor had no history of travel outside the United States. All cultures taken from the donor and the graft were without growth (although this did not exclude the graft as the source of infection).
and no reports of disease transmission were received from any other recipients of organs from this donor. In addition, the patient had no history of receiving infusions of holistic or alternative medicines.

The organism was initially detected in the aerobic Bact/Alert blood culture system (bioMérieux, Inc., Durham, NC) after 72 h incubation at 35°C. Presumptive identification of the pleomorphic gram-positive bacillus as *Streptomyces* sp. was based on phenotypic characterization by using standard conventional tests and cellular fatty acid analysis. Species identification was determined by DNA sequencing of the 16S rRNA gene. DNA sequencing reactions were performed with the Tag Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA), and data were generated with an ABI 377 automated instrument. The sequence data were assembled, edited, and compared with published sequences for the 16S rRNA gene of *S. bikiniensis* (2).

The genus *Streptomyces* belongs to the order Actinomycetales, which includes *Mycobacterium*, *Nocardia*, and *Actinomyces*. *Streptomyces* are gram-positive, extensively branched, filamentous bacteria that form aerial hyphae with chains of spores. Their natural habitat is soil, and each species has a defined geographic distribution. None are common in the United States. With the exception of specimens from actinomycotic mycetoma, the isolation of *Streptomyces* from clinical specimens frequently is considered laboratory contamination (3). Rare cases of clinical disease attributed to *Streptomyces* have been published, including bloodstream infection (1,4) and focal invasive infections (5–9). *Streptomyces* was not the only potential pathogen isolated from some of the clinical specimens in these studies.

Scant data are available on effective treatment of *Streptomyces* infection. Mycetoma caused by *Streptomyces* is often treated with penicillin, sulfonamides, or tetracycline; however, the cure rate is low. The recommended duration of therapy is lengthy (up to 10 months). Isolates of *S. griseus* referred to the Centers for Disease Control and Prevention were frequently resistant to ampicillin (80%), sulfamethoxazole (43%), cotrimoxazole (29%), and ciprofloxacin (57%) (10). Resistance to doxycycline (19%) and minocycline (10%) was lower. Vancomycin susceptibility was not tested. Resistance patterns must be interpreted cautiously because *Streptomyces* can synthesize antibiotics, potentially confounding results of in vitro susceptibility testing.

The patient described in this report had no signs or symptoms of infection. The transient fever that prompted the first blood culture was probably due to the methotrexate infusion and not infection with *S. bikiniensis*. That the fever was of short duration despite persistently positive blood cultures supports this conclusion. The potential for causing minimal symptoms may contribute to assignment of *Streptomyces* as a contaminant. Clinical correlation is difficult if the infection is silent. *Streptomyces* isolated from blood cultures should not be dismissed as contaminants without careful consideration of the clinical situation; the isolation of *Streptomyces* from repeat blood cultures strongly suggests a pathogenic role.

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**Drug-Resistant Mycobacterium tuberculosis among New Tuberculosis Patients, Yangon, Myanmar**

To the Editor: Spread of drug-resistant tuberculosis (TB) and disastrous rates of HIV-TB co-infection pose serious threats to TB-control programs around the world (1). The World Health Organization/International Union Against Tuberculosis and Lung Diseases urges all national TB programs to practice the Directly Observed Treatment-Short Course (DOTS) strategy as well as to closely monitor the patterns and trends of anti-TB drug resistance (2). Such data allow an assessment of the quality of TB control, help forecast future trends...
of drug-resistance, and serve as guidelines for suitable therapy.

