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On the Cover: México prehispánico, El antiguo mundo indígena by Diego Rivera.

page 450

Perspectives

Synopsis

Historical Review

Research

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Feline Host Range of *Canine parvovirus*: Recent Emergence of New Antigenic Types in Cats

Yasuhiro Ikeda,*† Kazuya Nakamura,† Takayuki Miyazawa,† Yukinobu Tohya,† Eiji Takahashi,† and Masami Mochizuki‡

Since the emergence of *Canine parvovirus* (CPV-2) in the late 1970s, CPV-2 has evolved consecutively new antigenic types, CPV-2a and 2b. Although CPV-2 did not have a feline host range, CPV-2a and 2b appear to have gained the ability to replicate in cats. Recent investigations demonstrate the prevalence of CPV-2a and 2b infection in a wide range of cat populations. We illustrate the pathogenic potential of CPV in cats and assess the risk caused by CPV variants.

Human health and animal welfare continue to be challenged by rapidly evolving pathogens. Although many details about specific host-parasite systems have been reported, our understanding of host range alteration and the evolution of virulence remains rudimentary. We reviewed the evolution of carnivore parvoviruses with particular reference to *Canine parvovirus* (CPV) infection in cats. These parvoviruses' molecular and phenotypic evolutionary pattern provides an exemplary system to study pathogen-host relationships and the evolution of virulence, both essential factors for understanding newly emerging infectious diseases.

Emergence of *Mink enteritis virus* and CPV Type 2 (CPV-2)

Infection by *Feline parvovirus* was thought only to occur in cats (*Feline panleukopenia virus,* FPLV) or raccoons until the mid-1940s, when a similar disease with a mortality of up to 80% was observed in infected mink kits in Canada (1). The disease caused by the mink agent, named *Mink enteritis virus* (MEV), was thereafter observed throughout many regions of the world (2). Since MEV was indistinguishable from FPLV by conventional methods such as serum-neutralization assay, MEV isolates have been differentiated from FPLV primarily on the basis of the host from which they are isolated. Using a panel of monoclonal antibodies (MAbs), we now classify FPLV and MEV isolates into three antigenic types, FPLV and MEV type 1 (MEV-1) group, MEV type 2 (MEV-2), and MEV type 3 (2,3) (Figure 1). MEV-1 and MEV-2 have repeatedly been isolated from the mink in the United States, Europe, Japan, and China (2,3; Y. Ikeda and M. Mochizuki, unpub. data).

In the late 1970s, another virus emerged in dogs (4,5). The new virus, designated CPV-2 to distinguish it from an unrelated *Canine parvovirus (Canine minute virus*), spread around the world within a few months (6,7). CPV-2 spread rapidly, killing thousands of dogs. Polyclonal antibody and in vivo cross-protection studies soon showed that CPV and FPLV were closely related antigenically, while CPV-2 and FPLV were distinguishable from each other when examined with a panel of MAbs (Figure 1). Subsequent extensive genetic analysis of numerous CPV-2, FPLV, and MEV isolates showed that the viruses form two distinct clusters represented by FPLV-type viruses from cats (FPLV), raccoons, and mink (MEV), and by CPV-type viruses from dogs and raccoon dogs. At least 11 conserved nucleotide differences (7 nonsynonymous and 4 synonymous changes) were seen between CPV-2 isolates and FPLV-type viruses in the capsid VP2 sequence; in contrast, CPV and FPLV isolates differ in <2% of their genomic DNA sequences (8) (Figure 2; Table).

Hypotheses on the Ancestor of CPV-2

Retrospective investigations to detect CPV antibodies in sera collected from dogs or related canids showed that the first positive titers were present in European dogs around 1975, while the first positive sera in the USA, Japan, and Australia were seen in early 1978. Various hypotheses on the

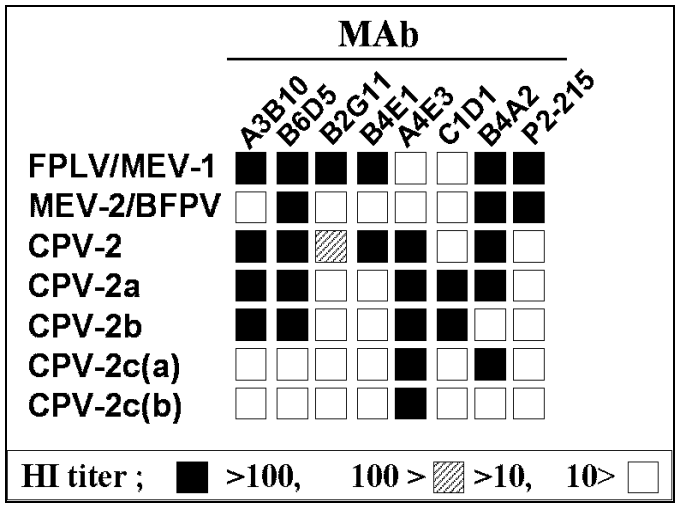

Figure 1. Antigenic profile of feline parvoviruses, including *Canine parvovirus* 2c (CPV-2c) types. Subtype-specific monoclonal antibodies are used to type the viruses in a hemagglutinin-inhibition test (HI). *Mink enteritis virus* (MEV-3) shows similar patterns to MEV-2 (2). FPLV = *Feline panleukopenia virus*; BFPV = blue fox parvovirus.

*University College London, London, United Kingdom; †University of Tokyo, Bunkyo-ku, Tokyo, Japan; and ‡Kyoritsu Shoji Corporation, Chiyoda-ku, Tokyo, Japan

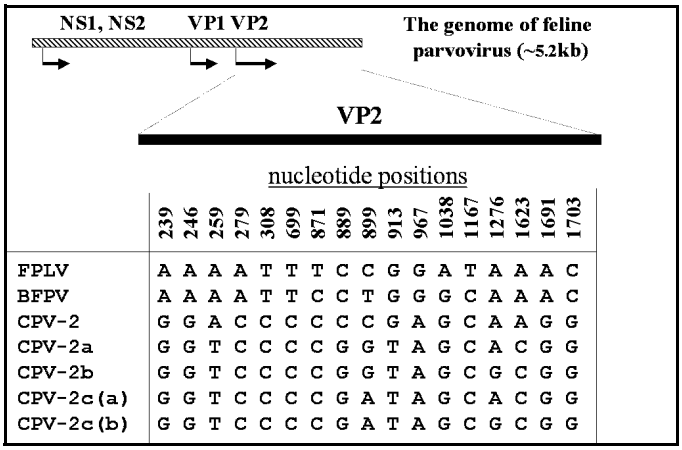

Figure 2. Conserved nucleotide differences between the *Feline panleukopenia virus* (FPLV)- and *Canine parvovirus* (CPV)-type viruses. Nucleotide positions in the VP2 gene are numbered above the sequences; BFPV = blue fox parvovirus.

mechanism of virus evolution in this group have been developed. The most widely accepted hypothesis is the emergence of CPV-2 from a variant of FPLV or of a closely related virus infecting another carnivore, such as mink or foxes (9,10).

Several intriguing observations support the latter hypothesis. First, based on the sequence analyses of the capsid VP-2 and the nonstructural NS1 genes, MEV is closer to CPV-2 than FPLV (9,11). More importantly, the virus isolated from an Arctic fox from Finland (blue fox parvovirus, BFPV) in 1983 appeared to be an intermediate between the FPLV- and CPV-type viruses. BFPV had three synonymous nucleotide changes in the VP2 gene that were specific for the canine sequence (12) (Figure 2), while the fox virus was classified antigenically as typical MEV-2-type (13) (Figure 1). These findings indicate that some animals in the family *Canidae*, such as mink or foxes, which are susceptible to FPLV-like viruses, might play a role as a reservoir for the ancestor of CPV. Recently, Truyen et al. (14) reported that the intermediate parvovirus sequence from a German red fox was CPV-2-like but had one FPLV-specific nonsynonymous substitution. This suggests that German red foxes could harbor the direct ancestor of CPV, although it remains possible that the intermediate red fox parvovirus emerged from conventional CPV-2 by one point natural mutation (Figure 3).

Emergence of CPV Types 2a and 2b (CPV-2a and CPV-2b)

Since the emergence of CPV-2, two new antigenic types of CPV, designated CPV-2a and CPV-2b, have arisen consecutively. These new virus types have now almost completely replaced CPV-2 viruses as the dominant infectious agents (15) (Figure 3). At least four conserved nonsynonymous substitutions have been observed between CPV-2 and CPV-2a isolates in the VP2 gene (Table). CPV-2b isolates have another two nonsynonymous changes from CPV-2a (Table). Although the exact mechanisms of these evolutions are not clear, the emergence of these new antigenic types of CPV can likely be ascribed to the adaptation of CPV-2-type viruses in dogs. Of interest, each new antigenic type has lost at least one neutralizing epitope compared with the former serotype (16).

Clinical Features of FPLV and CPV in Their Original Hosts

Parvoviruses replicate most efficiently in rapidly dividing cells. Replication is generally lytic, and tissue damage at these sites can be observed (17). Infection with FPLV causes two typical syndromes. When infection occurs in fetuses or very young kittens, a distinct cerebellar ataxia is observed when they become actively ambulatory (18,19). When older kittens are infected, illness characterized by loss of appetite, pyrexia, diarrhea, and leukopenia of both lymphocytes and neutrophils appears (20). On the other hand, two typical syndromes observed in CPV-infected dogs are acute myocarditis in young puppies with a high mortality (21) and hemorrhagic enteritis in older puppies (4,22).

Mortality from FPLV infection is likely to depend on the general condition of the animals before infection. Experimental infection of specific pathogen-free (SPF) or germfree cats with FPLV generally leads to mild diseases (23,24). No or slight intestinal lesions can be observed in infected germfree cats (23), which suggests that the intestinal lesions are caused by secondary bacterial, rather than primary viral, infection.

Host Range of FPLV- and CPV-Type Viruses

The host ranges of FPLV- and CPV-type viruses have been extensively studied in vitro. In general, CPV-type viruses replicate efficiently in feline and canine cell lines, while most

Table. Phylogenetically informative amino acid sequences in the VP2 gene

Virus	80	87	93	103	232	297	300	305	323	426	555	564	568
FPLV/MEV-1	Lys	Met	Lys	Val	Val	Ser	Ala	Asp	Asp	Asn	Val	Asn	Ala
MEV-2/BFPV	Lys	Met	Lys	Val	Val	Ser	Val	Asp	Asp	Asn	Val	Asn	Ala
CPV-2	Arg	Met	Asn	Ala	Ile	Ser	Ala	Asp	Asn	Asn	Val	Ser	Gly
CPV-2a	Arg	Leu	Asn	Ala	Ile	Ser/Ala	Gly	Tyr	Asn	Asn	Ile	Ser	Gly
CPV-2b	Arg	Leu	Asn	Ala	Ile	Ser/Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly
CPV-2c(a)	Arg	Leu	Asn	Ala	Ile	Ala	Asp	Tyr	Asn	Asn	Val	Ser	Gly
CPV-2c(b)	Arg	Leu	Asn	Ala	Ile	Ala	Asp	Tyr	Asn	Asp	Val	Ser	Gly

FPLV = *Feline panleukopenia virus*; MEV = *Mink enteritis virus*; BFPV = blue fox parvovirus; CPV = *Canine parvovirus.*

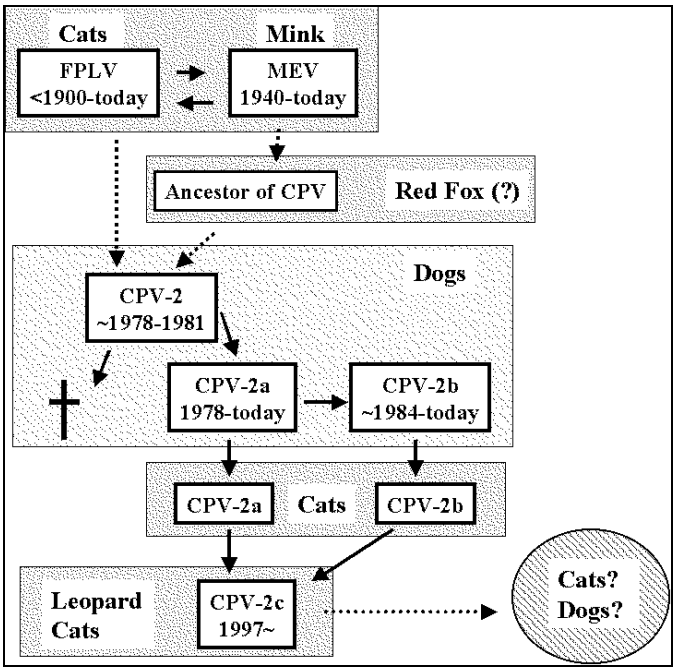

Figure 3. The apparent evolutionary processes of feline parvoviruses.

FPLV and FPLV-like viruses can replicate efficiently only in feline cells (11,25-27). Subsequent recombination mapping and site-directed mutagenesis experiments have clearly shown that the VP2 gene (including the differences of VP2 residues 93, 103, and 323; Table) is important in controlling canine host range, although a part of the nonstructural NS gene of CPV also participates in FPLV replication in canine cells (27,28). Recently, Ikeda et al. (11) reported a unique FPLV isolate from a domestic cat, which could replicate efficiently in a canine cell line. Interestingly, this isolate was antigenically FPLV-type but had a natural mutation of VP2 residue 323 Asp to CPV-specific Asn, supporting previous site-directed mutagenesis studies. Moreover, FPLV-type virus actually has the potential to acquire canine host range by natural mutation, although phylogenetic analyses indicate that the isolate is unlikely to be a direct ancestor of CPV-2 (11).

The in vivo host ranges of FPLV and CPV seem to be more complicated. FPLV can replicate in feline tissues, such as lymph nodes, thymus, spleen, or intestinal epithelial cells, and high titers of virus are shed in feces. In dogs, however, FPLV replication is seen only in the thymus and bone marrow, not in the gut or mesenteric lymph nodes (26), resulting in no virus shedding in feces (29). In terms of viral evolution, the CPV ancestor had only to gain the ability to infect the gut in order to be shed and spread in the dog population (26). Indeed, Mochizuki et al. (30) report the isolation of FPLV-type virus from diarrheic feces of a clinically diseased dog. Although the reason why the antigenically FPLV-type virus could gain canine host range remains to be determined, the virus possibly had some genetic mutation(s) that did not change its antigenic properties yet rendered the virus able to infect canine gut cells.

Until recently, the feline host range of CPV has been controversial. Goto et al. report that CPV replicates in cats in a pattern similar to FPLV (31); other studies find no detectable CPV replication in any feline tissue (26,32). This discrepancy, however, is due to the antigenic differences of the examined viruses. The virus (Kushiro strain) used in the first study (31) was shown to be CPV-2a (27), whereas the other studies used CPV-2 (26,32). Truyen et al. (33) directly compared the feline host ranges among CPV-2, CPV-2a and CPV-2b and showed efficient replication of both CPV-2a and CPV-2b in experimentally infected cats. CPV-2a and CPV-2b isolates replicate to high titers in lymphoid and intestinal tissues, while the CPV-2 isolate used in this study did not replicate in experimentally infected animals (33).

Feline Host Range of CPV in the Wild

In late 1980s, CPV was first recognized in cats in a natural setting (30). Mochizuki et al. (30) examined eight feline isolates collected during 1987 to 1991 in Japan and demonstrated that three were antigenically and genetically identical to CPV-2a viruses. The first isolation of CPV-2a-type virus from a cat was in 1987 (30). All three CPV-2a-type viruses were isolated from the feces of clinically healthy cats, while the isolates from cats with typical feline panleukopenia were all conventional FPLV-type. Subsequently, CPV-2a and CPV-2b viruses were recognized in cats in the USA (2 of 20 isolates) and Germany (3 of 36 isolates) (33).

Recently, Ikeda et al. (11) examined 18 isolates from unvaccinated cat populations and demonstrated that 15 of the isolates could be classified as CPV-2a- or 2b-related viruses (11). Since carnivore parvoviruses are likely to spread freely and rapidly in the environment when few cats and dogs are vaccinated against FPLV or CPV, CPV-2a/2b-type viruses seem to have more advantages over conventional FPLV in cats under natural conditions. It is therefore possible that CPV-2a/2b-type viruses will replace FPLV-type viruses as the dominant infectious agents in domestic cats even in developed countries, where FPLV vaccines are commonly used.

Emergence of New Antigenic Types of CPV (CPV-2c) in Cats

Feline parvoviruses continue to evolve. CPV-2a and 2b have been detected not only from domestic cats but also from wild felids worldwide (11,34). Steinel et al. (34) report the detection of CPV-2b-type viral DNA from one fecal sample of a Namibian farm-raised cheetah and the tissue sections of four captive cheetahs in the United States. CPV-2a-type sequence was also found in the fecal sample of the Siberian tiger from a German zoo (34).

During 1996 to 1997, CPV-2a/2b-related viruses were isolated from Asian small wildcats, leopard cats *(Felis bengalensis*), in Vietnam and Taiwan (11,35). These viruses were designated as leopard cat parvovirus (LCPV). Three of the six isolates were demonstrated to be new antigenic types of CPV;

the other three isolates were essentially identical to CPV-2a or 2b. Subsequently, the new antigenic type viruses were shown to have a natural mutation of VP2 in common (11) (residue 300 Gly to an Asp, Table), which results in remarkable changes of their antigenic properties. The new antigenic type, characterized by the loss of the VP2 epitopes recognized by the reference MAbs A3B10, B6D5, and C1D1, is currently designated as CPV-2c (Figure 1) (11). The reactivity against MAb B4A2, which distinguishes CPV-2b from the other serotypes, further classifies the CPV-2c-type isolates into two serotypes, CPV-2c(a) and CPV-2c(b) (Figure 1).

CPV-2c-type viruses have been isolated only from leopard cats but not from domestic cats in the same area. Since the phylogenetic analysis indicated that CPV-2a and CPV-2b-type viruses were likely to evolve to CPV-2c(a) and CPV-2c(b)-type viruses, respectively, the mutation at the residue 300 Gly to Asp is probably ascribed to the adaptation of CPV-2a/2b-type viruses to leopard cats. Similar to the emergence of CPV-2a and CPV-2b, CPV-2c has lost neutralizing epitopes compared with the former serotypes, CPV-2a and 2b.

Virulence of CPV-2a and -2b in Cats

The pathogenicity of CPV-2a and 2b in cats remains debatable. Mochizuki et al. reported the isolation of CPV-2a from a cat manifesting clinical signs of feline panleukopenia (36). The detection of CPV-2a/2b-type DNA sequences from the cheetahs with chronic diarrhea and enteritis or the tiger with anorexia and diarrhea (34) strongly suggests CPV-2a's and CPV-2b's pathogenic potential in large felids. In sharp contrast, recent studies using SPF cats experimentally infected with CPV-2a or CPV-2b showed no or slight illness, such as mild lymphopenia, in the infected animals (31,33,37,38). Moreover, the fact that many CPV-2a- and CPV-2b-type viruses were isolated from clinically healthy cats (11,30,35,39) seems to indicate their relatively low pathogenicity.

At present, this discrepancy remains to be resolved. Note, however, that the experimental infection of SPF cats with FPLV generally leads to mild disease (23,24). In this regard, the study reported by Goto et al. (31) is intriguing. These researchers compared the clinical signs of five SPF and four conventional cats experimentally infected with CPV-2a. The infected five SPF cats showed neither clinical signs nor leukopenia through the observation period, while depression (four cases), vomiting (two), diarrhea (one), and severe leukopenia (four) were observed in the four conventional cats. One cat died 4 days after infection (31). These data indicate that the illness from CPV-2a/2b infection highly depends on the general condition of the cats before infection.