In 1997 the national TB programs of Myanmar introduced DOTS in the capital city, Yangon, which has approximately 5 million inhabitants. All new case-patients in national TB program clinics are routinely treated with isoniazid, rifampicin, ethambutol, and pyrazinamide without drug susceptibility testing of Mycobacterium tuberculosis. However, isolates from previously treated patients are frequently tested for drug susceptibility, and treatment is guided by the results. Myanmar is one of the 22 countries that account for 80% of the world’s new TB cases (3), yet little is known about drug-resistant TB in that country. We report on the pattern of drug resistance to first-line anti-TB drugs among M. tuberculosis complex isolates from Zone 1 TB center in Yangon, which receives approximately 70% of the national TB programs’ TB cases in Yangon. Of the 864 patients who attended this center in July 2000, a total of 202 were diagnosed as having pulmonary TB on the basis of medical history, clinical signs, two smear-positive sputum samples, and chest x-ray, if necessary. Approximately half of these cases were new pulmonary TB patients, i.e. smear-positive patients who had never been treated previously. Sputum specimens from 72 consecutive, new pulmonary TB case-patients were injected on Ogawa medium according to standard procedure (2); samples from 68 patients (94%) were culture-positive. Isolates from 17 patients were lost for further study because of bacterial contamination and failure to grow on subculture. Thus, isolates from 51 patients were available for the current investigation. By using the AccuProbe Mycobacterium tuberculosis complex test (Gen-Probe, San Diego, CA), all isolates were found to belong to the M. tuberculosis complex. Testing of isolates for susceptibility to isoniazid, rifampicin, ethambutol, and streptomycin was performed by using the standard Mycobacteria Growth Indicator Tube manual system, as recommended by the manufacturer (Becton Dickinson, Sparks, MD). The Wayne assay (4), which measures the activity of pyrazinamidase, was used for pyrazinamide susceptibility testing. This assay was performed according to World Health Organization guidelines for speciation within the M. tuberculosis complex (5). Eighteen isolates (35%) were resistant to any one of the five anti-TB drugs. Thirteen isolates (26%) were resistant to isoniazid, nine isolates (18%) to streptomycin, four isolates (8%) to ethambutol, one isolate (2%) to rifampicin, and one isolate (2%) to pyrazinamide. Only one isolate (2.0%) was multidrug resistant (MDR)-M. tuberculosis, i.e., resistant to both isoniazid and rifampicin.

The World Health Organization/International Union Against Tuberculosis and Lung Diseases global survey in the year 2000 (6) showed that the prevalence of resistance to at least one anti-TB drug (isoniazid, rifampicin, ethambutol, and streptomycin) among new cases ranged from 1.7% to 36.9%. In our study, 33.3% of the isolates from new pulmonary TB patients were resistant to at least one of these drugs. The finding shows that a relatively high frequency of drug resistance exists among our patients. If pyrazinamide is included in the calculation, the proportion of drug resistance among our patients is 35.3%. In 1994, Ti et al. reported that MDR-TB among 400 patients with newly diagnosed pulmonary TB who attended the Zone 1 TB center (7). When one considers the corresponding figure of 2.0% in the current material, frequency of MDR-TB in Yangon does not seem to have changed dramatically during the period 1994–2000. MDR-TB among new patients appears to be less common in Yangon than in big cities in Thailand (4.2%) (8) and in China (4.5%) (6). However, a substantial number of our isolates (15.7%) were resistant to two or more anti-TB drugs, in most cases to both isoniazid and streptomycin (9.8%). In the 1994 report by Ti et al., mono-resistance to streptomycin (6.5%) or isoniazid (5.8%) predominated, and 2.0% of the isolates were resistant to both isoniazid and streptomycin (7). Our present results, therefore, indicate that drug resistance is an imminent threat to TB-control efforts in Yangon, although MDR-TB still seems to be relatively rare.

The low number of MDR cases in our study could partly be explained by demographic features of the studied population, which is composed predominantly of people residing in satellite townships. These townships usually attract young people who immigrate to Yangon from village areas. These immigrants are less likely to have previous exposure to TB than the permanent population since the prevalence of TB infection is lower in rural than in urban areas (9). Moreover, population densities of the satellite townships are 2- to 10-fold lower than in inner Yangon city (Myanmar Central Statistical Organization). The high number of drug-resistance cases among our patients with newly detected TB could be explained by an undisclosed past exposure to anti-TB drugs. The case detection rate reported by the Myanmar national TB programs is 48% (3), suggesting that many TB patients receive their treatment elsewhere. A World Health Organization report (10) indicates that >80% of the health-care expenditure in Myanmar and other Asian countries such as India, Vietnam, and Cambodia is spent in the private sector. In such countries, poor treatment practices in the private sector may lessen the impact of the DOTS implemented by national TB programs and contribute to a growing incidence of drug-resistant TB. This problem will undoubtedly be escalated by the availability of anti-TB drugs without prescription. HIV-TB coinfection often results in increased frequency of adverse drug effects, which may reduce compliance and increase induction of drug resistance. Although
the prevalence of HIV positivity among our patients is unknown, a pre-
liminary study from Yangon shows that the prevalence of drug-resistant TB among HIV-seropositive and -seronegative patients is the same (pers. comm., Myanmar national TB programs). To our knowledge, this report is the first to describe drug-resistant patterns in M. tuberculosis isolates from Myanmar.

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Pneumocystis carinii vs. Pneumocystis jiroveci: Another Misnomer (Response to Stringer et al.)

To the Editor: The proposal by Stringer et al. to change the name of Pneumocystis carinii found in humans to Pneumocystis jiroveci requires critical consideration (1). First, their rationale for the choice of Jirovec is not compelling. Principle III of the International Code of Botanical Nomenclature (ICBN) states: “the nomenclature of a taxonomic group is based upon priority of publication” (2). Jirovec’s publication in 1952 was not the first to report P. carinii infection in human lungs. In 1942, two Dutch investigators, van der Meer and Brug, described P. carinii as the infecting organism in a 3-month-old infant with congenital heart disease and in 2 of 104 autopsy cases (a 4-month-old infant and a 21-year-old adult) (3). Their description, photomicrographs, and drawings of P. carinii are unequivocal. They also described the typical “honeycomb” patterns in alveoli. In 1951, Dr. Josef Vanek at Karls-Universität in Praha, Czechoslovakia, reported his study of lung sections from 16 children with interstitial pneumonia and demonstrated that the disease was caused by P. carinii (4). Vanek notes in his report, “In man the parasite was for the first time established as a cause of pneumonia in a child by G. Meer and S. L. Brug (1942).” In 1952, Jirovec reported P. carinii as the cause of interstitial plasmacellulmonary pneumonia in neonates (5). A year later, in a coauthored publication, Vanek, Jirovec, and J. Lukes acknowledged and referenced the earlier reports of van der Meer and Brug and Vanek (6). If principle III is to be followed, as well as fairness to the investigators, both van der Meer and Brug and Vanek hold priority over Jirovec, assuming the designation of the species name should be based on the name of the first person to discover P. carinii in humans.

The nomenclature of P. carinii has actually been fraught with errors from the beginning. In the earliest publications, Carlos Chagas and Antonio Carini mistook the organism for stages in the life cycle of trypanosomes. Chagas placed it in a new genus, Schizotrypanum (7,8). In 1912, Delanoë and Delanoë at the Pasteur Institute in Paris published the first description of the organism as a new entity unrelated to trypanosomes (9). They proposed the name “Pneumocystis carinii” as a tribute to Carini. The Delanoë paper has remained unchallenged as the original description of P. carinii. Both Chagas and Carini later acknowledged their errors and the validity of the Delanoës’ conclusion. By current ICBN principles, P. carinii is acceptable nomenclature because the authors of the first publication proposed the name of Carini, rather than their own.

In addition, changing the name to P. jiroveci will create confusion in clinical medicine where the name P. carinii has served physicians and microbiologists well for over half a
century. I was moved to write this letter because of a call from a knowledgeable oncologist asking for information on “the new strain of P. carinii that has just been reported from the Centers for Disease Control and Prevention,” referring to the report by Stringer et al (1).

AIDS patients are well informed about P. carinii pneumonia and avidly monitor medical news about their disease. Without doubt, the name change will cause confusion and undue anxiety among the many thousands of HIV-infected patients who attend clinics. Health-care workers will have an added burden of explaining why the name was changed, but the organism and infection are unchanged. Also, versions of the pronunciation of jiroveci (yee row vet zee) by American patients, physicians, and health-care workers will be interesting to hear.

The tone of the article by Stringer et al. implies that the change of P. carinii to P. jiroveci is final, which is not the case. The nomenclature of fungi is governed by ICBN under the auspices of the International Botanical Congress and is not based solely on molecular genetics. Neither P. carinii nor P. jiroveci have been submitted for ICBN scrutiny. In another paper, Stringer et al. outline the mechanics for submission, but indicate that no application has been submitted for their proposal (10). In fact, P. carinii has not been acknowledged as a fungus by ICBN or any other authoritative taxonomic system. Only when nomenclature is registered in ICBN, can a name be referred to as “formally accepted.” In the meantime, the workable terminology proposed earlier by Stringer et al. in 1994 (11) will suffice for clinical use.