Pathogenic Potential of CPV-2c

Since feline parvoviruses shed in the feces survive in the environment for up to several months, a fecal-oral route is considered to be the predominant means of their transmission. Although CPV-2c-type viruses have been isolated only from leopard cats (13,38), the new serotype viruses will likely spread to domestic cat and dog populations. Nakamura et al. (38) compared the virulence between FPLV, CPV-2a, and CPV-2c in SPF cats. In this experiment, diverse pathogenicity of the CPV-2a for individual cats was observed. One cat had symptoms frequently associated with parvovirus infection, including leukopenia and diarrhea; the other cats remained asymptomatic. One cat showed no evidence of infection. In contrast to the results obtained with CPV-2a-inoculated animals, all cats inoculated with CPV-2c developed diseases, although the symptoms were relatively milder than those observed in FPLV-inoculated cats. These data indicate that CPV-2c and CPV-2a both have the potential to cause diseases in cats, with some variations of symptoms. CPV-2c appears to be more infectious in cats than CPV-2a and to induce a higher frequency of disease than CPV-2a, although the numbers of cats tested in the experiment were small. Since CPV-2a did not produce any clinical symptoms in the infected SPF cats, yet demonstrated strong virulence in the infected conventional cats (31), it is also possible that CPV-2c infection results in severe illness in conventional cats.

The virulence of CPV-2c in dogs remains to be determined. The most probable hypothesis is that the new antigenic viruses can infect dogs and cause some illness, as seen in the emergence of CPV-2a and 2b in 1980s. However, the CPV-2c-type viruses may also have lost their canine host range. The latter hypothesis is based on the fact that CPV-2, which is believed to have emerged from FPLV-related viruses, fails to infect cats. The pathogenic potential of CPV-2c in dogs needs to be addressed (Figure 3).

Persistent Infection of CPV in Cats

Animals that recover from feline parvovirus infection retain high specific neutralization antibodies and show no virus shedding. Although isolation of FPLV from apparently healthy cats has been reported, feline parvoviruses are generally believed to be completely eliminated from recovered animals.

As mentioned, CPV-type viruses have been isolated from the fecal samples of apparently healthy cats (30). Moreover, many CPV-type viruses were isolated from the peripheral blood mononuclear cells (PBMC) of cats with high specific neutralizing antibodies (11,35,39), suggesting that CPV could persistently infect cats irrespective of the presence of the neutralizing antibodies. Although precise mechanisms of the persistent infection of CPV remain to be determined, PBMC probably play a role as a reservoir for the viruses. If one assumes that CPV actually infects cats persistently, examination will be needed to determine whether sporadic shedding of the virus occurs in recovered cats.

The Efficacy of Conventional FPLV Vaccines against CPV

The study of an attenuated live FPLV vaccine for CPV-2b infection has shown that vaccinated SPF cats are protected from challenge with CPV-2b at 2 weeks after vaccination (37).

A cross-neutralization study of the antibodies induced by an inactivated FPLV vaccine demonstrated that the vaccinated cats actually develop neutralizing antibodies against CPV-2a, 2b, and 2c as well as FPLV (40). These data indicate that commercially available FPLV vaccines can be used for protection against CPVs, at least in the short term. However, antibody titers induced by a FPLV vaccine are significantly lower against CPVs than FPLV (40). Indeed, CPV infection was observed in the cheetahs vaccinated with a killed FPLV vaccine (34). We therefore suggest that FPLV vaccines are not always sufficient to protect cats from CPV infection in the long term. Steinel et al. (34) have proposed the need for inactivated vaccines that use CPV-2a or 2b for cats. CPV-2a/2b-based vaccines are expected to protect cats more efficiently from CPV infection than conventional FPLV vaccines. Recently, Nakamura et al. reported that cats experimentally infected with CPV-2a develop high titers of neutralizing antibodies against CPV-2a and 2b but show relatively low titers against FPLV (40). Thus, like FPLV vaccines for CPV infection, CPV-2a/2b-based vaccines may be less efficient for FPLV infection, which would be a major concern. Interestingly, CPV-2c-infected cats showed similar neutralization antibody titers against FPLV, CPV-2a, and 2b as well as CPV-2c (40). An inactivated CPV-2c-based vaccine for cats could be a promising vaccine candidate against both CPV and FPLV infection.

Problems with the Current Parvovirus Nomenclature

Finally, we point out problems with the current nomenclature of carnivore paroviruses, including FPLV, MEV, and CPV. As we mentioned, all carnivore parvovirus isolates are known to be genetically closely related to each other; interspecies transmissions readily occur among carnivores. On the other hand, field isolates have been distinguished on the basis of the host from which they are isolated. According to this system, CPV-2a- and 2b-type isolates from cats should be designated as FPLV types 2 and 3, even though they are essentially indistinguishable from CPVs from dogs. To solve this problem, a new nomenclature is needed. Naming any field carnivore isolate as feline parvovirus or carnivore parvovirus, irrespective of their original hosts, and using the names such as FPLV and CPV-2a to distinguish antigenic or genetic properties would be more appropriate.

Conclusion

The evolutionary pattern of FPLV in cats differs from that of CPV in dogs. Since FPLV is in evolutionary stasis in cats, FPLV mainly evolves with random genetic drift (9). In contrast, CPV appears to evolve in dogs under certain positive selection on the VP2 protein (9), which may be because of its short history in dogs. How CPVs are evolving in cats remains relatively obscure. Since CPV-2a and 2b are likely to act as newly emerging parasites in cats, some cat-specific positive selection(s), such as relative in vivo fitness and immune surveillance, could operate as a driving force of CPV evolution. The emergence of CPV-2c in leopard cats is a good example of the evolution of CPV in new hosts. Similarly, since specific antibodies against CPV have been detected in a wide range of wild animals, such as large felids, wildcats, civets, otters, and even bears, such interspecies transmissions probably result in accelerated emergence of other new antigenic types of CPVs because of the new host-specific positive selection.

Elucidating how feline parvoviruses are evolving and how newly emerging variants behave may help to prevent a possible outbreak of the new variant. Assuming that a new virulent CPV variant emerges in cats in the future, what can we expect? Fortunately, the newly emerging variant will not likely cause rapid outbreaks in cats or dogs, since FPLV and CPV-2a/2b have been actively circulating in carnivore populations. Commercially available FPLV or CPV-2-based vaccines might also protect animals from the new virus infection. However, if the new virus gains wider host ranges, deadly outbreaks could be observed, as when CPV-2 emerged in dogs. In any case, recent isolates need to be investigated to anticipate and assess the risk caused by newly emerging viruses.

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Dr. Ikeda is a postdoctoral fellow in the Department of Immunology, University of London, United Kingdom. He has a research background in veterinary virology and current interests in the application of genetically modified dendritic cells as therapeutic vaccines as well as the host range restriction of infectious diseases.

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Address for correspondence: Yasuhiro Ikeda, Department of Immunology and Molecular Pathology, Windeyer Institute of Medical Sciences, University College London, 46 Cleveland Street, London W1T 4JF, UK; fax: 0207-679-9357; e-mail: ikedayasuhiro@hotmail.com

Antimicrobial Use and Antimicrobial Resistance: A Population Perspective

Marc Lipsitch* and Matthew H. Samore†

The need to stem the growing problem of antimicrobial resistance has prompted multiple, sometimes conflicting, calls for changes in the use of antimicrobial agents. One source of disagreement concerns the major mechanisms by which antibiotics select resistant strains. For infections like tuberculosis, in which resistance can emerge in treated hosts through mutation, prevention of antimicrobial resistance in individual hosts is a primary method of preventing the spread of resistant organisms in the community. By contrast, for many other important resistant pathogens, such as penicillin-resistant *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant *Enterococcus faecium* resistance is mediated by the acquisition of genes or gene fragments by horizontal transfer; resistance in the treated host is a relatively rare event. For these organisms, indirect, population-level mechanisms of selection account for the increase in the prevalence of resistance. These mechanisms can operate even when treatment has a modest, or even negative, effect on an individual host's colonization with resistant organisms.

The growth of antimicrobial resistance has prompted calls to reduce unnecessary antibiotic use and to improve treatment protocols to maximize the lifespan of these drugs. These calls rest on the well-supported idea that the use of antimicrobial agents is a powerful selective force that promotes the emergence of resistant strains.

To reduce antimicrobial resistance, multiple, and often conflicting recommendations, have been made. For example, strategies to minimize the burden of resistance in hospitals have included reduction of all antimicrobial classes, increased use of prophylactic antimicrobials to reduce colonization, rotation of different antibiotic classes in a temporal sequence, and simultaneous use of different antimicrobials for different patients (1-6).

Underlying these often varying recommendations for improving antimicrobial use is frequently conflicting evidence about the relationship between antibiotic treatment and antibiotic resistance. In some pathogens, showing that antibiotic treatment puts treated persons at a greater risk for acquiring resistant organisms has been difficult (7-8); nonetheless, the cumulative effect of using these antibiotics has clearly been to increase the prevalence of resistance in the population as a whole.

For many pathogens of current concern, especially organisms for which asymptomatic colonization typically precedes infection (e.g., *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus* spp., and the gram-negative enteric bacteria), the relationship between antimicrobial use and resistance differs in fundamental ways from the relationship found in *Mycobacterium tuberculosis*, for which many modern principles of chemotherapy were developed. Furthermore, we argue that the selective effects of antibiotic use on these organisms are poorly understood, and we make specific suggestions for studies that could improve understanding of the mechanisms by which antibiotics exert natural selection on these organisms. Such an understanding will be crucial for the design of rational policies of antibiotic use to maximize the lifespan of existing drugs and to minimize the impact of resistant infections.

*Harvard School of Public Health, Boston, Massachusetts, USA; and †University of Utah School of Medicine, Salt Lake City, Utah, USA

Resistance in People and Populations

Ehrlich's advice that treatment of infections should "hit hard and hit early," formulated in the earliest days of antimicrobial chemotherapy, presciently summarized the principles of treatment for infections such as tuberculosis (TB) (9). These principles are embodied in modern protocols of directly observed, short-course chemotherapy, where the goal is to treat with adequate concentrations of multiple drugs and maintain treatment until the bacterial population is extinct. Resistance to each of the major antituberculosis drugs is mediated by single point mutation; therefore tuberculosis treatment is designed to prevent the ascent of subpopulations of mutant bacilli that are resistant to any one of the drugs. Similar principles have been suggested for other infections in which resistance can arise by simple mutation, most notably HIV (9), although there has been some controversy on this topic (11). In these infections, the relationship between treatment, resistance in the treated person, and resistance in the community at large is relatively clear. Inadequate therapy (owing to subtherapeutic drug concentrations, too few drugs, or poor adherence to therapy) results in the emergence of resistance, and possibly treatment failure, in the treated host. Following the emergence of resistance in the treated host, resistant infections may be transmitted to others. (Figure, A; Table).

For many pathogens, both the genetics and the epidemiology of resistance differ from those of TB in important ways.

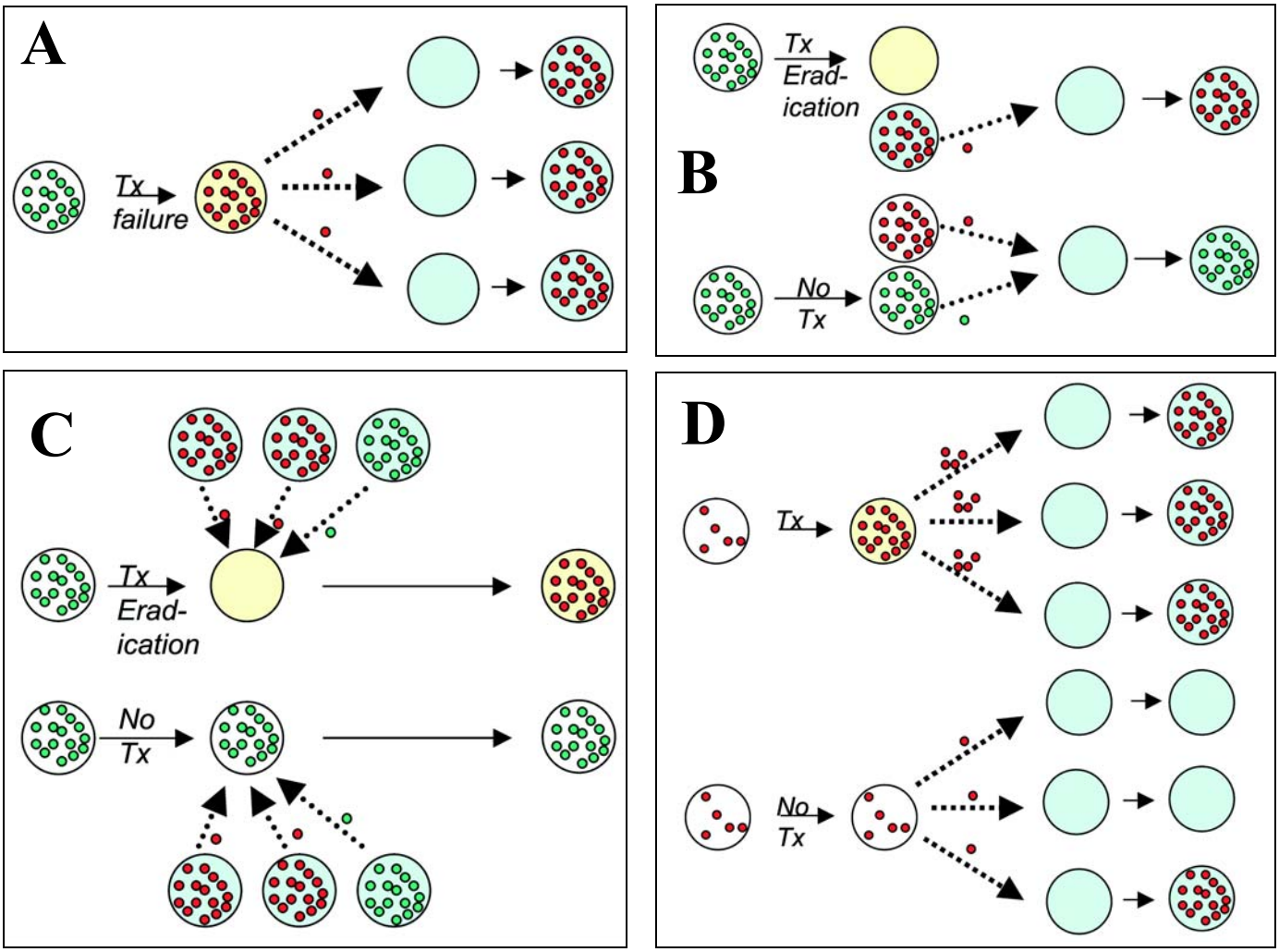

Figure. Four mechanisms by which antibiotic treatment can create selection for resistance in the population, showing direct effects—increased resistance in treated (yellow) vs. untreated (white) hosts, and indirect effects—increased resistance in others (turquoise) due to treatment of specific hosts. (A) Subpopulations (usually mutants) of resistant (red) bacteria are present in a host infected with a predominantly susceptible (green) strain; treatment fails, resulting in outgrowth of the resistant subpopulation, which can then be transmitted to other, susceptible hosts (turquoise). (B) Successful treatment of an individual infected with a susceptible strain reduces the ability of that host to transmit the infection to other susceptible hosts, making those hosts more likely to be infected by resistant pathogens than they would otherwise have been, and shifting the competitive balance toward resistant infections. (C) Treatment of an infection eradicates a population of susceptible bacteria carried (often commensally) by the host, making that host more susceptible to acquisition of a new strain. If the newly acquired strain has a high probability of being resistant (as in the context of an outbreak of a resistant strain), this can significantly increase the treated individual's risk of carrying a resistant strain, relative to an untreated one. (D) Treatment of an infection in an individual who is already colonized (commensally) with resistant organisms may result in increased load of those organisms if competing flora (perhaps of another species) are inhibited—leading to increased shedding of the resistant organism and possibly to increased individual risk of infection with the resistant organism.

For example, methicillin resistance in *S. aureus* and vancomycin resistance in *Enterococcus* are mediated by the acquisition of one or several new genes, rather than by point mutations in existing genes. In *Streptococcus pneumoniae*, penicillin resistance occurs when segments of wild-type penicillin-binding protein genes are replaced with alleles whose sequences differ from the wild-type at multiple positions. These new resistance mechanisms arose and spread in large populations under conditions of antibiotic selection pressure, but they are unlikely to occur de novo in any single person because of the multiple changes involved. Organisms (or plasmids) bearing these types of resistance must be acquired, generally as a consequence of cross-transmission. Furthermore, most of these organisms are not obligate pathogens such as HIV or TB; as a result, much of their exposure to antibiotics occurs during treatment directed at infections caused by other, unrelated organisms.

Because of these genetic and epidemiologic differences, the paradigm for tuberculosis treatment, minimizing resistance in the treated host and the community by preventing the emergence of resistant subpopulations during treatment, is often inapplicable to these organisms (12). Antibiotic treatment promotes the spread of these organisms, as suggested by the rapid increases in resistance in many of the organisms after the new drug classes are introduced. However, the effects of treatment in promoting resistance occur by less direct mechanisms, which depend on competitive interactions between drug-resistant and drug-susceptible strains.

Table. Mechanisms by which antimicrobial treatment has direct and indirect effects on resistance

Mechanism (effect of treatment)	Relationship between selection for resistance and treatment success	Relationship between no./dose of antibiotics and selection	Examples	Figure
Emergence of resistance during treatment (D[a], I[b])	↓[d]	↓	TB, HIV, *Pseudomonas aeruginosa*, *Enterobacter* spp.	1a
Reduced transmission of susceptible strains (I)	↑[e]	↑	May occur for nearly every infection	1b
Increased susceptibility to colonization (D, I)	?[c]	?	Commensals of skin, intestinal and respiratory tracts	1c
Increased density of colonization in individuals already colonized with resistant organisms, by inhibiting competitors (D,I)	?	↑- ?	VRE[f] and anti-anaerobic treatments	1d

[a]D=direct.
[b]I=indirect.
[c]?=relationship uncertain.
[d]↓inverse relationship.
[e]↑positive relationship.
[f]VRE=vancomycin-resistant *Enterococci*.

Indirect Effects on Resistance

For any infectious disease, the infection or colonization status of any one (index) patient affects the risk of infection or colonization of others. Measures (such as vaccination or antibiotic treatment) that change the incidence or duration of infection in one person will affect that person's contacts (13-14). Just as vaccination programs benefit those who are not vaccinated because of the phenomenon of herd immunity, antibiotic usage by some persons may increase the risk of colonization or infection with resistant organisms in people who have not received antibiotics. Members of a population experience indirect effects of antimicrobial use, defined as the enhancement of risk for acquiring a resistant organism, because of the use of antimicrobials by other persons in the group or population.

For example, simply by eradicating susceptible organisms, and thereby reducing the opportunities for transmission of susceptible strains, antibiotics received by treated hosts can increase the probability that other hosts will acquire resistant variants (Figure, B; Table). For many pathogens, acquisition of one strain reduces a person's chances of acquiring other strains, either via immune responses, via direct interference (15-17), or both. These inhibitory interactions create competition between resistant and susceptible strains. As a result, treatment of some patients, by eradicating susceptible strains and thereby reducing their ability to transmit to other hosts, is advantageous to resistant strains in the population. Mathematical models (18-22) and epidemiologic studies (23) suggest that this mechanism of shifting the competitive balance in favor of resistant strains can increase the prevalence of resistant organisms in the community, alone or in combination with other mechanisms. An important feature of this kind of indirect effect is that it need not involve an increase in a patient's own risk of carrying resistant organisms, only a reduction in the duration or probability of carrying susceptible ones.

In these organisms, the increase in transmission of resistant pathogens is a consequence of successful treatment of the infected host, resulting in the eradication of drug-susceptible pathogens that colonize or infect that host. As a consequence, the more effective a treatment is at eradicating drug-susceptible populations of these organisms, the more it will promote the spread of resistant ones. This spread contrasts with TB, in which treatment failure is often associated with the emergence of resistance in treated hosts, so unsuccessful treatment is seen as a factor promoting the spread of resistance (although, over a time scale of decades, this type of indirect mechanism described here may play a role even in tuberculosis [21]).