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A New Name (Pneumocystis jiroveci) for Pneumocystis from Humans (Response to Hughes)

Reply to W.T. Hughes: We appreciate Dr. Hughes’ letter of concern regarding our article endorsing the name Pneumocystis jiroveci (1). When working with well-known disease agents and syndromes, these types of changes are more difficult to adopt because of the effect they have on daily communication, patient care, record keeping, and other important routines of health-care providers. However, in this case, new information and understanding dictate that a change be made.

For some time, scientists have known that humans are infected by a particular species of Pneumocystis and that this species does not infect other host species. In recognition of these facts, Frenkel named the human pathogen Pneumocystis jiroveci, using the procedure prescribed by the International Code of Botanical Nomenclature (ICBN) (2). Although Dr. Hughes raised a number of issues, none justifies rejecting the new, valid name.

Dr. Hughes suggested that the name P. jiroveci is incorrect on the basis of principal III of ICBN, which holds that “the nomenclature of a taxonomic group is based upon priority of publication.” He indicated that Jirovec was not the first investigator to report Pneumocystis in humans. Although this situation may be the case, principal III has not been violated because “priority of publication” refers to the time when a name is validly published, not to the time when an organism is first described. The name P. jiroveci was validly published in 1999, and this name therefore has priority. To be valid, all of the following steps must be completed: a
name must be published in a scientific journal, the name must be a binary Latin name, the organism must be described in Latin, the rank of the organism must be indicated, and the new species must be called by the term “typus or holotypus,” and the specimen or microscope slides must be placed in a public holding (details are available from: URL: http://www.bgbm.fu-berlin.de/iapt/nomenclature/code/SaintLouis/0000St.Luis-title.htm). Dr. Frenkel was the first to fulfill these requirements in his 1999 publication (2).

The 1912 publication by Delanoë and Delanoë does not have priority in naming Pneumocystis from humans because the organism studied by the Delanoës was from the rat. The rat-derived Pneumocystis organism continues to be known as P. carinii, in keeping with principal III. As an additional historical note, Dr. Frenkel was the first investigator to understand the clear differences between human and rat-derived Pneumocystis, which were described in a landmark publication in 1976 (3). He proposed a change in nomenclature in which the name Pneumocystis jiroveci n. sp. applied to the human organisms and Pneumocystis carinii was retained for the rat organism. However, Frenkel did not attempt to follow ICBN procedures because at the time Pneumocystis was thought to be a protozoan. Nevertheless, this early paper established the idea of naming human Pneumocystis, P. jiroveci.

Dr. Hughes stated a concern over the possibility that the name change may cause “confusion and undue anxiety among the many thousands of HIV-infected patients who attend clinics.” Such concern is understandable. However, patients will have guidance in understanding the significance of the name change. Health-care providers will allay any fears that might be elicited by the application of the new name. The level of anxiety experienced by persons at risk of acquiring Pneumocystis carinii pneumonia (PCP) is more likely to decline than to increase. People may be relieved to learn that they are not going to catch PCP from a pet, for example.

Dr. Hughes suggested that the name P. jiroveci is unofficial because it has not yet been sanctioned by a body of experts that scrutinizes proposed name changes and has the power to either accept or reject them. This situation is not the case. The process by which new names are validated does not directly involve a body of experts. The International Botanical Congress does not evaluate names. Instead, the congress has established ICBN, which sets forth the procedures authors must follow to publish a valid new name. The scientific basis for the new name is included in the publication. In the case of P. jiroveci, abundant evidence shows that P. carinii and P. jiroveci are different species. This evidence, which also indicates that the genus Pneumocystis contains many additional species, has been reviewed extensively (4, 5). Dr. Hughes gave the impression that this evidence is exclusively molecular genetic data. In fact, the molecular genetic evidence is mirrored by clear biologic differences, the most dramatic being host species specificity.

As our knowledge of the biology and genetics of disease-causing microorganisms grows, openness to changes in the taxonomy and classification is needed. Given the impact such changes can have outside of the realm of basic science, the decision to accept the proposed changes in the nomenclature used for Pneumocystis has not been made frivolously. This decision is the result of a long and deliberate process that began almost 10 years ago, when data demonstrating that different mammalian hosts harbor different Pneumocystis species first began to appear. In 1994 and 2001, nomenclature issues were discussed at international meetings of the Pneumocystis community, with both physicians and research investigators present. In 1994, the data supporting new species were relatively limited. Consequently, a provisional tripartite nomenclature was adopted in lieu of recognizing new species (6). By 2001, however, the existence of multiple species and the necessity of assigning new species names were accepted by consensus. Because Frenkel had already published the name P. jiroveci, the suitability and validity of this name were also discussed. The new name was approved by consensus (4). We recognize that the results of these meetings do not necessarily reflect all opinions on the matter of Pneumocystis nomenclature, but we know of no better way to assess the majority opinion.

In endorsing the name Pneumocystis jiroveci, we hope to foster scientific understanding and communication. The tripartite name formerly used to denote the distinctness of this organism is not only cumbersome, it is inadequate because its meaning is not apparent and must be defined every time it is used. The arcane nature of the tripartite name tended to deprive the broad audience of persons interested in PCP of vital information, namely, a unique species of Pneumocystis infects humans. By contrast, the new species name clearly states the uniqueness of P. jiroveci; a distinction is needed when assessing the significance of findings obtained by studies on other members of the genus. Recognition of this uniqueness will undoubtedly stimulate more research on this species. Communication will best be served by uniformity in nomenclature.

Frenkel has assigned a valid name to the Pneumocystis species found in humans. Ignoring this name on the grounds of inconvenience is not only unjustified, it is impractical. If names published in accordance with ICBN are not accepted, the field will have no recognized mechanism for conferring names, fostering the use of idiosyncratic, inadequate, and misleading names. Communication and progress will suffer as a result.
LETTERS

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Correction, Vol. 9, No. 1

In “Transfusion-Associated Babesiosis after Heart Transplant” by Joseph Z. Lux et al., errors occurred in the text. On page 118, left column, lines 20–22, the sentence should read “he became symptomatic during the typical 2- to 8-week incubation period for transfusion-transmitted B. microti infection (7).”

The corrected text appears online at http://www.cdc.gov/ncidod/eid/vol8no12/02-0149.htm.
Field Epidemiology, 2nd edition

Michael B. Gregg, editor

ISBN 0-19-514259-4
Pages: 451 pp
Price: $49.95

Before the first edition of Field Epidemiology was published in 1996, no single authoritative textbook or manual existed for public health epidemiologists engaged in active field investigation and outbreak control. Those needing preparation or guidance in responding to acute problems in the community (infectious or noninfectious) were left to read a variety of disparate materials and exercises (many from the Centers for Disease Control and Prevention [CDC]), learn on the job, or find a mentor.

In the current revised and augmented edition of Field Epidemiology, the first chapter defines field epidemiology as “the constellation of problems faced by epidemiologists who are called upon to investigate urgent public health problems…” and further states, “public health epidemiologists must travel to and work in the field to solve the problem.” Thus, for those practicing in this arena, the motivation is not primarily research oriented but rather geared to those problems for which government agencies usually are given the primary mandates and responsibilities. I would imagine that those involved in “nonacute” epidemiology or investigating non-urgent public health problems also consider themselves to be carrying out “field work” (such as doing a nutritional or behavioral survey or assessment in the community) and might object to the all-inclusive title used for this book. Perhaps the title of “The Field of Acute Public Health Epidemiology,” although awkward, is a better description of the content of this book.

How will readers of Emerging Infectious Diseases benefit from such a book? Clearly this book provides an excellent primer in basic epidemiologic techniques, including discussions of principles and surveillance; collecting, analyzing, interpreting, and presenting data; surveys and sampling; even the more advanced topics, such as confounding, the role of chance, and the exploration of interactions, are discussed. More importantly, I believe the many chapters describing the operational aspects of epidemiologic field investigations—including the legal considerations, special problems in health-care settings, current public health laws, and laboratory support—provide insights into both the methods and investigational steps for many of the diseases that eventually result in publication in journals like Emerging Infectious Diseases. Thus, such a book provides readers with a greater appreciation and understanding for work behind the resulting publication.

Overall, I find the writing to be lucid and readable. Moreover, one can read any chapter independently. A large number of examples and case studies are used. For example, before the reader has progressed more than a sentence or two into the book, the authors describe the events that unraveled at the American Legion Convention in Philadelphia, which led to the discovery of Legionella pneumophila. Throughout the book, chapters focus on practical and real-world situations.