Combinations of Direct and Indirect Effects on Resistance

A third mechanism by which antimicrobial use increases the number of patients colonized or infected with resistant organisms is by modifying the treated host's colonization resistance (Figure, C; Table). Eradication or reduction of drug-susceptible normal flora by antibiotic treatment may increase vulnerability to acquisition of new strains. This effect will increase the patient's probability of being colonized with a resistant organism if, during or shortly after treatment, he or she is exposed to others with resistant organisms. This mechanism is direct in the sense that it increases the treated patient's risk of colonization with resistant organisms but is also associated with indirect effects because of the requirement for transmission. An index host given antibiotics is placed at greater risk for colonization with resistant organisms (direct effect), but this risk is amplified by his or her exposure to other patients harboring resistant organisms, which is in turn enhanced by their use of antibiotics (indirect effect).

A fourth mechanism by which antimicrobial use increases antimicrobial resistance is by increasing the density of resistant organisms within a patient who already harbors such organisms at a lower density (Figure, D; Table). Enhanced shedding of these organisms, resulting in an increased risk to other patients (an indirect effect), has been documented (i.e., in the case of anti-anaerobic agents that increase shedding of vancomycin-resistant *Enterococci* (VRE) (24). An increased risk of resistant infection to the treated patient (a direct effect)

may occur if a higher density of resistant organisms places the patient at higher risk of infection with his or her own flora. Unlike the other three ways by which antimicrobial use promotes resistance, this mechanism is mediated through antimicrobial treatment of patients already colonized with the resistant organism.

There are a number of other cases in which direct and indirect effects of antibiotic treatment are combined. Due to the diversity of genetic mechanisms of resistance, the risk of emergence of resistance during treatment represents a continuum, with TB at one end and VRE (or MRSA) at the other. Fluoroquinolone resistance in *S. pneumoniae* mediated by the accumulation of mutations in the DNA gyrase and topoisomerase IV genes (25), or resistance to third-generation cephalosporins in Enterobacteriaceae mediated by mutations in TEM and SHV beta-lactamases located on plasmids (26), lie between these two extremes. In these cases, multiple mutations are required to turn a fully susceptible strain into a clinically resistant one. For a patient colonized or infected with a fully susceptible strain, emergence of resistance during treatment may be highly unlikely because of the requirement for selecting multiple mutations. However, in such cases, there may be selection in consecutive hosts for small increases in levels of resistance to a particular compound, resulting eventually in the emergence of clinical resistance (27). Patients may be colonized with a mixed flora of resistant and susceptible organisms, and eradication of the drug-susceptible flora may permit outgrowth of the resistant subpopulation (28). This mechanism has some formal similarity to what occurs in TB, except that for a colonizing bacterium such as the pneumococcus or the enteric colonizers, outgrowth of resistant organisms in the site of colonization need not be associated with treatment failure. In these cases, the treated patient is at increased risk of carrying resistant organisms (direct effect), but an indirect effect on the population occurs because the treated patient no longer carries susceptible organisms and is, therefore, unable to transmit them.

Treatment with one antimicrobial drug can select for resistance to a number of other, unrelated agents, by several means. If individual organisms are resistant to multiple drugs, then use of any one of these may promote resistance to others (29). Additionally, by altering the balance of different components of the indigenous microbial flora, treatment with one agent may increase the load of a pathogen resistant to another agent, simply by killing off competing flora of different species; this has been observed, for example, with anti-anaerobic treatments that increase the load of VRE (24). These complexities increase the number of relationships that need to be studied in assessing the effects of antimicrobial use on resistance and also the number of potential confounders in any study.

Implications for Evaluating Treatment Strategies

Variation in mechanisms of resistance has implications for the choice of antimicrobial therapy and the evaluation of strategies to minimize resistance. Adopting the individual and population-level perspective informs therapeutic decision-making, clinical study design, and public policy.

In TB, preventing the emergence of resistance in a treated host is a sound policy for averting the emergence of resistance at the population level as well (although once resistant strains have emerged, special measures are required to contain them [30]). With respect to antimicrobial resistance, what is good for the patient is good for the population.

In contrast, for other types of resistance, antimicrobial treatment may exert individual-level effects that are substantially different in magnitude or even opposite in direction to that of population-level effects. Treatment with a beta-lactam may produce only a small, short-lived increase in the treated patient's odds of carrying or being infected by a resistant pneumococcus (7). In some cases, treatment may actually eradicate carriage of a resistant organism, thereby reducing the individual's risk of resistant carriage. Small or unobservable effects on individual risk have been observed in other cases as well, such as vancomycin use for VRE (8, 31) and the use of various antibiotics for infections with resitant gram-negative rods (32). In these cases, preventing resistance in the treated patient may not be the central goal of a prudent antibiotic use policy; instead, treatment should seek to minimize the advantage it provides to resistant organisms in the community or the hospital as a whole, subject to the constraint of providing effective treatment for the patient.

The considerations of the distinctive biologic and epidemiologic mechanisms of antibiotic resistance in different pathogens lead to several broad suggestions for future studies. First, the optimal study design to estimate individual-level effects of antibiotics on colonizing organisms such as VRE and beta-lactam resistant *S. pneumoniae* is to measure acquisition and loss rates in an observational cohort or experimental study where subjects are serially cultured before, during, and after antibiotic therapy (23, 33). Time-to-event statistical models (e.g., Cox proportional hazards regression) are appropriate analytic methods for these kinds of studies (23, 31, 34). This design allows investigators to distinguish between the effects of antimicrobials on the risk for acquisition (colonization) and their effects on the risk for clinical infection once an patient has been colonized with a resistant organism.

As a consequence of the mechanisms we have described, the magnitude of an antibiotic's effect on a patient's risk of resistant colonization or infection may be dependent on his or her exposure to potential transmission of resistant organisms (13). Stated differently, the frequency of contact with others carrying the resistant organisms is likely an important effect-modifier of antibiotic effects for pathogens that do not follow the simple model of emergence of resistance exhibited by organisms such as *M. tuberculosis*. Individual-level antibiotic effects mediated by alterations in colonization resistance or killing of susceptible bacteria may be greater in settings of high exposure to resistant organisms, for example, during outbreaks (7). Controlling for transmission risk or measuring effects conditional on a specified level of transmission risk is

advised, when possible. Standard analytic approaches make the assumption that outcomes in different subject are independent, but this assumption is violated in the case of infectious diseases. Use of one of these strategies to model exposure to transmission will help to account for this nonindependence of outcomes in different persons in the same study (13, 35-37).

One practical result of quantifying direct, individual-level antibiotic effects is to provide information on the short-term risk of infection with a resistant organism to a person about to initiate antibiotic treatment. This hazard needs to be taken into account when weighing the risks and benefits of use of antimicrobial agents in individual patients. However, analogous to the evaluation of vaccine programs, combined direct and indirect antibiotic effects carry increased importance from the public health and policy management perspective (38, 39). The measurement of population-level effects of antimicrobials also has educational value in demonstrating to clinicians and patients the extent to which individual antibiotic use choices have negative consequences for the population as a whole. Such a conflict between individual benefit and the population's harm is an example of what economists term an "externality" or what environmentalists have called the "Tragedy of the Commons" (40).

To estimate overall antibiotic effects from data requires group-level studies. Observational group-level studies may lack sufficient data to avoid confounding and other causes of ecologic bias (41). For this reason, studies that estimate the effects of individual- and group-level antimicrobial use are generally preferable to ones that contain group-level data alone. Depending on the context, the appropriate group(s) may include the family, the community, the hospital, or the hospital unit or department (42-44). Further research is necessary to evaluate hierarchical regression methods and compare results obtained from different levels of analysis (44).

For the most accurate measurement of overall antibiotic effect on resistance in communities, a cluster-randomized intervention trial is appropriate (45). In cluster-randomized trials, the unit of randomization is a group such as a community or a hospital, and multiple units (sometimes as few as six, but often more) are assigned to each of two (or more) treatment arms. We are not aware of published studies using this design to evaluate antibiotic resistance, although we know of two in progress (R. Platt, pers. comm.) (12). However, this design has been used in other areas of infectious disease epidemiology for which group level effects are important (such as vaccination programs), and it is considered the standard design for investigations of the effects of insecticide-impregnated bednets in preventing malaria (45-47). In the context of antimicrobial resistance, cluster-randomized trials have two key advantages. First, unlike studies that gather individual-level data alone, they provide the opportunity to observe the indirect effects of treatment on resistance. Second, they provide a clean way to avoid the statistical problems of nonindependence between patients in a study that may reduce the power or increase the false-positive rate of observational studies. In cluster-randomized studies of antimicrobial resistance, both the incidence rate of infection with resistant organisms in the population and the ratio of resistant to susceptible (or proportion of total organisms that are resistant) would be appropriate study endpoints.

Role of Mathematical Models

Transmission-dynamic modeling can also play an important role in bridging the gap between individual- and group-level effects (20, 21, 48-50). These models take information about individual-level effects as parameters and make predictions about the response of the population to changes in such parameters as transmission risk or antibiotic usage. Although models cannot substitute for empirical intervention studies, they can be particularly valuable in at least four ways: 1) generating hypotheses about the relationship between antibiotic use and resistance that can be used in designing and prioritizing empirical studies; 2) defining the conditions under which a particular intervention is likely to work, thereby suggesting how empirical results can (and cannot) be extrapolated to other settings; 3) providing explanations for phenomena that have been observed but whose causes were uncertain; and 4) identifying biological mechanisms that, while important, remain poorly understood.

An example of models for generating hypotheses comes from the question of antimicrobial rotation or "cycling." Cycling of antimicrobial classes in hospitals has been suggested and is currently being evaluated for its ability to curtail resistance in major nosocomial pathogens (5, 51-54). One mathematical model of this process has suggested that using a mixture of different drug classes simultaneously (e.g., if two drug classes are available for empiric therapy of certain infections, treat half of the patients with one drug class and half with the other) will reduce resistance more effectively than cycling under a broad range of conditions (19). This suggests that such mixed regimens would be good candidates for comparison with cycling in controlled trials.

As a second example, levels of resistance in hospital-acquired pathogens may change rapidly within a matter of weeks or months after changes in antimicrobial use. By contrast, studies of reductions in antimicrobial use in communities have shown slow and equivocal effects on resistance in community-acquired pathogens (55). Mathematical models suggest that, in communities, the key factor driving the change in resistance levels may be the "fitness cost" of resistance, i.e., resistance will decline after a reduction in antimicrobial use if resistant organisms in untreated patients are at a disadvantage for transmission or persistence (20, 50, 56-57). This cost may be small in many bacteria, accounting for the slow response (55, 58). In contrast, a model indicates that, in hospitals, changes in resistance may be driven primarily by the admission of new patients who often bring with them drug-susceptible flora, and this may rapidly "dilute" levels of resistance in the absence of continuing selection by antibiotics (59). If correct, this explanation suggests that the success of antimicrobial

control measures should be evaluated differently for hospitals and for communities.

The use of mathematical models, and more generally the attempt to predict the relative merits of different interventions, will depend on an improved understanding of the mechanisms of antibiotic selection in particular organisms. For example, two recently published models for the nosocomial spread of resistant pathogens made contrasting assumptions about whether antimicrobial treatment increased an patient's susceptibility to colonization only during treatment (60) or for a period following treatment (59), and about the importance of colonization with drug-susceptible strains in protecting against acquisition of resistant ones. As a result of these differences in assumptions, predictions differed in important ways: one model suggested that reduction of antibiotic use would be a comparatively poor intervention when endemic transmission is high and that resistant organisms could persist endemically even in the absence of input from admitted patients or antibiotic selection (60). The other model predicted rapid declines in the level of resistance when use is reduced, and a more complicated relationship between the effectiveness of interventions and the level of transmission within the hospital (59). Testable predictions will permit the evaluation of different models for particular settings and provide a basis for refining the assumptions of these models.

Conclusion

The relationship between antibiotic usage and antibiotic resistance for many types of pathogens is largely mediated by indirect effects or population-level selection. When resistant and susceptible organisms compete to colonize or infect hosts, and use of an antibiotic has a greater impact on the transmission of susceptible bacteria than resistant ones, then increasing use of the antibiotic will result in an increase in frequency of organisms resistant to that drug in the population, even if the risk for treated patients is modest. Antimicrobial use and patient-to-patient transmission are not independent pathways for promoting of antimicrobial resistance, rather they are inextricably linked.

Study designs to assess the effect of antimicrobial use on resistance should reflect these diverse pathways of direct and indirect effects. Estimates of direct effects of antimicrobial use on treated patients will be most informative if clinical cultures are combined with measurements of colonization. Use of time-to-event (e.g., Cox proportional hazards) models provides a natural way of controlling for the patient's length of stay when assessing the effect of treatment on acquisition of resistant organisms. Analyses that control for a person's exposure to other patients carrying resistant organisms will help to capture the effect modification because of varying transmission pressures during a study. Inclusion of data on antimicrobial use by the group to which others are exposed (siblings, fellow patients on a hospital unit, total use in a community) and to individual-level data will provide one method of estimating both direct and indirect effects of antibiotic use. Nonindependence of individual outcomes makes the interpretation of intervention studies problematic unless measures are taken to account for this nonindependence; cluster-randomized studies, used in other areas of infectious disease epidemiology, are an excellent solution to this problem. We have commented elsewhere on other aspects of study design for antimicrobial resistance, notably the importance of control group selection (7, 61-62).

Understanding in detail, for each pathogen, the mechanisms by which antimicrobial use selects for antimicrobial resistance in treated patients and in the population is of more than academic importance. For practitioners, these mechanisms matter for making well-informed decisions about the design of treatment protocols, the choice of antibiotics and doses for particular indications. For policymakers, these issues have direct bearing on the design of campaigns to encourage more rational antibiotic use and on the priorities in regulating the use of antimicrobial agents for human and animal use (63-64).

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Marc Lipsitch is Assistant Professor of Epidemiology at Harvard School of Public Health. He uses experimental (animal model), epidemiologic, and mathematical modeling approaches to study the population biology of antimicrobial resistance and to assess the effects of vaccination on the population structure of *Streptococcus pneumoniae*.

Matthew Samore is Associate Professor of Internal Medicine at the University of Utah and Chief of the Division of Clinical Epidemiology. He attended the University of Wisconsin School of Medicine, completed his residency in Internal Medicine at Washington University in St. Louis, and a fellowship in Infectious Diseases at Brigham and Women's Hospital and Beth Israel Hospital in Boston. His research interests include antibiotic resistance in hospitals and communities, computer-decision support for antibiotic prescribing, and surveillance of errors and adverse events.

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Address for correspondence: Marc Lipsitch, Department of Epidemiology, Harvard School of Public Health, 677 Huntington Avenue, Boston, MA 02115; fax: 617-566-7805; e-mail: mlipsitc@hsph.harvard.edu

Perspectives. Articles should be approximately 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.

Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Baylisascaris procyonis: An Emerging Helminthic Zoonosis

**Frank Sorvillo,* Lawrence R. Ash,* O.G.W. Berlin,*† JoAnne Yatabe,†
Chris Degiorgio,‡ and Stephen A. Morse§**

Baylisascaris procyonis, a roundworm infection of raccoons, is emerging as an important helminthic zoonosis, principally affecting young children. Raccoons have increasingly become peridomestic animals living in close proximity to human residences. When *B. procyonis* eggs are ingested by a host other than a raccoon, migration of larvae through tissue, termed larval migrans, ensues. This larval infection can invade the brain and eye, causing severe disease and death. The prevalence of *B. procyonis* infection in raccoons is often high, and infected animals can shed enormous numbers of eggs in their feces. These eggs can survive in the environment for extended periods of time, and the infectious dose of *B. procyonis* is relatively low. Therefore, the risk for human exposure and infection may be greater than is currently recognized.

Baylisascaris procyonis, a ubiquitous roundworm infection of raccoons (*Procyon lotor*), is increasingly being recognized as a cause of severe human disease (1,2). *B. procyonis* has a widespread geographic distribution, with infection rates as high as 70% in adult raccoons and exceeding 90% in juvenile raccoons (3). As with other ascarids, eggs are excreted in feces and must develop externally, typically in soil, to become infectious. When raccoons ingest infective eggs, larvae will hatch, enter the wall of the small intestine, and subsequently develop to adult worms in the small bowel. However, ingestion of eggs by other host animals, especially rodents and other small mammals, results in extraintestinal migration of larvae (4); an estimated 5%-7% of larvae invade the brain (5). The migration of helminth larvae through tissue in suboptimal hosts is termed larva migrans and may affect the viscera (visceral larva migrans [VLM]), the eye (ocular larva migrans [OLM]), or the nervous system (neural larva migrans [NLM] (6). Raccoons may also become infected when they eat larvae that have become encapsulated in the tissues of rodents and other animals (3).

More than 90 species of wild and domesticated animals have been identified as infected with *B. procyonis* larvae (3). Outbreaks of fatal central nervous system disease caused by *B. procyonis* have occurred on farms and in zoos and research animal colonies and have affected commercial chickens, bobwhite quail, guinea pigs, commercial pheasants, and domestic rabbits (7-11). Natural infections have also been recognized in dogs, rodents, porcupines, chinchillas, prairie dogs, primates, woodchucks, emus, foxes, and weasels (12-16). Experimental infection of a variety of nonhuman primates has also been reported (17).

*University of California Los Angeles School of Public Health, Los Angeles, California; †Specialty Labs, Santa Monica, California, USA; ‡University of California Los Angeles School of Medicine, Los Angeles, California, USA; and §Centers for Disease Control and Prevention, Atlanta Georgia, USA

Human Infection

B. procyonis infection of humans typically results in fatal disease or severe sequelae (1,2,18-24; pers. comm., W. Murray). Clinical manifestations include eosinophilic encephalitis, ocular disease, and esoinophilic cardiac pseudotumor. Elevated peripheral cerebrospinal fluid eosinophilia can be detected in cases of meningoencephalitis. Eleven recognized human cases, four of them fatal, have been reported (Table). The first human case was reported in 1984 in a 10-month-old infant with fatal eosinophilic meningoencephalitis (18). At autopsy, numerous granulomas containing larvae of *B. procyonis* were observed in several organs and tissues (18). The brain was the most heavily affected, with granulomas concentrated in the periventricular white matter, around the dentate nuclei, and along the cerebral and cerebellar cortices. Numerous granulomas and larvae were also found in the mesentery and cardiac tissue. The infant's family lived in a rural, wooded area of Pennsylvania, and raccoons were nesting in unused chimneys at the time infection was acquired.

Four additional cases of eosinophilic encephalitis with similar pathologic characteristics have been documented. Magnetic resonance images from a human case of *Baylisascaris* encephalitis are shown in Figure 1. In patients who have survived central nervous system (CNS) invasion, severe neurologic sequelae have resulted. In a fatal case, an eosinophilic cardiac pseudotumor, affecting principally the left ventricle, was observed at autopsy; no larvae or granulomas were found in any other tissue examined.

No effective therapy exists for the visceral form of *B. procyonis* larval infection. In an experimental model, mice treated with albendazole and diethylcarbamezine within 10 days after infection were protected from CNS disease (25); however, several anthelminthic agents have been used to treat human cases without success. Laser photocoagulation has been successful in treating ocular infection (26).

Because the disease is transmitted by the fecal-oral route, human cases of *B. procyonis* infection typically occur in younger age groups, mainly infants, who often engage in oral

Table. Reported human cases of larval *Baylisascaris procyonis* infection

Year[a]	Location	Age	Sex	Clinical	Outcome	Reference
1980	Pennsylvania	10 mo	Male	Eosinophilic meningoencephalitis	Fatal	17
1984	Illinois	18 mo	Male	Eosinophilic meningoencephalitis	Fatal	18
1990	New York	13 mo	Male	Eosinophilic meningoencephalitis	Severe neurologic sequelae	19
1992	California	29 yr	Male	Diffuse unilateral subacute neuroretinitis	Ocular sequelae	21
1991	Germany	48 yr	Female	Diffuse unilateral subacute neuroretinitis	Ocular sequelae	22
1995	Massachusetts	10 yr	Male	Esoinophilic cardiac pseudotumor	Fatal	20
1996	Michigan	6 yr	Male	Chorioretinitis, neurologic deficits	Severe neurologic sequelae	23
1996	Michigan	2 yr	Male	Eosinophilic meningoencephalitis, chorioretinitis	Severe neurologic sequelae	23
1997	California	13 mo	Male	Eosinophilic meningoencephalitis	Severe neurologic sequelae	2
1998	California	11 mo	Male	Eosinophilic encephalitis	Severe neurologic sequelae	1
1999	California	17 yr	Male	Eosinophilic meningoencephalitis	Fatal	[b]

[a]Year of onset or report.
[b] Pers. comm., W. Murray.

exploration of their environment and are therefore more likely to be exposed to *B. procyonis* eggs. Raccoon activity near the patient's residence is often described. All but one of the reported patients to date have been male, however; there is no reason to believe that females are less susceptible to infection.

Diagnosis and Underrecognition of Infection

Diagnosis of *B. procyonis* infection is typically done through morphologic identification of larvae in tissue sections (27). However, accurate diagnosis requires experience in recognizing larval morphologic characteristics and differentiating among a number of possible larval nematode agents, including *Toxocara canis*, *T. cati*, *Ascaris lumbricoides*, and species of *Gnathastoma*, *Angiostrongylus*, and *Ancylostoma,* as well as larval cestode infections such as cysticercosis and echinococcosis (6,27). Characteristic features of *B. procyonis* larvae in tissue include its relatively large size (60 μ) and prominent single lateral alae (27) (Figure 2). While serologic testing has been performed in some cases as supportive diagnostic evidence, no commercial serologic test is currently available (28,29). However, a presumptive diagnosis can be made on the basis of clinical (meningoencephalitis, diffuse unilateral subacute neuroretinitis [DUSN], pseudotumor), epidemiologic (raccoon exposure), radiologic (white matter disease), and laboratory results (blood and CNS eosinophilia).

Human baylisascariasis is probably underrecognized, and the full spectrum of clinical illness is unclear. The agent is unknown to most clinicians and typically is not considered in a differential diagnosis. In addition, confirming the diagnosis requires an effective biopsy specimen that must contain an adequate cross-section of a larva. Since small numbers of larvae can cause severe disease and larvae occur sporadically in tissue, a biopsy may frequently fail to include larvae; such a specimen will result in a negative finding. Moreover, larval morphologic characteristics may not be recognized or may be misidentified. The accurate diagnosis of parasites in tissues can be difficult even for trained microscopists, and mistaken identification, particularly of helminth larvae, is not uncommon (27). Finally, no commercial serologic test exists for the diagnosis of *B. procyonis* infection, and the sensitivity, specificity, and predictive value of available serologic tests are unknown. Evidence for underrecognition of larval *B. procyonis* infection can be found in several reported cases of DUSN caused by larvae compatible with *B. procyonis* and a case of eosinophilic meningoencephalitis reported in an infant in 1975 (26,30,31).

Infection Potential and Human Risk

Although relatively few human cases of baylisascariasis have been reported, several factors suggest that the likelihood of exposure and infection may be greater than is currently recognized. Raccoons have a widespread geographic distribution, and infection with *B. procyonis* is common in raccoon populations, with typically high prevalence rates observed. An infected raccoon can harbor numerous adult worms and may excrete large numbers of eggs. A single adult female worm may produce an estimated 115,000 to 877,000 eggs per day, and an infected raccoon can shed as many as 45,000,000 eggs daily (3,4,32). In light of the relatively low infectious dose of *B. procyonis* (estimated to be $\leq$5,000 eggs) and the viability of the eggs in the environment for months to years, the infection potential is not insubstantial. Raccoons have increasingly become peridomestic animals living in close proximity to human residences and are among the fastest growing wildlife

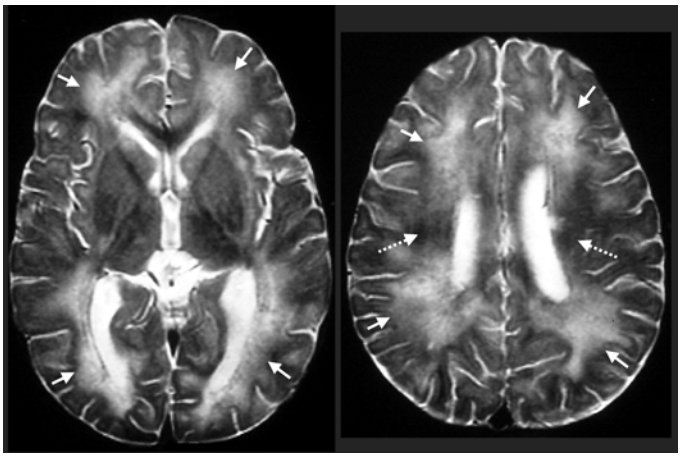

Figure 1. Biopsy-proven *Baylisascaris procyonis* encephalitis in a 13-month-old boy. Axial T2-weighted magnetic resonance images obtained 12 days after symptom onset show abnormal high signal throughout most of the central white matter (arrows) compared with the dark signal expected at this age (broken arrows).

populations nationwide. These animals benefit from feeding on abundant pet food left accessible, either accidentally or intentionally, and their populations can thrive under such conditions. In one suburban area near the residence of a recent patient in northern California, the raccoon population was measured at 30 animals per quarter acre. Areas frequented by raccoons and used for defecation were found in close proximity to human dwellings, and *B. procyonis* eggs are routinely recovered from these areas (1). Children, particularly toddlers, may be at particular risk of exposure.

Although baylisascariasis may indeed be underdiagnosed, asymptomatic human infection may be the typical response, and the limited number of cases reported may indicate that an unrecognized immune defect is necessary for severe infection to occur. The prevalence of asymptomatic infection in human populations has yet to be determined.

A Possible Agent of Bioterrorism

In an era of increasing concern about bioterrorism (33), certain characteristics of *B. procyonis* make it a feasible bioterrorist agent. The organism is ubiquitous in raccoon populations and therefore easy to acquire. Enormous numbers of eggs can be readily obtained, and these eggs can survive in an infectious form for prolonged periods of time. As with other ascarids, the eggs can remain viable in a dilute (0.5%-2%) formalin solution for an indefinite period of time, and animal studies suggest that *B. procyonis* has a relatively small infectious dose. Moreover, the organism causes a severe, frequently fatal infection in humans, and no effective therapy or vaccine exists. Introduction of sufficient quantities of *B. procyonis* eggs into a water system or selected food products could potentially result in outbreaks of the infection. A similar agent, *Ascaris suum*, a roundworm of pigs, was used to intentionally infect four university students who required hospitalization after eating a meal that had been deliberately contaminated with a massive dose of eggs (34). Contamination of community water sources would be difficult since the eggs of *B. procyonis* are relatively large (80 μm long by 65 μm wide) and would be readily removed by standard filtration methods or the flocculation and sedimentation techniques used by municipal water systems in the United States. However, posttreatment contamination or targeting of smaller systems could be possible.

Conclusion

Baylisascariasis is an emerging helminthic zoonosis with the potential for severe infection that may be a more important public health problem than is currently recognized. Educating the medical community is of paramount importance in helping to define the extent of infection. Physicians should consider *B. procyonis* infection in the differential diagnosis of patients with eosinophilic meningoencephalitis, DUSN, and eosinophilic pseudotumor. While infants and children have a higher probability of infection, all age groups are at risk. The public should be made aware of the potential risks of exposure to raccoons and raccoon feces. Raccoons should be discouraged as pets or should be routinely evaluated for *B. procyonis* infection and treated. However, screening and treatment may not be sufficient to prevent exposure, since the likelihood of reinfection

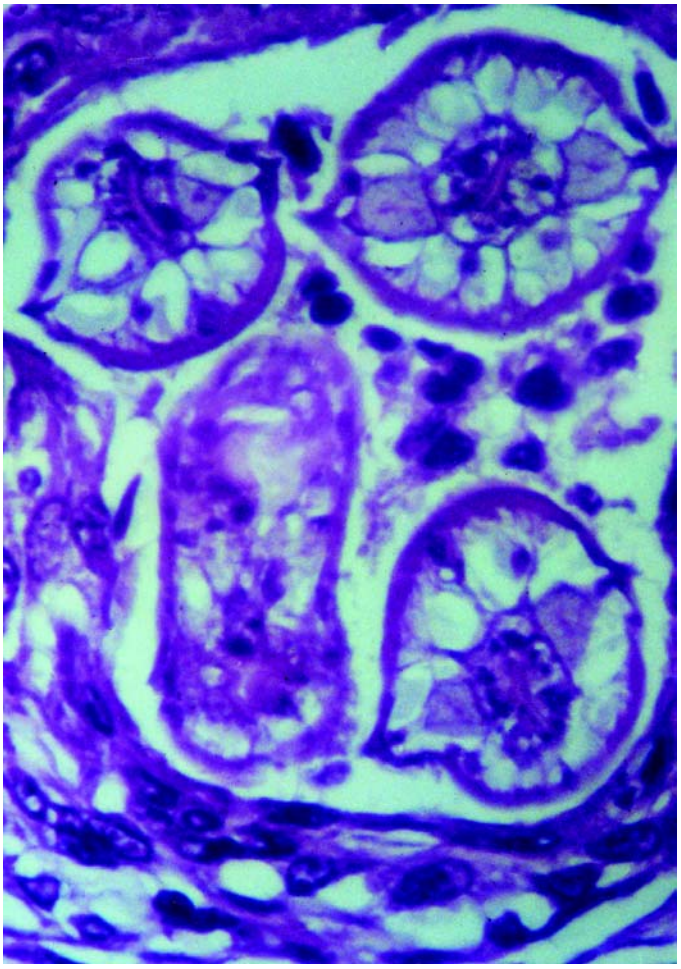

Figure 2. Cross-section of *Baylisascaris procyonis* larva in tissue section of brain, demonstrating characteristic diagnostic features including prominent lateral alae and excretory columns.

is high. The public should be discouraged from feeding raccoons and should ensure that possible food sources (such as pet food, water, and garbage) are protected from raccoon access. Further study of the impact of larval *B. procyonis* infection on human health is warranted. Development of a standardized serologic test for *B. procyonis* would allow epidemiologic studies of its prevalence and incidence and help determine factors associated with infection. A sensitive and specific test would also provide a noninvasive method of diagnosis. Finally, a better understanding of the pathogenesis of *B. procyonis* infection and efforts to develop effective treatment approaches are warranted.

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Dr. Sorvillo is Associate Professor, Department of Epidemiology, UCLA School of Public Health. His research interests include the epidemiology and control of infectious diseases, particularly parasitic agents.

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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.

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Address for correspondence: Frank Sorvillo, UCLA School of Public Health, Box 951772, Los Angeles, CA 90024, USA; fax: 714-816-9099; e-mail: fsorvill@ucla.edu

Megadrought and Megadeath in 16th Century Mexico

Rodolfo Acuna-Soto,* David W. Stahle,† Malcolm K. Cleaveland,† and Matthew D. Therrell†

The native population collapse in 16th century Mexico was a demographic catastrophe with one of the highest death rates in history. Recently developed tree-ring evidence has allowed the levels of precipitation to be reconstructed for north central Mexico, adding to the growing body of epidemiologic evidence and indicating that the 1545 and 1576 epidemics of cocoliztli (Nahuatl for "pest") were indigenous hemorrhagic fevers transmitted by rodent hosts and aggravated by extreme drought conditions.

The native people of Mexico experienced an epidemic disease in the wake of European conquest (Figure 1), beginning with the smallpox epidemic of 1519 to 1520 when 5 million to 8 million people perished. The catastrophic epidemics that began in 1545 and 1576 subsequently killed an additional 7 million to 17 million people in the highlands of Mexico (1-3). Recent epidemiologic research suggests that the events in 1545 and 1576, associated with a high death rate and referred to as cocoliztli (Nahuatl for "pest"), may have been due to indigenous hemorrhagic fevers (4,5). Tree-ring evidence, allowing reconstructions of the levels of precipitation, indicate that the worst drought to afflict North America in the past 500 years also occurred in the mid-16th century, when severe drought extended at times from Mexico to the boreal forest and from the Pacific to Atlantic coasts (6). These droughts appear to have interacted with ecologic and sociologic conditions, magnifying the human impact of infectious disease in 16th-century Mexico.

The epidemic of cocoliztli from1545 to 1548 killed an estimated 5 million to 15 million people, or up to 80% of the native population of Mexico (Figure 1). In absolute and relative terms the 1545 epidemic was one of the worst demographic catastrophes in human history, approaching even the Black Death of bubonic plague, which killed approximately 25 million in western Europe from 1347 to 1351 or about 50% of the regional population.

The cocoliztli epidemic from 1576 to 1578 killed an additional 2 to 2.5 million people, or about 50% of the remaining native population. Newly introduced European and African diseases such as smallpox, measles, and typhus have long been the suspected cause of the population collapse in both 1545 and 1576 because both epidemics preferentially killed native people. But careful reanalysis of the 1545 and 1576 epidemics now indicates that they were probably hemorrhagic fevers, likely caused by an indigenous virus and carried by a rodent host. These infections appear to have been aggravated by the extreme climatic conditions of the time and by the poor living conditions and harsh treatment of the native people under the encomienda system of New Spain. The Mexican natives in the encomienda system were treated as virtual slaves, were poorly fed and clothed, and were greatly overworked as farm and mine laborers. This harsh treatment appears to have left them particularly vulnerable to epidemic disease.

Cocoliztli was a swift and highly lethal disease. Francisco Hernandez, the Proto-Medico of New Spain, former personal physician of King Phillip II and one of the most qualified physicians of the day, witnessed the symptoms of the 1576 cocoliztli infections. Hernandez described the gruesome cocoliztli symptoms with clinical accuracy (4,5). The symptoms included high fever, severe headache, vertigo, black tongue, dark urine, dysentery, severe abdominal and thoracic pain, large nodules behind the ears that often invaded the neck and face, acute neurologic disorders, and profuse bleeding from the nose, eyes, and mouth with death frequently occurring in 3 to 4 days. These symptoms are not consistent with known European or African diseases present in Mexico during the 16th century.

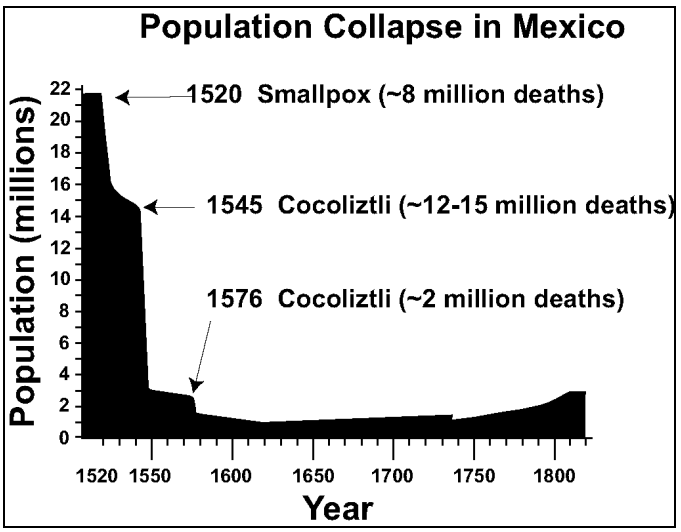

Figure 1. The 16th-century population collapse in Mexico, based on estimates of Cook and Simpson (1). The 1545 and 1576 cocoliztli epidemics appear to have been hemorrhagic fevers caused by an indigenous viral agent and aggravated by unusual climatic conditions. The Mexican population did not recover to pre-Hispanic levels until the 20th century.

*Universidad Nacional Autonoma de Mexico, Ciudad Universitaria, Mexico and † University of Arkansas, Fayetteville, Arkansas, USA

The geography of the 16th century cocoliztli epidemics supports the notion that they may have been indigenous fevers carried by rodents or other hosts native to the highlands of Mexico. In 1545 the epidemic affected the northern and central high valleys of Mexico and ended in Chiapas and Guatemala (4). In both the 1545 and 1576 epidemics, the infections were largely absent from the warm, low-lying coastal plains on the Gulf of Mexico and Pacific coasts (4). This geography of disease is not consistent with the introduction of an Old World virus to Mexico, which should have affected both coastal and highland populations.

Tree-ring evidence, reconstructed rainfall over Durango, Mexico during the 16th century (6), adds support to the hypothesis that unusual climatic conditions may have interacted with host-population dynamics and the cocoliztli virus to aggravate the epidemics of 1545 and 1576. The tree-ring data indicate that both epidemics occurred during the 16th century megadrought, the most severe and sustained drought to impact north central Mexico in the past 600 years (Figure 2; [7]). The scenario for the climatic, ecologic, and sociologic mediation of the 16th-century cocoliztli epidemics is reminiscent of the rodent population dynamics involved in the outbreak of hantavirus pulmonary syndrome caused by *Sin Nombre Virus* on the Colorado Plateau in 1993 (8,9). Cocoliztli was not pulmonary and may not have been a hantavirus but may have been spread by a rodent host. If true, then the prolonged drought before the 16th-century epidemics would have reduced the available water and food resources. The animal hosts would then tend to concentrate around the remnants of the resource base, where heightened aggressiveness would favor a spread of the viral agent among this residual rodent population. Following improved climatic conditions, the rodents may have invaded both farm fields and homes, where people were infected through aspiration of excreta, thereby initiating the cocoliztli epidemic. The native people of Mexico may have been preferentially infected because they worked the agricultural fields and facilities that were presumably infested with infected rodents.

Ten lesser epidemics of cocoliztli began in the years 1559, 1566, 1587, 1592, 1601, 1604, 1606, 1613, 1624, and 1642 (10). Nine of them began in years in which the tree-ring reconstructions of precipitation indicate winter-spring (November-March) and early summer (May-June) drought (8). But the worst epidemic of cocoliztli ever witnessed, 1545-1548, actually began during a brief wet episode within the era of prolonged drought (Figure 3). This pattern of drought followed by wetness associated with the 1545 epidemic is very similar to the dry-then-wet conditions associated with the hantavirus outbreak in 1993 (Figure 3; [9]), when abundant rains after a long drought resulted in a tenfold increase in local deer mouse populations. Wet conditions during the year of epidemic outbreak in both 1545 and 1993 may have led to improved ecologic conditions and may also have resulted in a proliferation of rodents across the landscape and aggravated the cocoliztli epidemic of 1545-1548.

The disease described by Dr. Hernandez in 1576 is difficult to link to any specific etiologic agent or disease known today. Some aspects of cocoliztli epidemiology suggest that a native

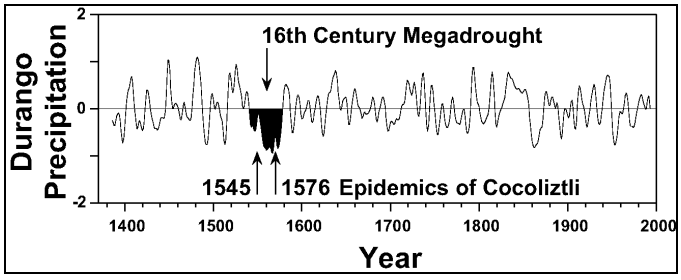

Figure 2. Winter-spring precipitation reconstructed from tree ring data, Durango, Mexico (normalized and smoothed to highlight decennial variability). The tree-ring estimates explain 56% of the variance in precipitation for Durango and are consistent with independent precipitation data. This reconstruction is well correlated with the all-Mexico rainfall index ($r = 0.76$; $p < 0.001$) and with precipitation over north central Mexico, where the cocoliztli epidemics appear to have been most severe. Note the unprecedented 16th-century megadrought during both cocoliztli epidemics.