Over 25 years ago, I started work in Los Angeles as an epidemiologist in the Acute Communicable Disease Control program and later for the Toxics Epidemiology program. This book would have been worth its “weight in gold” if it had been available to me as a novice epidemiologist. Even today, 200–300 outbreaks and investigations later, I found much to learn, particularly those chapters providing new and updated information on bioterrorism preparedness and response and field investigations of occupational disease and injury. I especially found intriguing the chapter on surveillance, which includes a number of case studies, giving the reader an appreciation for the many biases and limitations in analyzing and interpreting incidence and prevalence data. The book includes an excellent appendix with current interesting Web sites. Despite the large number of authors, almost all experts in their fields (a veritable who’s who of well-respected and accomplished epidemiologists), with slightly different writing styles, most of the book flows evenly. Some oddities do appear now and then, such as the mnemonic or acronym called “SLACK OFF” used in the chapter on analysis. This term is meant to help the investigator remember to describe the methods and techniques in an investigation (S is for Shells, which actually means setting up tables for analysis at the start of an investigation). I found the chapter on sampling lacking in examples, unlike most chapters in the book. Particularly well-written and of interest to the computer and technologically minded is the chapter by Andy Dean, the father of Epi Info Software. This robust freeware product provided by CDC is one of the best-kept secrets of epidemiologists, and researchers in allied fields can benefit from reading about this comprehensive database and statistics software for public health professions (available from: URL: http://www.cdc.gov/epiinfo).

Overall, this comprehensive volume can be viewed as a “Prolegomena for any Future Public Health Epidemiology.” I highly recommend it for those who will be practicing field epidemiology and for those who wish to know more about the application of epidemiologic methods to acute and urgent public health problems.

Marc A. Strassburg*

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Self-Portrait with Monkey (1938).
Oil on masonite, 16” x 12”
Albright-Knox Art Gallery, Buffalo, New York, USA
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Frida Kahlo Museums Trust. Av. Cinco de Mayo No. 2,
Col. Centro, Del. Cuauhtémoc 06059, México, D.F.

Frida Kahlo was born in Mexico City, the third daughter of a German father and a Spanish and Native American mother. Her life was marred by physical trauma—from childhood polio that left her with a limp, to serious injury in 1926, when a bus she was riding collided with a streetcar. Lifelong pain and its psychological aftermath had a profound effect on her artistic development (1).

Kahlo was well educated and fiercely independent. A frail girl with a limp, she set out to be a tomboy, an intellectual, a heartbreaker, and a communist. Relentless physical pain, marital strife, and emotional rejection marked the course of her life. Her work, which incorporates Mexican folk motifs and particularly the small votive pictures known as retablos, exudes powerful feeling and is unlike that of any of her contemporary Mexican muralists (2). Characterized by boundless energy and strength, her paintings represent her passion for meaning and truth, her feistiness and defiance of limits, her intimate acquaintance with suffering, and finally her poignant acknowledgment of things as they are.

Kahlo’s artistic talent was recognized by the French poet and critic André Breton in 1938, when he visited Mexico. Breton, who had studied medicine and worked on psychiatric wards during World War I, was a founder and chief promoter of the surrealist movement. The movement, partly borne of post–World War disillusionment, promoted a “revolution of the mind” against a civilization that seemed to be lowering human aspirations and proliferating human misery. Surrealism sought to synthesize humans and their world, eliminating the barriers between dream and reality, reason and madness, persons and things (3).

During her early association with Breton in Mexico (which he termed a “naturally surrealist country”). Kahlo worked alongside the surrealists, yet she denied any connection with them: “They thought I was a surrealist…but I wasn’t. I never painted dreams, I painted my own reality” (2). Even if she never espoused surrealist ideology, Kahlo seemed to embody it. She transcended her physical suffering and delved into untapped emotional depths for universal truth, which she uncovered and brought to the viewer in raw, brilliant color. In a surrealist manner, Kahlo’s work was permeated by her tempestuous life and cannot be fully understood apart from it.

From her vivid self-portrait on the cover of this month’s Emerging Infectious Diseases, Frida Kahlo casts a pensive but challenging look at a world that denied her the comforts of health. Like an exotic flower, she embellishes the luscious tropical tableau. Yet, in spite of her regal demeanor and the scene’s vibrant hues, something is troubling about the picture. Menace lurks in nature itself, which though seemingly embracing, is not unqualifiedly benign. The enigmatic presence of the monkey heightens the portrait’s uneasiness. Might it be the devil, as purported in Kahlo’s native Mexico? Non-human primates are frequent human playmates in the arts, the circus, and the streets—always amusing, romantic, and mysterious, and sometimes dark. Might the monkey on Kahlo’s back be the harbinger of ill health? Like her contemporaries, Kahlo knew little of her close phylogenetic kinship with her pet or the extreme caution prescribed by this kinship. The threat signaled by the presence of a primate, be it turbulence in Kahlo’s life or herpes B viruses in ours, remains uncharted. The monkey on our back is to decipher the zoonotic puzzle of infection that perpetuates suffering and limits the immense capacity of the human spirit.