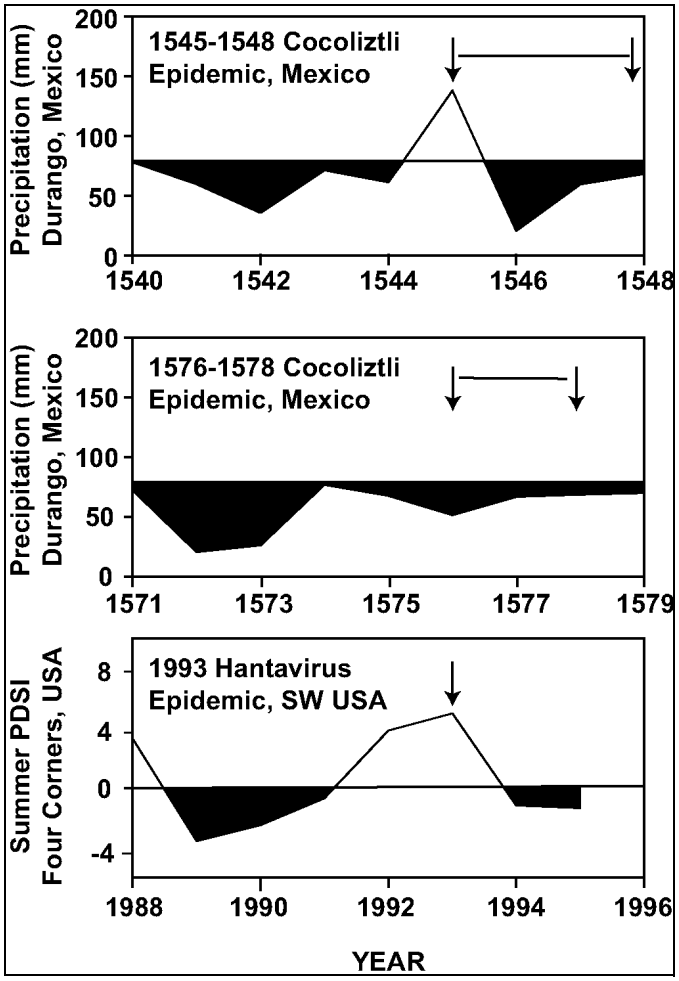

Figure 3. The winter-spring precipitation totals estimated for each year in Durango, 1540–1548 (top), 1571–1579 (middle), compared with the Palmer drought index, southwestern USA 1988–1995 (bottom). A tenfold increase in deer mice was witnessed in the southwestern USA during the 1993 outbreak, a year of abundant precipitation following a prolonged drought. The similar dry-wet pattern reconstructed for the 1545 epidemic of cocoliztli may have impacted the population dynamics of the suspected rodent host to aggravate the epidemic.

agent hosted in a rain-sensitive rodent reservoir was responsible for the disease. Many of the symptoms described by Dr. Hernandez occur to a degree in infections by rodent-borne South American arenaviruses, but no arenavirus has been positively identified in Mexico. Hantavirus is a less likely candidate for cocoliztli because epidemics of severe hantavirus hemorrhagic fevers with high death rates are unknown in the New World. The hypothesized viral agent responsible for cocoloztli remains to be identified, but several new arenaviruses and hantaviruses have recently been isolated from the Americas and perhaps more remain to be discovered (11). If not extinct, the microorganism that caused cocoliztli may remain hidden in the highlands of Mexico and under favorable climatic conditions could reappear.

Acknowledgments

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Dr. Acuna-Soto is a professor of epidemiology on the Faculty of Medicine at Universidad Nacional Autonoma de Mexico. He is particularly interested in the history and environmental context of disease in Mexico.

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Address for correspondence: David Stahle, Tree-Ring Laboratory, Department of Geosciences, Ozark Hall 113, University of Arkansas, Fayetteville, AR 72701 USA; fax: 479-575-3846; e-mail address: dstahle@mail.uark.edu

Sampling Bias in the Molecular Epidemiology of Tuberculosis

Megan Murray*

Among the goals of the molecular epidemiology of infectious disease are to quantify the extent of ongoing transmission of infectious agents and to identify host- and strain-specific risk factors for disease spread. I demonstrate the potential bias in estimates of recent transmission and the impact of risk factors for clustering by using computer simulations to reconstruct populations of tuberculosis patients and sample from them. The bias consistently results in underestimating recent transmission and the impact of risk factors for recent transmission.

Molecular epidemiology makes use of the genetic diversity within strains of infectious organisms to track the transmission of these organisms in human populations. It is used extensively to differentiate reactivation tuberculosis (TB), which is due to a remote infection, from disease caused by recently transmitted organisms. This approach is based on the concept that epidemiologically related organisms share similar or identical genetic fingerprints, while unrelated organisms differ at some genetic loci. Isolates of *Mycobacterium tuberculosis* that occur in clusters sharing similar fingerprints are thought to be caused by recently transmitted infection; those with unique fingerprints are thought to result from distantly acquired infection. Since the extent of recent transmission of an infectious disease often directly reflects the success of control measures (1,2), accurately assessing this quantity is of considerable public health importance.

In addition to distinguishing primary TB from reactivation disease, these molecular techniques have been used to identify risk factors for recent transmission in population-based epidemiologic studies (3). The goals of these investigations have been both to quantify the extent of ongoing transmission of *M. tuberculosis* and to identify host- and strain-specific risk factors for disease spread. Typically, these researchers have studied a specific population at risk for the disease by enrolling a cohort of persons with incident clinical TB, assessing these patients' individual risk factors, and fingerprinting the TB isolates obtained from them (4-11). TB cases are then categorized as either clustered or unique; a cluster is usually defined as two or more patients whose isolates share an identical or near-identical DNA fingerprint, while unique cases are those with unmatched patterns (12). Clustered cases are assumed to share fingerprints as a result of recent spread of the organism among those in the cluster, while cases with unique patterns are assumed to be TB resulting from reactivated latent infection. These studies usually report the proportion of cases that are clustered within the cohort and use this result to infer the relative proportions of clustered and unclustered cases in the community from which the cohort was drawn.

Two different methods have been used to estimate the proportion of clustered cases. The first method, usually referred to as the "n" method, uses the number of all cases that fall into clusters as the estimator of clustered cases. The "n minus one" method assumes that one case per cluster is a case of reactivation TB and thus removes one case per cluster from the counts of "clustered" cases. The "n minus one" approach gives a number of clustered cases that is always less than that calculated by the "n" approach. Covariates associated with clustered fingerprints are taken to be host-specific risk factors for recent transmission of *M. tuberculosis*. The identification of these risk factors may provide specific targets for interventions designed to interrupt disease transmission.

These population-based molecular studies are often based on random or convenience samples drawn from available clinical isolates of *M. tuberculosis*. Implicit in the "population-based" approach to molecular epidemiology is the assumption that the results of studies based on these samples are reliable estimates of the parameters of interest in the population from which the sample was drawn. The criteria by which an estimate is judged to be reliable require that it be precise and unbiased, or, in other words, free from both major random and systematic error (13). Small samples usually render parameter estimates imprecise, or more vulnerable to the effects of chance, but do not specifically cause them to be systematically biased. When the parameter in question is a measure of clustering, however, the correct classification of each clustered case depends on other cases that share identical fingerprints being included in the sample. If these cases are not included because the sample is too small, clustered cases will be misclassified as unique and the resulting proportion of clustered cases will be underestimated. This, in turn, results in underestimation of the extent of recent transmission and overestimation of the extent of reactivation TB, as well as biased estimation of the effects of risk factors for transmission.

The magnitude of the bias incurred by sampling strategies depends both on the sampling fraction and the frequency distribution of sizes of clusters in the population. A recent simulation study of the influence of sampling on estimates of recent TB transmission demonstrated that an increase in sampling fraction yields an increase in the proportion of isolates identified as clustered (14). These simulations further showed that underestimation of clustering is more marked in populations of

*Harvard School of Public Health, Boston, Massachusetts, USA

isolates that include small clusters than those within which large clusters predominate. For this study, I extended this approach by using analytic methods in addition to simulations to estimate the magnitude of the bias introduced by commonly used sampling strategies in assessing the relative proportion of clustered and unclustered cases and in estimating the relative effect of potential risk factors for recent transmission.

Methods

The purpose of this study is to investigate biases inherent in estimating measures of clustering and risk factors for clustering when common sampling strategies are used to collect the empirical data. Since the true distributions of cluster sizes cannot be directly observed if sampling is not complete, I used a Monte Carlo simulation model to generate a variety of hypothetical cluster distributions based on simple assumptions about TB transmission. These distributions represent a wide range of potential data structures reflecting heterogeneous transmission parameters, contact networks, and sociodemographic variables. Accordingly, my aim here is not to model TB transmission dynamics with precision but to generate a collection of heterogeneous cluster distributions that could be used to demonstrate the effects of sampling, given a variety of potential transmission settings.

Generally, the microsimulation model enumerates a population of discrete individuals, each of whom is characterized by a vector of variables that affect risk for TB infection, for clinical disease, and for transmitting infection once infected. Persons are assigned to a series of social and physical spaces such as households, neighborhoods, and multineighborhood communities. The model also specifies the stochastic processes by which latent disease reactivates, infection progresses to primary TB, immunity is conferred by vaccination or by previous infection, and duration of disease is determined. Persons to whom disease is transmitted during the simulation acquire a variable reflecting the strain number of the source of their infection; thus, chains of disease transmission can be identified as "clusters" of cases sharing a specific strain number. The model is run over a time period during which these stochastic processes may occur. Output of the model includes standard measures of the incidence of infection and disease, the prevalence of infectious TB over time, and a count of cluster sizes. Five different cluster distributions were generated on the basis of running the model for 4 years with input variables specific to the different geographic and social settings in which TB is transmitted. The assumptions and baseline input variables for the model have been described (15).

Estimation of Bias in Proportions of Unique and Clustered Cases

The proportion of unique cases calculated after sampling and the variance of that proportion were estimated as follows. Using the "n" method to estimate the proportion of clustered cases, we assume that the true set of isolates is composed of n_k clusters of size k for $k = 1,2,\ldots,k_{\max}$. Further, we assume that each subject in the true set of isolates is sampled independently with a common sampling probability p.

Let I_{ijk} be the indicator of whether the i^{th} subject $i = 1,\ldots,k$ from the cluster, $j=1,\ldots, n_k$, of size k has been sampled. Under our assumptions, the I_{ijk} are i.i.d. *Bernoulli* (p) random variables. The total number of subjects sampled is

$$N = \sum_{k=1}^{k_{\max}} \sum_{j=1}^{n_k} \sum_{i=1}^{k} I_{ijk} \quad .$$

Therefore, the expected value of the number of isolates is

$$E(N) = \sum_{k=1}^{k_{\max}} n_k kp \quad .$$

The variance of N is $var(N) = \left[\sum_{k=1}^{k_{\max}} n_k k \right] p(1-p).$

Now let $U_{jk} = 1$ if the number of isolates sampled from the j^{th} cluster of size k is precisely 1 and $U_{jk} = 0$ if otherwise. Then the total number of unique isolates is

$$U = \sum_{k=1}^{k_{\max}} \sum_{j=1}^{n_k} U_{jk} \quad .$$

Now U_{jk} is a Bernoulli random variable with success probability $kp(1-p)^{k-1}$ equal to the probability of choosing exactly one member from the j^{th} cluster of size k. Hence,

$$E(U) = \sum_{k=1}^{k_{\max}} n_k kp(1-p)^{k-1}$$

$$var(U) = \sum_{k=1}^{k_{\max}} n_k kp(1-p)^{k-1}(1-pk(1-p)^{k-1})$$

The expectation and large sample variance of the random variable (U/N) are derived in Appendix 1. Using these formulae for $E(U/N)$ and $var(U/N)$, estimates of the biased results for a range of sampling fractions were calculated for each of the five transmission scenarios described above. The results of this analysis were verified against a computer simulation that counted cluster sizes after random draws without replacing a proportion p of the true populations of isolates. For each true data set, the simulated data collection process was repeated 1,000 times. The mean value of the estimates obtained is reported, in addition to simulated confidence intervals expressed as values that represented the 0.05th and 0.95th largest estimates. The variance of the empirical distribution for the set of 1,000 simulations was nearly identical to that obtained by the large sample variance formula for a ratio expressed above.

These simulations were repeated using the "n minus one" approach, in which one case per cluster is removed from the count of clustered cases and added to the count of

reactivation cases. The analytic solution follows the same logic (Appendix 2).

Estimation of Bias in the Relative Risks and Odds Ratios of Risk Factors for Recent Transmission

The magnitude of bias in the odds ratios of potential risk factors introduced by the misclassification of clustering due to sampling error was also assessed. Risk factors for clustering were postulated to which were assigned "true" odds ratios of 2, 5, and 10. The prevalence of these risk factors in the absence of clustering was set at 0.1. This exposure was thus randomly assigned to 10% of the unclustered cases and proportions of the clustered cases to obtain the specified odds ratios in each of the modeled data sets. The odds ratios were recalculated after sampling by moving the clustered cases that were sampled as unique from the category of recently transmitted cases to the category of reactivated cases and reassessing the respective exposure status for these outcomes.

Results

Bias in Estimates of Proportion of Unique and Clustered Cases

Output from the transmission model (Table 1) includes estimates of the incidence of TB infection and clinical TB disease, as well as a summary of the frequency distribution of cluster size for each scenario. The proportion of unique isolates that would be observed after sampling a given fraction of the isolates in the complete data sets for each of the five scenarios was estimated analytically and verified by computer simulation. These methods produced nearly identical results, demonstrating that there is often substantial bias in the estimated proportions of unique and clustered cases of TB when sampling is based on sampling fractions consistent with those used in common epidemiologic practice. Table 2 summarizes the error in the measurement of the proportion of unique and clustered cases of TB introduced by sampling various fractions of the data from the range of transmission scenarios. These results are given for both the "n" and "n minus one" methods of counting clustered cases; they show that the error in these estimates depends on both the "true" transmission pattern and the fraction of the total data sampled. Transmission scenarios in which there is a higher "true" proportion of unique cases and those in which transmission is concentrated in large clusters tend to demonstrate less error than those in which there are fewer unique cases and more small clusters. In all cases in which the estimate is biased, the estimated proportion of unique cases is an overestimate of the true value, indicating that the error in these estimates tends to inflate the proportion of TB cases due to reactivation and minimize the proportion due to recent transmission. In many simulations, all of the 1,000 estimates obtained were less than the true proportion.

Bias in Odds Ratios for Risk Factors for Clustering

The bias in the proportions of clustered and unclustered cases results from misclassification of cluster status due to inadequate sampling; this misclassification also biases the results of analyses of risk factors for recent transmission in the direction of the null hypothesis of no effect. Table 2 also presents estimates of the odds ratios for the effect of a range of hypothetical risk factors for recent transmission. These results show that the odds ratios of a risk factor for clustering are markedly underestimated in the transmission scenarios in which there are lower proportions of unique cases or in which smaller cluster predominate. This bias is especially marked when odds ratios are high; in the worst-case scenario described in Table 2, an odds ratio of 10 could be estimated as 1.58 when only 10% of the isolates are sampled.

Discussion

The recent development of molecular methods to accurately type infectious organisms has led to a marked proliferation in studies of the molecular epidemiology of infectious diseases, especially of TB. The goals of many of these studies have been to address the longstanding problem of assessing the relative proportions of incident TB cases due to recent transmission and to chronic or reactivated disease and to identify risk factors for recent transmission. A systematic bias that consistently underestimates the proportion of cases due to recent transmission could present a serious impediment to the

Table 1. Model-based output statistics from a microsimulation of tuberculosis transmission

	High burden		Moderate burden		Low burden
Output statistics	Sudan	NY prison	Algeria	US prison	Netherlands
Tuberculosis incidence[a]	190	581	32	82	14
Consensus incidence estimates	200	NA[b]	44	NA[b]	10
ARI[c]	0.025	0.046	0.003	0.005	0.001
Maximum cluster size	87	19	9	17	15
Mean cluster size	10.2	3.2	1.7	2.9	1.7
Proportion of unique isolates	0.181	0.253	0.432	0.289	0.490

[a]Incidence per 100,000. Consensus incidence estimates are shown for comparison with estimates obtained from the model.
[b]No data available.
[c]ARI = Annual risk of infection.

Table 2. Monte Carlo means and 95% ranges for the proportion of unique isolates and for odds ratios after sampling a fraction of the complete data set.

	Sampling fraction			
Country/Specific Statistics	1	0.7	0.5	0.1
Sudan				
Proportion of reactivated isolates				
n method	0.18	0.19 (0.18-0.19)	0.21 (0.19-0.23)	0.37 (0.30-0.43)
"n minus one" method	0.28	0.32 (0.30-0.34)	0.35 (0.30-0.41)	0.54 (0.47-0.62)
Odds ratios[a]	2	1.88 (1.87-1.97)	1.77 (1.66-1.88)	1.34 (1.28-1.45)
	5	4.18 (4.13-4.78)	3.51 (3.01-4.18)	1.84 (1.60-2.18)
	10	7.52 (7.38-9.27)	1.84 (1.67-1.84)	2.37 (2.08-2.99)
New York prisons				
Proportion of reactivated isolates				
n method	0.12	0.14 (0.12-0.16)	0.16 (0.13-0.20)	0.45 (0.28-0.62)
"n minus one" method	0.33	0.36 (0.33-0.38)	0.39 (0.32-0.45)	0.67 (0.61-0.73)
Odds ratios	2	1.77 (1.60-1.95)	1.62 (1.45-1.83)	1.16 (1.12-1.29)
	5	3.51 (2.73-4.58)	2.78 (2.18-3.83)	1.37 (1.25-1.37)
	10	5.79 (4.07-8.66)	4.17 (2.99-6.58)	1.58 (1.39-2.12)
Algeria				
Proportion of reactivated isolates				
n method	0.43	0.48 (0.45-0.51)	0.16 (0.13-0.20)	0.45 (0.28-0.62)
"n minus one" method	0.65	0.71 (0.69-0.74)	0.76 (0.69-0.83)	0.92 (0.81-0.99)
Odds ratios	2	1.81 (1.75-1.92)	1.67(1.45-1.97)	1.29 (1.18-1.73)
	5	3.68 (2.79-4.82)	3.02 (2.99-4.73)	1.98 (1.77-2.33)
	10	6.58 (5.55-8.23)	5.05 (4.17-6.58)	2.62 (2.26-3.28)
U.S. prisons				
Proportion of reactivated isolates				
n method	0.29	0.33 (0.29-0.39)	0.37 (0.29-0.48)	0.68 (0.35-1.00)
"n minus one" method	0.33	0.35 (0.31-0.38)	0.37 (0.32-0.41)	0.62 (0.50-0.73)
Odds ratios	2	1.8 (1.62-1.98)	1.67 (1.45-1.97)	1.29 (1.18-1.73)
	5	3.68 (2.79-4.82)	3.02 (2.99-4.73)	2.11 (1.62-5.31)
	10	6.86 (5.30-9.66))	4.67 (3.02-9.13)	2.11 (1.62-5.31)
Netherlands				
Proportion of reactivated isolates				
n method	0.49	0.62 (0.55-0.69)	0.62 (0.55-0.69)	0.89 (0.77-1.00)
"n minus one" method	0.65	0.78 (0.72-0.85)	0.78 (0.72-0.85)	0.93 (0.79-1.00)
Odds ratios	2	1.8 (1.62-1.98)	1.67 (1.57-1.81)	1.40 (1.31-1.49)
	5	3.68 (2.79-4.82)	3.05 (2.63-3.78)	2.02 (1.79-2.32)
	10	6.86 (5.30-9.66))	4.76 (3.86-6.47)	3.86 (2.39-3.25)

[a]Confidence intervals for odds ratios are based on the results of 2 by 2 tables, with data adjusted for the mean misclassification introduced by sampling.

constructive use of molecular typing techniques for studying the epidemiology of infectious disease.

The results of this study show the extent to which bias can be introduced by sampling strategies commonly used in the molecular epidemiology of TB. Depending on the underlying distribution of cluster sizes, the error involved in underestimating the proportion of unique TB isolates in a sample may be sizable, even when up to 70% of the complete data is sampled. The odds ratios for risk factors for clustering are also consistently and markedly underestimated with this approach. The findings of this study support the conclusions of previous investigators (14) who have shown that the extent of error in

these estimates is a function of both sampling fraction and underlying cluster distribution in the complete data sets. These results imply that reasonable predictions of the extent of error can be made, given knowledge of both the true distribution of cluster sizes in the population of persons with TB and the size of the population of TB patients from which the sample was drawn. Although the true distribution of cluster sizes cannot be observed in the absence of complete sampling, epidemic models such as this one may elucidate factors that contribute to these distributions and help investigators arrive at prior expectations of cluster distributions in the specific transmission scenarios under study.

I considered how much impact this kind of sampling bias might have had on the studies of the molecular epidemiology of TB published to date. Many researchers report on a convenience sample of cases drawn from one or more clinical sites, without providing an estimate of the number of incident cases in the area in question during the period in which the cases were collected (16-19). In areas with high TB prevalence, the number of cases in these series is often <1% of the number of cases expected in that region on the basis of national reporting or World Health Organization predictions. These results suggest that the bias expected in these studies is so extreme that the findings are useful only as lower bounds for the proportion of recently transmitted cases and for risk factors for recent transmission. Nonetheless, lower bounds may be informative in situations in which undetected transmission is incorrectly attributed to reactivation disease alone or when a new risk factor for transmission is identified.

In industrialized countries with lower rates of incident TB, researchers have tried to enroll a compete cohort of patients by making use of public health reporting systems to identify and fingerprint all new cases of clinical TB in a defined geographic region during a specified time period (4,5,20-22). Although this approach leads to much more complete and systematic sampling, it may not always ensure that the resulting estimates are free from bias. For these series of cases to be complete samples, one would have to assume that none of the cases in the sample had transmission links to cases that did not appear in the study population or were reported before the onset of the study. Furthermore, the most rigorously documented TB fingerprinting studies have reported 15%-40% loss of data as a result of difficulties in culturing, fingerprinting, and interpreting fingerprint patterns (4,5,20-22). Even if the patients excluded from these studies resemble those retained in every other respect, their exclusion will result in a biased outcome of the study.

The "complete" data sets used to estimate bias in this study were generated through stochastic epidemic modeling that outputs cluster distributions in addition to estimates of the incidence of TB infection and disease. Multiple demographic and disease-specific parameters have been found to affect cluster distributions, and many potential "transmission scenarios" could be generated by varying these parameters. In addition, the length of the study period and the stability of the molecular markers used will impact the observed patterns of clustering (23,24). Given that true cluster distributions cannot be known in the absence of complete sampling, the model cannot be validated by using it to derive known cluster distributions. Since the purpose of this study is to explore the bias in measures commonly used in empirical studies of molecular epidemiology, sets of parameters were chosen from a variety of specific areas in which the burden of TB disease has been described or projected based on the information currently available. Although the true transmission patterns in any particular population may be inadequately captured by the epidemic model used, these results do provide some perspective on the potential misinterpretation of molecular data on TB. The simulations may also differ from data sets obtained in the field in that sampling was random and the very real problem of selection bias in the collection of isolates was not addressed. Finally, in the assessment of the bias in the estimates of the effect of risk factors for clustering, I assigned risk status randomly within groups of clustered and unique cases. If cluster size were correlated with a risk factor for clustering, so that, for example, incarceration was more common among cases in large clusters than small ones, the bias in the odds ratio of incarceration would be less than the estimates reported here.

These results demonstrate that estimates of clustering based on molecular fingerprinting of a population of isolates of infectious agents may be severely biased. When these methods are used to estimate the extent of primary and reactivation disease in a community, they consistently underestimate recent transmission. In circumstances in which the error is greatest, the bias may undermine the value of an investigation by providing a community with false reassurance that ongoing transmission is being curtailed and therefore that control measures are adequate.

The findings of this study further suggest that molecular methods in epidemiology require the development of both appropriate epidemiologic study design and analytic tools to yield meaningful assessments of disease transmission. In particular, they imply that estimates of recent transmission obtained by molecular methods cannot be compared across studies which have used different sampling fractions and in which the distribution of cluster size can reasonably be expected to vary. One way for molecular epidemiologists to approach this problem is to provide sensitivity analyses estimating the potential error involved, given prior expectations of cluster distributions and an estimate of the fraction of cases sampled in a particular study. The analytic solution presented here can be easily programmed and used to explore the range of potential error under a variety of hypothetical transmission scenarios.

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Dr. Murray is an assistant professor of epidemiology at the Harvard Medical School and an infectious disease clinician at the Massachusetts General Hospital. Her research interests include epidemiologic methods, the molecular epidemiology of tuberculosis, and the transmission dynamics of infectious diseases.

Appendix 1

We wish to derive the expectation and variance of the random variable *U/N,* denoting the proportion of all sampled isolates that form a unique cluster of size 1 in the sample. In large samples, the mean of *U/N* is approximately the ratio of the mean of *U* to the mean of *N*:

$$\mathrm{E}\left[U/N \cong E(U)/E(N)\right.$$

and the variance of *U/N* is approximated by the large sample variance formula for a ratio.

$$var(U/N) \cong \frac{\mathrm{var}(U)}{\{E(N)\}^2} + \frac{\{E(U)\}^2\,\mathrm{var}(N)}{\{E(N)\}^4} - \frac{2E(U)\,\mathrm{cov}(U,N)}{\{E(N)\}^3}$$

It only remains to evaluate *cov*(*U,N*), which is done in the following lemma.

Lemma: Under our assumptions,

$$\mathrm{cov(U,N)} = \sum_{k=1}^{k_{\max}} n_k \{1 - n_k kp\} kp(1-p)^{k-1}$$

Proof: by independence, $cov(U,N) = \sum_{k=1}^{k_{\max}} \sum_{j=1}^{n_k} \mathrm{cov}(U_{jk}, N_{jk})$

where $N_{jk} = \sum_{i=1}^{k} I_{ijk}$. Now, $cov(U_{jk},N_{jk}) = E(U_{jk}, N_{jk}) - E(U_{jk})\text{-}E(N_{jk}) = E(U_{jk})\text{-} E(U_{jk})\text{-}E(N_{jk}) = E(U_{jk})[1\text{-} E(N_{jk})]$ where $E(U_{jk}) = kp(1\text{-}p)^{k-1}$ and $E(N_{jk}) = n_k p$. The result then follows.

Appendix 2

The bias in the proportion of reactivated cases after sampling when the clustered cases are counted by using the "n minus one" method is described below. The number of cases considered to be due to reactivation is the sum of the unique cases and the source cases. The "true" number of source cases is equal to the number of clusters in the complete data set,

$$\sum_{k=2}^{k_{\max}} n_k \quad .$$

We are interested in finding the number of source cases after sampling. Since the number of source cases in a sample is equal to the "true" number of source cases minus the source cases that are not sampled or are sampled as unique, we need to estimate the expected value of the numbers of clusters not sampled and the expected value of the clusters sampled as unique. Let E(CL0) and E(CL1) be the expected values of the numbers of clustered not sampled or sampled as unique, respectively. Then, by using the nomenclature defined in the text and following the logic there described:

$$\mathrm{E(CL0)} = \sum_{k=2}^{k_{\max}} n_k (1-p)^k \quad \text{and} \quad \mathrm{E(CL1)} = \sum_{k=2}^{k_{\max}} n_k k (1-p)^{k-1}$$

The expected number of source cases after sampling a fraction *p* of the complete set of isolates is equal to

$$\sum_{k=2}^{k_{\max}} n_k \quad \sum_{k=2}^{k_{\max}} n_k (1-p)^k \quad \sum_{k=2}^{k_{\max}} n_k k (1-p)^{k-1}$$

The overall estimate of the proportion of reactivated cases can then be obtained by summing the number of unique cases after sampling with the number of source cases and dividing by the expected number of sampled isolates, *p*(N).

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Address for correspondence: Megan Murray, Department of Epidemiology, Harvard School of Public Health, 677 Huntington Ave., Boston, MA 02115, USA; fax: 617-566-7805; e-mail: mmurray@hsph.harvard.edu

A Waterborne Outbreak of *Escherichia coli* O157:H7 Infections and Hemolytic Uremic Syndrome: Implications for Rural Water Systems[1]

Sonja J. Olsen,* Gayle Miller,† Thomas Breuer,* Malinda Kennedy,* Charles Higgins,‡ Jim Walford,† Gary McKee,† Kim Fox,# William Bibb,* and Paul Mead*

In the summer of 1998, a large outbreak of *Escherichia coli* O157:H7 infections occurred in Alpine, Wyoming. We identified 157 ill persons; stool from 71 (45%) yielded *E. coli* O157:H7. In two cohort studies, illness was significantly associated with drinking municipal water (town residents: adjusted odds ratio=10.1, 95% confidence intervals [CI]=1.8-56.4; visitors attending family reunion: relative risk=9.0, 95% CI=1.3-63.3). The unchlorinated water supply had microbiologic evidence of fecal organisms and the potential for chronic contamination with surface water. Among persons exposed to water, the attack rate was significantly lower in town residents than in visitors (23% vs. 50%, p<0.01) and decreased with increasing age. The lower attack rate among exposed residents, especially adults, is consistent with the acquisition of partial immunity following long-term exposure. Serologic data, although limited, may support this finding. Contamination of small, unprotected water systems may be an increasing public health risk.

Escherichia coli O157:H7 is now a well-recognized cause of human illness. Although outbreaks of *E. coli* O157:H7 infections are frequently associated with food or milk derived from cattle, other sources, including fresh fruits and vegetables and water, have been implicated (1). In the United States, the first reported drinking water outbreak of *E. coli* O157:H7 infections occurred in 1989 in rural Missouri (2). Since this outbreak, six others have been associated with drinking water. Three were small and occurred in a camp, a recreational vehicle park, and a well (Centers for Disease Control and Prevention [CDC], unpub. data). More recently, three highly publicized drinking water outbreaks of *E. coli* O157:H7 infections (one each in Wyoming, New York, and Canada), have focused increased attention on the safety of drinking water (3,4). Here we summarize the results of the outbreak investigation in Wyoming.

During late June 1998, physicians near Alpine, Wyoming, noted an increase in bloody diarrhea among town residents. Alpine is a small town (pop. <500) on the Wyoming-Idaho border that is frequented by tourists to Grand Teton and Yellowstone National Parks. By July 9, *E. coli* O157:H7 had been isolated from stool samples from 14 persons, including residents of Wyoming, Utah, and Washington. On July 11, CDC and Wyoming health officials began an investigation to determine the magnitude of the outbreak and the source of *E. coli* O157:H7 infections.

Methods

Case Finding and Hypothesis Generation

To identify patients, Wyoming health officials contacted area physicians and health officials in neighboring states. Interviews identified ill persons in Alpine, as well as ill persons from outside Wyoming who had attended a large family reunion in Alpine June 26-28. Patients were routinely interviewed about a wide variety of potential exposures, and stool samples were collected for laboratory confirmation. Environmental health inspectors in Wyoming conducted an assessment of retail food distribution in Alpine. This information was used to develop questionnaires for the subsequent cohort studies.

Family Reunion Cohort Study

On July 11 and 12, we conducted a cohort study of persons from out-of-state families who had attended the family reunion in Alpine from June 26 to 28. Using a questionnaire administered by telephone, we asked reunion attendees about illness and exposure to certain foods and municipal water during their stay in Alpine. All family members were questioned individually except children too young to be interviewed; parents responded for these children. A case was defined as diarrhea

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Wyoming Department of Health, Cheyenne, Wyoming, USA; ‡Wyoming Department of Agriculture, Cheyenne, Wyoming, USA; and #United States Environmental Protection Agency, Cincinnati, Ohio, USA

[1]This information was presented at the 36th Annual Meeting of the Infectious Disease Society of America, Denver, CO, November 1998 (Abstract #782).

(≥3 loose stools in a 24-hour period) with onset after June 26 in a person attending the family reunion.

Alpine Cohort Study

To confirm the results of the smaller family reunion cohort and to estimate the attack rate in the community, we conducted a cohort study of all residents of Alpine during July 13-16. Using a questionnaire administered by telephone, we asked residents about recent gastrointestinal symptoms and exposure to various meats and Alpine municipal water during the 7-day period June 25 to July 1. We collected information on consumption of tap water or beverages made with tap water, the average number of glasses drunk in the week, and water filtration practices. Telephone numbers were called at least twice (once during the day and once at night) before being deemed a "no answer." A case was defined as a) a stool culture yielding *E. coli* O157:H7, or b) diarrhea (≥3 stools in a 24-hour period) with onset after June 25 in an Alpine resident who was in town between June 25 and July 1.

Laboratory Investigation

All stool samples were sent on ice to the Wyoming state laboratory, where they were plated on sorbitol-MacConkey agar (5). Samples producing sorbitol-negative colonies were selected and tested for O157 antigen, and positive samples were then assayed for H7 antigen by latex agglutination (6). Specimens identified as *E. coli* O157:H7 were sent to the Utah Department of Health State Laboratory for subtyping by pulsed-field gel electrophoresis (PFGE) (7). Additional stool samples were collected serially from day-care attendees, food handlers, and health-care workers whose first stool culture yielded *E. coli* O157:H7; subsequent stool samples were tested for Shiga toxin by using only the Premier EHEC assay (Meridian Diagnostics Inc., Cincinnati, OH) (8). Serum was collected from town residents to test for immunoglobulin (Ig) G and IgM antibodies to O157 lipopolysaccharide by enzyme-linked immunosorbent assay (ELISA) at CDC (9). An antibody >1:320 for IgM or >1:160 for IgG was considered positive.

Environmental Investigation

Representatives from the Wyoming State Department of Agriculture and the U. S. Environmental Protection Agency (EPA) inspected the Alpine municipal water system and reviewed testing and safety measures. On July 14, 1998, water samples were collected from the outlet of the storage tank and several points in the distribution line. Deer and elk fecal samples were collected in the area above the spring collection system. Water samples were sent overnight to the Cincinnati EPA laboratory and analyzed for bacterial contamination within 24 hours of collection. Water samples were tested for total and fecal coliforms and *E. coli* by using m-ENDO Medium (10). Animal fecal samples were tested for *E. coli* O157:H7 at the Wyoming state laboratory as described above for human samples.

Statistical Analysis

In the univariate analysis, relative risks (RR) and 95% confidence intervals (CI) were computed by using Epi Info (version 6.04, CDC, Stone Mountain, GA). Variables found to be significantly associated with disease at the univariate level were examined in a logistic regression model (LogXact version 2, Cytel Software Corporation, Cambridge, MA). The minimum duration of fecal shedding was estimated as the time between the onset of symptoms to the last positive Shiga toxin result before two negative results. Data for children and adults were examined by using Kaplan-Meier survival analysis; curves were compared by using the log-rank test (SAS version 6.12, SAS Institute Inc., Cary, NC).

Results

Case Finding

We identified 157 ill persons from 15 states. The dates of illness onset ranged from June 25 to July 27 for patients with culture-confirmed infection and June 22 to July 30 for patients with culture-negative stools (Figure 1). Hemolytic uremic syndrome developed in four persons: a 15-month-old girl, a 17-month-old boy, a 4-year-old boy, and a 36-year-old woman. All have since recovered. Fifty-seven isolates were subtyped by PFGE; the patterns were indistinguishable.

Review of initial interviews showed that ill persons reported only infrequent exposure to some food and drink items known to be associated with *E. coli* O157:H7 transmission, including raw milk and apple cider. Wyoming state environmental health inspectors determined that no recently recalled food products, including ground beef, had been shipped to stores in Wyoming. This information was verified by U.S. Food and Drug Administration and U.S. Department of Agriculture officials.

Family Reunion Cohort

Twelve families consisting of 44 persons attended the family reunion in Alpine. Of the 41 (93%) persons interviewed, 12 met the case definition; one patient's stool sample yielded *E. coli* O157:H7. Initial interviews showed that all food and drink items, except water, were purchased out of state.

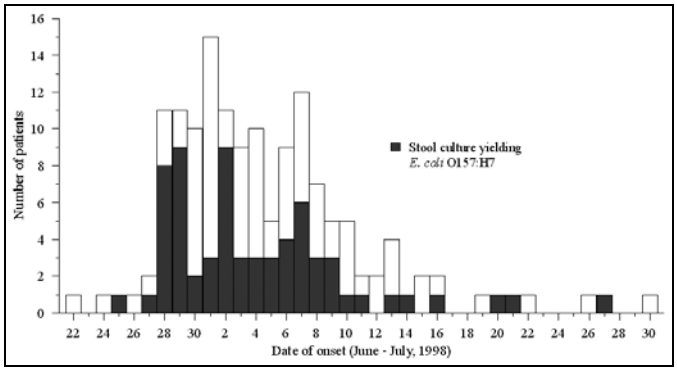

Figure 1. Cases of diarrhea by date of onset and *Escherichia coli* O157:H7 culture status, Alpine, Wyoming, June to July 1998.

The overall attack rate was 29%: 33% among males, 24% among females, and 50% among children <5 years old. The only exposure significantly associated with illness was drinking Alpine municipal water. Persons who drank Alpine municipal water were nine times more likely to become ill than were persons who did not drink the water (95% CI=1.3-63.3). The attack rate among reunion members who drank Alpine municipal water during the weekend was 50%. Attack rates for persons who drank Alpine municipal water did not vary by age (14) (Table 1)

Alpine Cohort

The 1997-98 Alpine telephone directory listed 490 phone numbers with the Alpine prefix; of the 287 household numbers, we reached 146 (51%). Twelve of the households reached were excluded because the family had been out of town during the exposure period, June 25 to July 1. A total of 319 persons were living in the remaining 134 households during the exposure period; 284 (89%) were available for interview. Of the 284 respondents, 139 (49%) were male, and the median age was 39 years (range <1 to 92). For 192 (68%) interviewees, Alpine municipal water was their source of tap water at home.

Fifty-four (19%) persons met the case definition; 18 (33%) had stool samples yielding *E. coli* O157:H7. The attack rate was almost two times higher in women (25%) than in men (13%); however, in the week before illness, women were no more likely than men to drink any or more water.

In univariate analysis, 5 of 16 exposure variables were significantly associated with illness (except when noted, all exposures refer to the period June 25 through July 1): playing in a sprinkler, playing with water guns, being serviced by municipal water at home, drinking municipal water, and drinking municipal water during June 26 to 28 (Table 2). Since drinking Alpine municipal water was a broader exposure than, and not as strong a risk factor for illness as, drinking municipal water during June 26 to 28, it was excluded in multivariate analysis. In the multivariate model, the only variable that remained significantly associated with illness was drinking municipal water on the weekend of June 26 to 28 (Table 3; OR 10.1; 95% CI=1.8-56.4).

Overall, the attack rates among residents who drank Alpine municipal water were 23% during June 25 to July 1 and 27% during the 3-day period of June 26-28. Attack rates among females or males for persons who drank Alpine municipal water did not differ significantly. However, although not significant, the risk for illness decreased with increasing age (Table 1). Among persons who drank municipal water, the average number of glasses of water and the risk for illness were not statistically associated (Kruskal-Wallis test, p=0.7).

Table 1. Attack rates among persons exposed to municipal water, by age group, Alpine, Wyoming, June–July 1998

Age group (years)	Resident cohort N (%)	Family reunion cohort (nonresidents) N (%)
All ages	48/207 (23)	11/22 (50)
≤9	10/30 (33)	3/6 (50)
10-19	7/26 (27)	2/5 (40)
20-39	11/55 (20)	3/5 (60)
40-59	16/63 (25)	3/6 (50)
≥60	4/33 (12)	0/0

Laboratory Investigation

Twenty persons with culture-confirmed infection were followed with serial stool cultures to examine the duration of fecal shedding; 14 were children <10 years old in day care and 6 were adults aged 16 to 60 years (including 4 adult food handlers and 1 health-care worker). The median length of fecal shedding was 9 days among children (range 1 to 35) and 7.5 days among adults (range 2 to 36), a difference that was not statistically significant (p=0.63). Among children <5 years old, the median length of fecal shedding was 9 days (range 1 to 35).