Polyxeni Potter

Upcoming Infectious Disease Conferences

March 17-20, 2003
27th National Immunization Conference
Chicago, IL
Contact: Suzanne Johson DeLeon
(404) 639-8225
E-mail: msj1@cdc.gov
Website: http://www.cdc.gov/nip/nic

April 6-8, 2003
Society for Healthcare Epidemiology of America (SHEA) Annual Meeting
Arlington, VA
Contact: SHEA Meetings Dept.
(856) 423-7222 ext. 350
Website: www.shea-online.org

May 7, 2003
Certificate of Knowledge in Travel Medicine Examination
International Society of Travel Medicine Conference
New York, New York
Contact: Brenda Bagwell
E-mail: exam@istm.org
Phone: 770-736-7060
Fax: 770-736-6732
Website: http://www.istm.org

May 7-11, 2003
8th Conference of the International Society of Travel Medicine
New York City
Contact: Lisa Astorga,
Conference Manager
E-mail: lastorga@talley.com
Website: www.istm.org

May 10-13, 2003
13th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)
Glasgow UK
Contact: Administrative Secretariat +41 61 686 77 11
E-mail: info@akm.ch
Website: www.escmid.org/eccmid2003

May 27-30, 2003
Global Health Council’s 30th Annual Conference
Our Future on Common Ground:
Health and the Environment
Washington, DC
Website: http://www.globalhealth.org/

May 29-30, 2003
ISC Disease Management Series
International Conference Surgical Infections: Prevention and Management
Moscow, Russia
Contact: Dr. Dr. Dmitry Galkin
PO Box 60, Smolensk, 214019, Russia
Tel.: 7 0812 611301/611327
Fax: 7 0812 611294
E-mail: galkin@antibiotic.ru
Website: http://www.antibiotic.ru

The CDC Foundation: Building partnerships between the community and the Centers for Disease Control and Prevention

Find out how you can become a CDC Foundation partner

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50 HURST PLAZA, SUITE 765
ATLANTA, GA 30303
(404) 653-0790
CDCFOUNDATION.ORG
Look in the March issue for the following topics:

- Enterovirus 71 Outbreaks, Taiwan: Occurrence and Recognition
- Human Metapneumovirus in Severe Respiratory Syncytial Virus Bronchiolitis
- Electron Microscopy for Rapid Diagnosis of Emerging Infectious Agents
- Reservoir Competence of North American Birds for the New York 1999 Strain of West Nile Virus
- Emergence of Ceftriaxone-Resistant Salmonella Isolates and Rapid Spread of Plasmid-Encoded CMY-2-Like Cephalosporinase
- *Bartonella henselae* in *Ixodes ricinus* (*acari ixodida*) Ticks Removed from Humans, Belluno Province
- New Lyssavirus Genotype from the Lesser Mouse-Eared Bat (*Myotis blythi*)
- Molecular Detection of *Bartonella quintana*, *B. koehlerae*, *B. henselae*, *B. claridgeiae*, and *Rickettsia felis* in Cat Fleas
- Amplification of the Sylvatic Cycle of Dengue Virus Type 2 Senegal, 1999–2000: Entomologic Findings and Epidemiologic Considerations
- Persistence of Virus-Reactive Serum Immunoglobulin M Antibody in Confirmed West Nile Virus Encephalitis Cases

For a complete list of articles included in the March issue, and for articles published online ahead of print publication, see http://www.cdc.gov/ncidod/eid/upcoming.htm
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.
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 eeditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and 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 purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Chinese, French, and Spanish translations of some articles can be accessed through the journal’s home page at http://www.cdc.gov/eid.

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 e-mail 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. Printed manuscript should be single-sided, beginning with the title page. 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 underlining) 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 as “personal communication.”

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 Aural 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).

Manuscript Types

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) 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.

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) 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) and a brief biographical sketch of first author—both authors if only two. Report the findings in a larger perspective (i.e., “Here is what we found, and here is what the findings mean”).

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, nor should they contain figures or tables. References (not 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.