Serum samples were collected from 129 town residents 16 to 27 days after the most likely time of exposure, July 27. Of the 129 persons, 57 (44%) were male, and the mean age was 42 years (range 3 to 78 years). Twenty-six (20%) persons had illness that met the Alpine cohort case definition (all drank Alpine municipal water), and nine of these were either culture positive or had bloody diarrhea. Of the other 103 (80%) well persons, 95 drank municipal water and 8 did not. IgM and IgG geometric mean titers were highest in persons with culture-positive *E. coli* O157:H7 or bloody diarrhea, followed by persons with diarrhea, and then well persons (Figure 2). The geometric mean IgM titer was <1:320 and the IgG titer >1:160 for ill persons. Three of eight well persons who did not drink Alpine municipal water during the outbreak had IgG titers >1:160.

Table 2. Univariate analysis of exposures in Alpine resident cohort study, Alpine, Wyoming, June–July 1998[a]

Selected exposures[a]	Proportion ill		Relative risk (95% CI)
	Exposed	Nonexposed	
Played in sprinkler	10/29	44/254	2.0 (1.1 - 3.5)
Played with water guns	7/19	47/264	2.1 (1.1 - 3.9)
Serviced by municipal water at home	45/192	9/92	2.4 (1.2 - 4.7)
Drank municipal water[a,b]	48/211	3/68	5.2 (1.7 - 16.0)
Drank municipal water June 26-28	48/181	2/62	8.2 (2.1 - 32.8)
Venison consumption	3/13	51/271	1.2 (0.4 - 3.4)
Elk consumption	10/44	44/239	1.2 (0.7 - 2.3)
Jerky consumption	4/25	49/256	0.8 (0.3 - 2.1)
Hamburger consumption	29/147	21/111	1.0 (0.6- 1.7)
Pink hamburger consumption	0/7	27/130	undefined, p=0.2

[a]Except when noted, all exposures refer to the period June 25 through July 1, 1998.
[b]Only 8 (4%) of 211 persons who drank municipal water reported boiling it.
CI = confidence intervals.

Table 3. Multivariate analysis of exposures in Alpine resident cohort study, Alpine, Wyoming, June–July 1998

Exposure	Odds ratio (95% CI)
Played in sprinkler	1.5 (0.6-3.9)
Played with water guns	1.4 (0.5-4.3)
Serviced by municipal water at home	1.2 (0.4-3.8)
Drank municipal water June 26-28	10.1 (1.8-56.4)

CI = confidence intervals.

Environmental Investigation

The Alpine municipal water system was supplied by an underground spring. A series of small boxes connected by perforated pipes 7 to 10 feet below ground level collected water from an unconfined aquifer and routed it to an underground concrete storage tank. Pipes feeding off this tank then delivered unchlorinated water to the town. Sanitary surveys conducted in 1992 and 1997 indicated a potential risk of contamination from wildlife as well as surface water. Although Alpine was in compliance with the Total Coliform Rule, which requires one safe total coliform result each month, there were several positive readings in April 1998 (1/5 positive), May 1998 (4/7 positive), and June 1998 (2/10 positive) just before the outbreak. On inspection after the outbreak, the spring was found to be under the influence of surface water; a large pool of water was found in the area over the water collection pipes, probably the result of a late snow melt combined with heavy rains and ground water outfalls. Numerous deer and elk feces were present in the area; the animals came to the pool to drink. *E. coli* O157:H7 was not isolated from any of the five deer or elk fecal samples taken on July 14. Water taken from the storage tank on July 14 yielded a total coliform count of 108 CFU/100 mL. *Enterococcus faecium* was isolated from the same water sample, indicating fecal contamination. *E. coli* O157:H7 was not isolated.

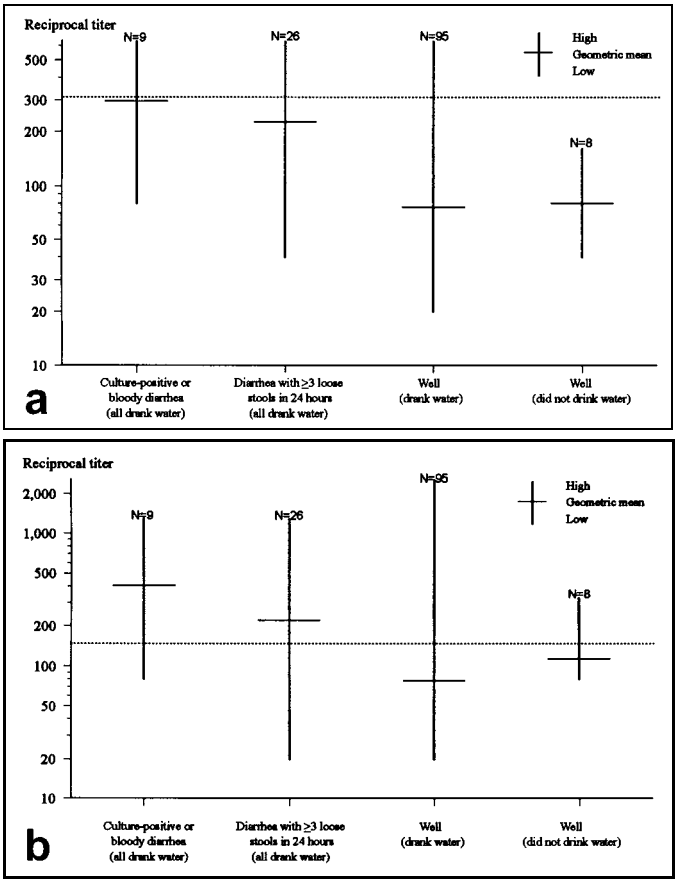

Figure 2. Reciprocal geometric mean and range of (a) immunoglobulin (Ig)M and (b) IgG antibody titers to O157 lipopolysaccharide. The dotted line indicates the positive cutoff (IgM >1:320 and IgG >1:160).

Discussion

In this investigation, we identified more than 150 cases of acute gastrointestinal illness among residents of Alpine and visitors from 14 other states. *E. coli* O157:H7 was confirmed by culture in 71 cases. Four persons, including three children, were hospitalized with hemolytic uremic syndrome; there were no deaths. Illness was significantly associated with drinking unchlorinated water from the Alpine municipal water system in each of two cohort studies.

We believe that the unchlorinated municipal water supply became contaminated when surface water, containing deer and elk feces, leached into the town's unconfined aquifer. Deer and other ruminants can harbor *E. coli* O157:H7 and shed the organism in their feces (11,12). Although EPA did not detect *E. coli* O157:H7 in water from the storage tank several weeks after the outbreak, the analysis did reveal high coliform counts and the presence of *Enterococcus faecium*. *Escherichia coli* O157:H7 has been shown to survive for prolonged periods in water, especially in cold water, by transforming into a viable but nonculturable state (13). In this state the pathogen cannot be isolated by traditional plating methods and therefore may not be detected.

Our serologic findings demonstrate the cross-sectional antibody profile of a population during an outbreak. IgG antibody titers to O157 lipopolysaccharide generally develop within the first week after illness onset and remain elevated for at least 2 months (9), whereas IgM antibody titers appear to increase more rapidly and decline within 8 weeks after illness onset (14). Consistent with a previous study (9), ill persons in our study demonstrated a markedly increased IgG antibody response but not IgM. Although this may reflect the fact that blood was drawn 2 to 4 weeks after exposure, when IgM antibodies may already be decreasing, it more likely reflects the lower specificity of this assay (9). While antibody titers to O157 lipopolysaccharide generally correlate with severity of disease (9), the titers of persons who do not develop clinical illness are thought to be low. Our findings suggest that among well persons, those exposed may have elevated titers compared with those not exposed.

Several lines of evidence suggest that Alpine residents may have been previously exposed to *E. coli* O157:H7 and as a result may have acquired a degree of immunity to symptomatic infection. Although the geometric mean titer of IgG antibodies was not above 1:160, three (38%) of eight well

residents who did not drink municipal water during the current outbreak had titers >1:160. In contrast, studies conducted to establish the specificity of the serologic assay have shown that only 3% to 4% of well persons in the United States have IgG antibodies titers >1:160 (CDC, unpub. data), suggesting a higher than expected baseline rate of seropositivity among Alpine residents. In addition, among persons who were exposed to municipal water on the weekend of June 26 to 28, the attack rate was significantly lower for town residents than for nonresident visitors (Table 1; 27% vs. 50%, $p<0.01$). Further, although not statistically significant, the attack rate among town residents who drank municipal water decreased with increasing age, whereas it was the same at all age groups among visitors, a finding consistent with older resident's having more opportunity for previous exposure to *E. coli* O157:H7. Although these findings suggest previous exposure to *E. coli* O157:H7, these data were based on small sample sizes. The mechanism of previous exposure may have been through episodic contamination of the water supply or eating wild game; 20% of persons interviewed reported having eaten elk, venison, or jerky during the week in question.

The median duration of shedding of *E. coli* O157:H7 by persons in this outbreak was slightly shorter than previously reported (15-18). The difference may be attributable to different methods used to estimate the endpoint of shedding; because stool samples were not collected at regular intervals in our study, we chose a conservative estimate of this endpoint, i.e., the last positive result.

We identified several methodologic limitations in this investigation. The first was nonresponse bias since only half the telephone numbers listed in Alpine were reached. However, since Alpine is a seasonal community with many part-year residents and rental properties, it is likely that we reached most of the households with members present at the time of the outbreak. An additional concern was the potential for household clustering in our town cohort study since several members within a household were interviewed. However, this effect was examined by using generalized estimating equation models and showed no appreciable difference from the simpler logistic regression models.

This outbreak highlights the importance of *E. coli* O157:H7 as a waterborne pathogen. The organism has a low infectious dose (19), which allows water to act as an efficient vector, and watersheds that are vulnerable to infiltration by animals run the risk of contamination. Of 18 waterborne outbreaks of *E. coli* O157:H7 infections reported to CDC from 1982 to 1998, five were caused by contaminated drinking water (CDC, unpub. data). All five of these outbreaks involved small water systems or wells that supplied rural townships or camps. More recently, in September 1999, a large waterborne outbreak of *E. coli* O157:H7 infections occurred at a county fair in New York (3). In that outbreak, the drinking water was likely contaminated when cow manure seeped into a shallow, unchlorinated well after a large rainstorm. Because of underreporting and underdiagnosis, reported outbreaks probably represent a small fraction of the true number of *E. coli* O157:H7 outbreaks associated with drinking water in the United States. In addition to data from domestic outbreaks, contaminated water has been implicated as the cause of at least four outbreaks in other countries (4,20-22), including a massive outbreak with over 1,400 illnesses in Canada in May 2000 (4), and as a risk factor for sporadic infection (23,24). These illnesses could have been prevented by properly protecting the water sources and adequate chlorination.

Small water systems, defined as those that serve fewer than 3,300 people, collectively serve approximately 40 million people, or 15% of the United States population (25). Small drinking water systems may be less likely to be adequately chlorinated and to routinely monitor for contaminants (25). The outbreak reported here confirms the potential of these small, unprotected and unchlorinated water systems to be an important source of infection with *E. coli* O157:H7 and other pathogens. Stronger enforcement of existing regulations and perhaps broadening of current regulations, such as the proposed ground water rule designed to prevent illness from drinking water from ground water sources through disinfection (26), are needed to protect rural drinking water systems in the United States.

Dr. Olsen is an epidemiologist in the Foodborne and Diarrheal Diseases Branch, Centers for Disease Control and Prevention. At the time of this investigation, Dr. Olsen was an officer in the Epidemic Intelligence Service. Her interests include infectious disease epidemiology and outbreak investigations.

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Address for correspondence: Sonja J. Olsen, Foodborne and Diarrheal Diseases Branch, Centers for Disease Control and Prevention, 1600 Clifton Rd., Mailstop A38, Atlanta, GA 30333, USA; fax: 404-639-2205; e-mail: sco2@cdc.gov

Biofilm on Ventriculoperitoneal Shunt Tubing as a Cause of Treatment Failure in Coccidioidal Meningitis

Larry E. Davis,*† Guy Cook,‡ and J. William Costerton§

We describe a case of recurrent coccidioidal meningitis in which a fungal biofilm on the tip of ventriculo-peritoneal shunt tubing was likely responsible for a 4-year persistence of *Coccidioides immitis,* despite the patient's taking an adequate dosage of fluconazole. Fungal biofilms should be considered as a cause for treatment failure and fungal persistence, especially when artificial prostheses or indwelling catheters are present.

Biofilms are matrix-enclosed populations of microorganisms adherent to each other or to surfaces or interfaces (1). Most reports are of bacterial biofilms, in which organisms have been shown to have an altered cell metabolism, slowed or suspended replication rates, and resistance to killing by antibiotics and macrophages (1-3). Bacterial biofilms are ubiquitous in nature. In streams, bacterial biofilm organisms are a thousandfold more common than free-living planktontic organisms (2). Biofilms also can produce chronic infections when they form on the surfaces of medical appliances such as catheters and prostheses (2,3).

Although less well studied, medically important fungal biofilms also exist and have similar properties of antimicrobial resistance and attachment to indwelling medical appliances (3-5). We report a case in which *Coccidioides immitis* produced a biofilm on a medical device, resulting in a persistent infection with major clinical consequences for the patient.

Case History

A 52-year-old man came to medical attention in 1993 with fever, confusion, lethargy, and leg weakness. He had noninsulin-dependent diabetes. On examination, the patient was obtunded, with a stiff neck, leg spasticity, hyperreflexia, Babinski signs, and a skin lesion. A punch biopsy of the lesion showed multinucleated giant cells and spherules with endospores consistent with *C. immitis*. Cranial computed tomography showed obstructive hydrocephalus, and a ventriculo-peritoneal shunt was placed. Ventricular cerebrospinal fluid (CSF) had four leukocytes (WBC)/mm^3, 40 mg/dL glucose, and 70 mg/dL protein. The ventricular CSF did not grow fungi, bacteria, or *Mycobacterium tuberculosis.* Both serum (1:128) and CSF (1:32) had positive microcomplement fixation antibody titers to *C. immitis*. Coccidioidal meningitis was diagnosed, and the patient received amphotericin B intravenously and intrathecally for 6 weeks, until elevated serum creatinine levels prevented further administration of the drug. The patient then was given 800 mg/day of fluconazole. Over the first 8 months, the patient slowly improved; his mental status returned to near normal, and he could walk and care for himself. He was discharged home on maintenance fluconazole (800 mg/day).

Four years later, the patient had recurring headaches, fevers, and declining mental status. The patient and his wife claimed that he rarely missed fluconazole doses. Neuroimaging showed no change in his previously enlarged ventricles. The ventriculo-peritoneal shunt was thought to be functioning normally. Ventricular CSF obtained from the shunt tubing contained 42 WBC/mm^3 (68% lymphocytes, 14% mononuclear cells, 17% atypical lymphocytes, 15% neutrophils, and 3% eosinophils), 51 mg/dL glucose, and 11 mg/dL protein and grew *C. immitis*. The original shunt tubing was surgically removed and replaced. Lumbar CSF and CSF obtained directly from the ventricle at the time of shunt replacement did not grow fungi. The patient was treated with fluconazole (1,200 mg per day) for several weeks, clinically improved over several months, and was discharged on fluconazole (800 mg per day). He has been stable at home for 2 years.

Methods and Results

C. immitis was isolated on Sabouraud dextrose agar at 30°C from the tip of the ventriculo-peritoneal shunt tubing. The initial growth of colonies was moist and gray and had a white, cottony, aerial appearance. The isolate was identified as *C. immitis* by DNA probes. The shunt tubing was then fixed in formalin, and a small scraping was taken from the tip of the tubing, stained with calcofluor, and examined under a dissecting microscope. Coarse, septate, branched hyphae, which had thick-walled, barrel-shaped arthrocondia along with empty-appearing cells, were seen, consistent with the hyphae of *C. immitis* (6) (Figure 1). The *C. immitis* isolate had a fluconazole MIC of 8 μg/mL obtained by the MacroBroth dilution technique. At the fungal reference laboratory, *C. immitis* strains

*New Mexico VA Health Care System, Albuquerque, New Mexico, USA; †University of New Mexico School of Medicine, Albuquerque, New Mexico, USA; ‡Bacterin, Bozeman, Montana, USA; and §Montana State University, Bozeman, Montana, USA

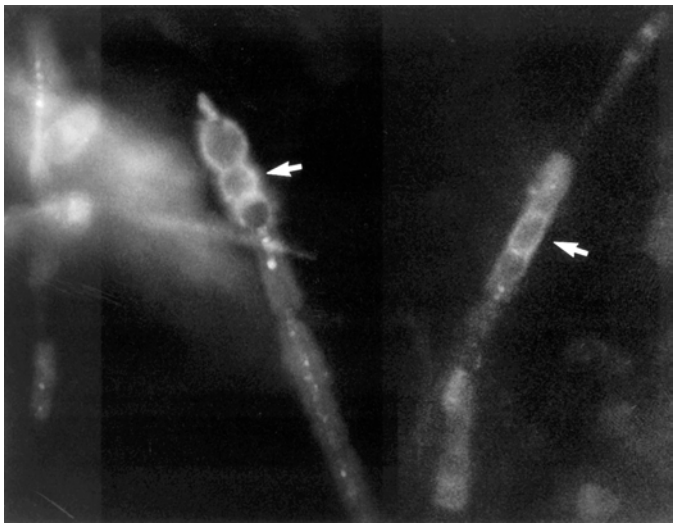

Figure 1. *Coccidioides immitis* hyphae dislodged from the tip of the ventriculo-peritoneal catheter tip, fixed in formalin and stained with Calcofluor. Stained barrel-shaped arthroconidia (arrows) are seen, along with empty cells (x400).

with a MIC ≤8 μg/mL are considered fluconazole sensitive. Material from the tip of the shunt tubing was stained with Molecular Probes SYTOX Green nucleic acid stain (Part #: S-7020, Molecular Probes, Eugene, OR), which is an impermeable, high-affinity, dead-cell stain. After brief incubation with SYTOX Green stain, the nucleic acids of dead cells fluoresce bright green when excited with the 488-nm spectral line of the argon-ion laser. The stain was prepared at 0.1% (v/v) in autoclaved double-filtered nanopure water. The material was directly stained with 0.4 mL of SYTOX Green and allowed to react for 5 minutes. Next, the sample was mildly washed with autoclaved nanopure water to remove excess stain and minimize background fluorescence. This material was directly imaged on a confocal laser scanning microscope (CLSM). The sample was then sectioned at each centimeter from the distal portion to the proximal portion. Monoclonal antibodies against *C. immitis* were not available for staining.

Scanning confocal microscopy was performed with a Leica TCS-NT confocal microscope (Heidelberg, Germany). A 63X 1.2 N.A Water Immersion Plan Apo lens objective was used for confocal laser microscope imaging. The confocal microscope was optimally configured for SYTOX Green analysis by using the 488-nm excitation laser with a 488-nm dichroic mirror and relative short pass filter of 580 nm in the first beam-splitter position. A band filter allowing wavelengths of 525 nm to 550 nm to pass to the first photo multiplier tube was used for imaging of the SYTOX Green stain.

CLSM analysis showed a layer of stained coccoid cells, 20 to 39 μm in depth, on the tip of the ventriculo-peritoneal shunt tubing (Figure 2). The depth of the colonization was indicative of a biofilm and is consistent with previous studies showing biofilm growth on explanted medical devices (3). The observed cocci were consistent with *C. immitis* spherule morphology and clearly formed a biofilm on the surface of the tubing.

Electron microscopy was performed on a Jeol 6100 Scanning Electron Microscope (Sundbyberg, Sweden). The sample was carbon coated by chemical vapor deposition for imaging. Analysis by electron microscopy revealed colonization of the tip of the ventriculo-peritoneal shunt tubing by unidentified cocci encapsulated in an exopolysaccharide matrix (Figure 3A). The amorphous mass was formed as the exopolysaccharide matrix material was condensed by dehydration, but the 4- to 6-μm spherical profiles of the *C. immitis* cells are clearly visible. Figure 3B shows a confluent biofilm formed on the tubing by *C. immitis*-containing hyphal elements as well as coccoid cells of *C. immitis* in the dehydration-condensed biofilm. The *C. immitis* biofilm was similar in structure to *Candida* sp. biofilms that also demonstrated hyphae and yeast organisms enveloped by an extracellular matrix (7).

Discussion

This case has several unusual features. First, the *C. immitis* in the ventricular shunt tubing was present in the hyphal phase. *C. immitis* is a dimorphic fungus that in nature is usually found in the mycelial phase (6). When the fungus infects humans, it is normally present in tissues in the yeast phase (6,8). With rare exceptions (9-11), autopsy studies have demonstrated *C. immitis* to be in the yeast phase in the CSF and meninges of patients with coccidioidal meningitis (8). The biofilm infection on the shunt tubing may have been responsible for

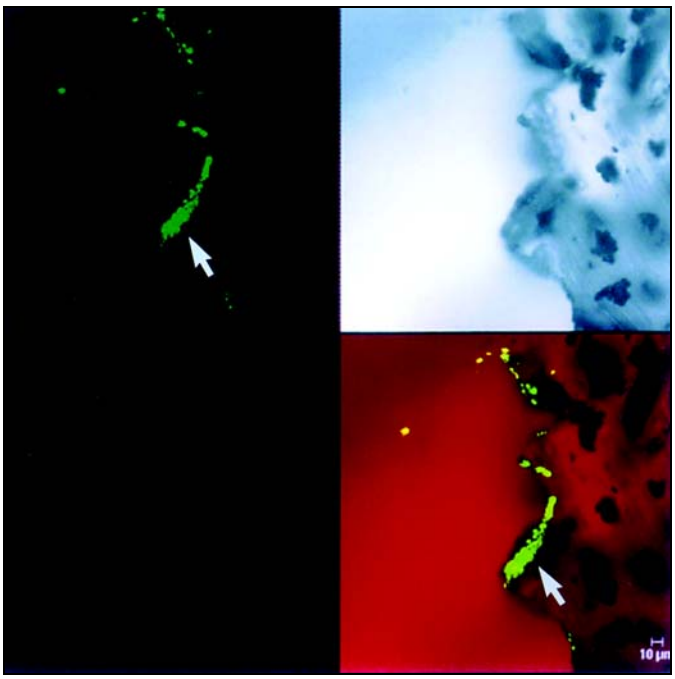

Figure 2. The upper left image shows a section from the tip of the shunt tubing stained with SYTOX Green nucleic acid stain and examined by scanning confocal microscopy with argon-ion laser light source. This specific staining for nucleic acids clearly shows the presence of a biofilm and some 4- to 6-μm cells. The upper right image shows an unstained, transmitted light microscopic image of the same area of the edge of the tubing. The bottom right image shows a recombined image with the nucleic acid stain colocalized with the transmitted light image. The recombined image shows that a substantial (~30 μm) biofilm composed of 4- to 6-μm cells has colonized the "scalloped" surface of this tubing. (x630 total magnification mosaic)

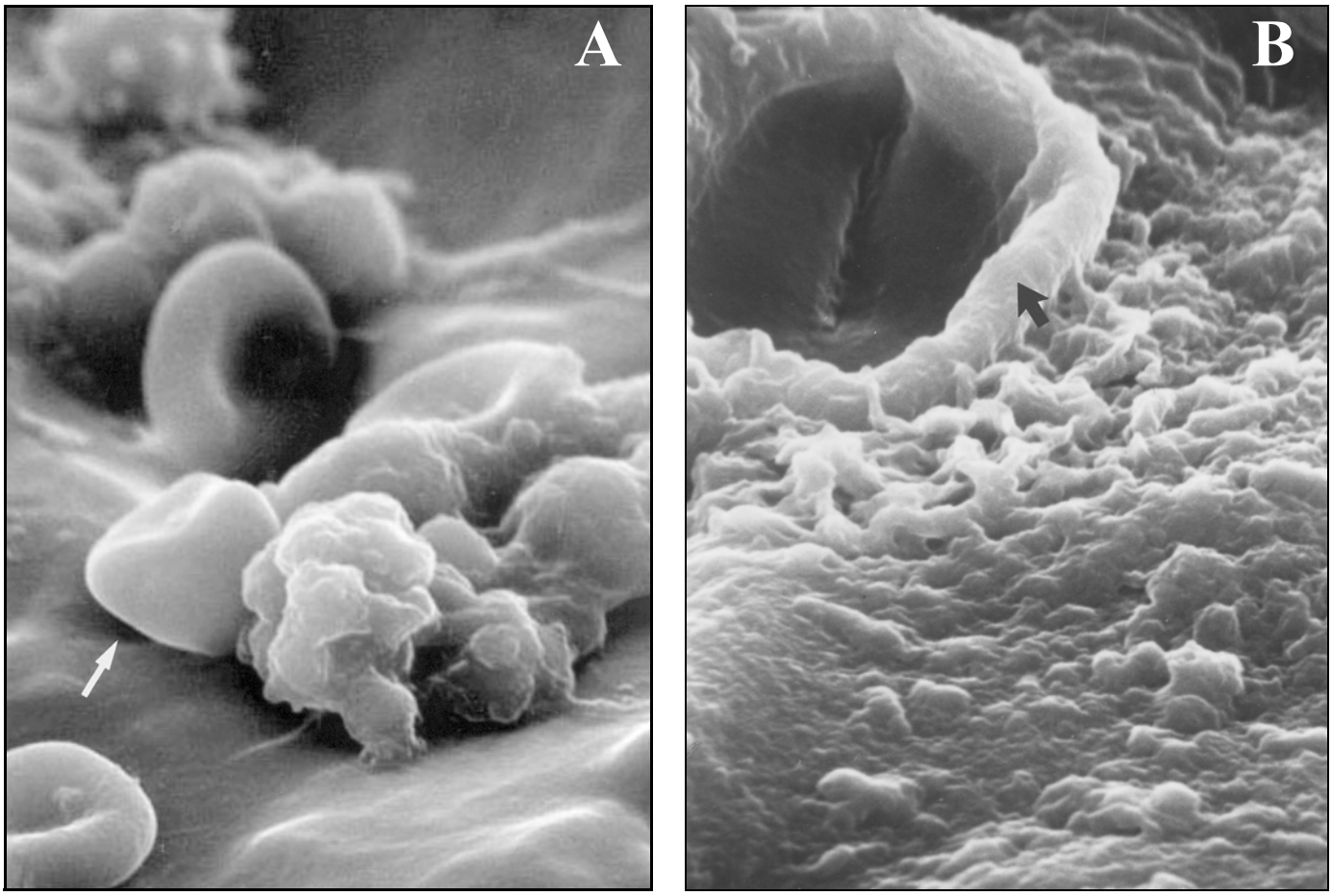

Figure 3. A. Scanning electron microscopy shows the presence of leukocytes and red blood cells on the tip of the ventriculo-peritoneal mass, within which coccoid cells can be visualized. The enclosing matrix material has condensed by dehydration, but the outline of the 4- to 6-μm coccoid cells (arrow), similar to those of *C. immitis,* can be resolved within the mass (x4,000). B. Scanning electron microscopy of the surface of the ventriculo-peritoneal shunt, showing complete colonization of the surface by a matrix-enclosed biofilm formed by the cells of *C. immitis*. Within the dehydration-condensed matrix of this biofilm, a hyphal element (arrow) and coccoid cells (4-6 μm) of the pathogen can be discerned (x5,000).

reversal of the normal human yeast phase. Second, in spite of the 4-year duration of the infection, *C. immitis* organisms recovered from the patient demonstrated an antimicrobial sensitivity to fluconazole. One possibility was that the hyphal form of *C. immitis* conveyed fluconazole resistance. However, this possibility was precluded since the Mycology Reference Laboratory routinely used the mycelial form of fungi in their antimicrobial testing. Third, fungal persistence continued in spite of the patient's taking fluconazole in a dosage that usually, but not always, produces clinical improvement (12). Since our patient did not have a fungal peritonitis or evidence of systemic coccidioidal infection from CSF containing *C. immitis* that would have normally exited the shunt tubing into the peritoneal cavity, we thought that noncompliance in taking the fluconazole could not account for the treatment failure. Instead, we concluded that the fungal biofilm was the most likely explanation for the fluconazole failure. Antimicrobial-drug resistance is known to develop when fungi or bacteria form a biofilm (2,13,14). In vitro studies with strains of pathogenic *Candida* spp. that were sensitive to fluconazole and amphotericin B in the planktonic state developed marked resistance to these antifungal drugs when the fungi were within a biofilm (13,15).

Candida spp. and *Cryptococcus neoformans* have been shown to produce biofilms on catheters (4,5); our case demonstrates that *C. immitis* can also produce a biofilm on indwelling catheters. Fungal biofilms should be considered as a potential cause for treatment failure of systemic fungal infections, especially if catheters or other artificial prostheses are indwelling in the patient.

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Dr. Davis is professor of neurology, neuroscience, and microbiology and immunology at the University of New Mexico. His research interest is infections of the nervous system.

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Address for correspondence: Larry E. Davis, New Mexico VA Health Care System, 1501 San Pedro Ave., Albuquerque, NM 87108, USA; fax: 505-256-2752; e-mail: LEDavis@UNM.edu

Experimental Infection of Horses With *West Nile virus*

Michel L. Bunning,*†‡ Richard A. Bowen,§ C. Bruce Cropp,† Kevin G. Sullivan,† Brent S. Davis,† Nicholas Komar,† Marvin S. Godsey,† Dale Baker,§ Danielle L. Hettler,† Derek A. Holmes,† Brad J. Biggerstaff,† and Carl J. Mitchell†

A total of 12 horses of different breeds and ages were infected with *West Nile virus* (WNV) via the bites of infected *Aedes albopictus* mosquitoes. Half the horses were infected with a viral isolate from the brain of a horse (BC787), and half were infected with an isolate from crow brain (NY99-6625); both were NY99 isolates. Postinfection, uninfected female *Ae. albopictus* fed on eight of the infected horses. In the first trial, Nt antibody titers reached ≥1:320, 1:20, 1:160, and 1:80 for horses 1 to 4, respectively. In the second trial, the seven horses with subclinical infections developed Nt antibody titers ≥1:10 between days 7 and 11 post infection. The highest viremia level in horses fed upon by the recipient mosquitoes was approximately 460 Vero cell PFU/mL. All mosquitoes that fed upon viremic horses were negative for the virus. Horses infected with the NY99 strain of WNV develop low viremia levels of short duration; therefore, infected horses are unlikely to serve as important amplifying hosts for WNV in nature.

West Nile virus (WNV), a flavivirus related to Japanese encephalitis, St. Louis encephalitis, and Murray Valley encephalitis viruses, was responsible for outbreaks of encephalomyelitis in humans and horses in New York (NY) during 1999 (1). The recognition of WNV in North America during 1999 was the first indication this virus was present in the Western Hemisphere. Continuing widespread virus activity in the northeastern USA during 2000 (2) suggests that WNV has become endemic. Previously, this virus was known from Africa, Europe, and south Asia; a subtype, Kunjin, is recognized in Australasia. During the peak transmission season, WNV cycles mainly between mosquito and wild bird species that differ according to geographic area. Before 1999, WNV had been reported to cause equine encephalomyelitis in Egypt (3), the Rhone River delta of France (4), Morocco (5), Israel (6), and Italy (7). Most equine infections were thought to result in mild clinical disease or inapparent infections, with only occasional cases of severe disease. However, during the 1999 epizootic, high illness and death rates in a cluster of equine cases centered around Riverhead, Long Island, Suffolk County, NY, indicated that not all infections resulted in mild disease (8). Within a radius of 10 km, 36 (43%) of 83 horses sampled from the Riverhead area were seropositive for WNV, and the clinical attack rate among the seropositive animals was 42%. The death rate for the 22 infected horses from Suffolk County was 36%. These observations raised the question of whether horses might be serving as amplifying hosts for WNV, exacerbating public health and veterinary problems.

Previous WNV experimental infection studies in equids are few, and attempts to induce and study clinical disease caused by WNV in equids have yielded equivocal results (3,9). In studies by Joubert et al. (10) and Oudar et al. (11), fever developed in four of nine equids (one jenny, one horse, and seven foals) after simultaneous needle inoculation of WNV by the subcutaneous and intravenous routes. In three of the four foals that became febrile, frank meningoencephalomyelitis and specific histopathologic lesions developed in central nervous system (CNS) tissue (12). One of 12 horses in our study developed encephalomyelitis.

Previous studies did not resolve the question of whether WNV-infected equids produce viremia levels of sufficient magnitude and duration to infect vector mosquitoes. Schmidt and Mansoury (3) reported transient (1-day) trace amounts of WNV in the blood of two of six donkeys infected by needle inoculation, but detectable levels of viremia did not develop in three horses in the study. Viremia titers and duration were not reported quantitatively in the studies by Joubert et al. (10) and Oudar et al. (11). A study conducted by U.S. Department of Agriculture (USDA) staff (J.Lubroth, pers. comm.) also reported low levels of viremia ($\leq 10^{2.5}$ $TCID_{50}$ per mL) in four horses infected by needle inoculation of a strain of WNV isolated from a horse during the 1999 epizootic.

Because of the lack of definitive studies on the potential for equines to serve as amplifying hosts for WNV following vector-borne transmission and the implications for public health and veterinary concerns, studies were needed of horses infected by mosquito bite with virus strains isolated during the 1999 outbreak in New York. Accordingly, we investigated the course of clinical disease, viremia levels and antibody responses, and the potential of viremic horses to infect vector mosquitoes (R. Bowen, unpub. data).

Methods

Equine selection and examinations

Mares and geldings of varying ages and breeds were used (Table 1). The horses were screened and shown to be negative

* Centers For Disease Control and Prevention, Atlanta, Georgia; † Centers For Disease Control and Prevention, Fort Collins, Colorado, USA; ‡United States Air Force; and §Colorado State University, Fort Collins, Colorado, USA

Table 1. Virus strain, age, sex, and day animal was euthanized after inoculation, May–July 2000

Identification no.	Virus strain	Age (yr)	Sex	Euthanized (days after inoculation)
1	BC787	14	Male	16
2	BC787	11	Female	14
3	NY99-6625	7	Female	14
4	NY99-6625	11	Female	16
9	BC787	11	Female	>90
10	BC787	10	Female	>90
11	BC787	13	Male	9
12	BC787	4	Male	>90
13	NY99-6625	5	Female	>90
14	NY99-6625	4	Female	>90
15	NY99-6625	6	Male	>90
16	NY99-6625	18	Female	27

for neutralizing antibodies to WNV and *St. Louis encephalitis viruses* (SLEV). Two days before infection, they were moved into a biocontainment building at Colorado State University and maintained under biosafety level-3 conditions for the duration of the project. The horses were fed mixed grain and hay twice a day. The horses were euthanized by pentobarbital overdose at varying times after infection (Table 1), necropsies were performed, and the carcasses were incinerated in the containment facility.

The horses were examined for signs of disease twice a day. Their body temperatures were recorded twice a day from day 0 (day of infection) to day 10, then daily through day 28, unless they were euthanized earlier. Pulse and respiration rates were recorded daily for the first 2 weeks after infection. Blood was collected for serum twice a day from day 0 through day 14, then daily through day 28, and twice a week for the duration of the project. Oral and rectal swabs were obtained daily from days 0 through 10, and the samples were placed into 1 mL of BA-1 medium (M-199 salts, 1% bovine serum albumin, 350 m/L sodium bicarbonate, 100 units/mL penicillin, 100 mg/L streptomycin, and 1 mg/L amphotericin in 0.05 M Tris, pH 7.6) and stored frozen for virus isolation. Blood samples were analyzed daily from day 0 through day 10 by using a QBC-V hematology analyzer (Clay Adams, Becton, Dickinson and Company, Franklin Lakes, NJ). The methods used for histopathology and immunocytochemistry have been described (R. Bowen, unpub. data).

Horses 5 through 8 were used to test a vaccine for WNV and were not part of this research project.

Mosquito Infection and Feeding Trials

Mosquitoes used for transmitting WNV to susceptible horses by bite were infected with either of two strains of WNV isolated from New York animals infected during the 1999 outbreak. Strain BC787 (1 passage in suckling mouse brain) was isolated from the brain of a horse, and NY99-6625 (1 passage in Vero cell culture) was isolated from the brain of a crow.

The *Aedes albopictus* mosquitoes used in these experiments were from a colony strain from Lake Charles, LA. Mosquitoes were reared in an insectary maintained at 26.7°C (± 0.5°) in approximately 80% relative humidity and with a long photophase (light:dark 16:8). Larvae were fed liver powder and rabbit chow as desired. Cohorts of 3- to 5-day-old adult female *Ae. albopictus* were inoculated intracoelomically with either of the two virus strains, placed in separate cages, given 5% sucrose for maintenance, and incubated under the insectary conditions described above. The virus dose per mosquito, based on appropriate dilutions of stock virus of known titer, was estimated to be approximately 170 Vero PFUs. Sucrose was withheld prior to feeding on equines.

Infected mosquitoes were allowed to feed on horses in two separate trials. A pilot experiment was conducted with four horses to determine probable periods of peak viremia before a more comprehensive experiment was conducted, which involved the feeding of large numbers of uninfected (recipient) mosquitoes. On day 8 postinoculation (May 10, 2000), caged infected mosquitoes were placed in a securely taped styrofoam container and transported to the equine holding facility. Feeding was accomplished by holding a cage of mosquitoes, with a gloved hand, against a shaved area immediately behind the left shoulder of each horse. Based on viremia profiles from the initial trial, a second trial was begun on July 10, 2000, in which infected mosquitoes that had been incubated 12 days postinoculation were allowed to feed on eight horses. Each horse was exposed to the mosquitoes for 5 minutes in each trial.

After feeding, the caged mosquitoes were sealed in a styrofoam container that was rinsed externally with diluted bleach and then transported to a laboratory. Mosquitoes were anesthetized with CO_2 and sorted on wet ice. Most blood-fed specimens were placed in stoppered vials and frozen at -70°C until they were triturated and tested for virus. Mosquitoes were ground individually in 1 mL each of BA-1 diluent in TenBroeck (Wheaton Science Products, Millville, NJ) tissue grinders. Samples were centrifuged for 4 minutes at 20,000 x *g* in a refrigerated microcentrifuge. Supernatants were poured into screw-cap vials, which were kept on wet ice until serial dilutions were made, and the samples were tested for virus by plaque assay.

During the second experiment, on days 3, 4, and 5 after infected mosquitoes had fed on the horses, uninfected 3- to 7-day-old *Ae. albopictus* females were permitted to feed on the 8 horses in lots of approximately 40 mosquitoes per cage. In addition, 3 lots of recipient mosquitoes were fed on horse 11 on days 8 and 9 postinfection, after the horse showed signs of clinical illness. Recipient mosquitoes were allowed to feed in an area behind the right shoulder of the animals on the side opposite from where the infected mosquitoes had fed. Fed mosquitoes were transported to a laboratory and sorted with a mechanical aspirator, and each cohort was placed in a separate 0.2-L cage. Blood-engorged mosquitoes were given 5%

sucrose for maintenance, and the 0.2-L cages were placed inside larger cages and incubated as described. Recipient mosquitoes that fed on horses on days 3 and 4 postinfection were incubated for 10 days. The 8 cohorts of recipient mosquitoes fed on day 5 postinfection were incubated only 7 days because a large number had died. Following incubation, mosquitoes were immobilized by being chilled briefly and were placed in tubes while still alive. They were then frozen at -70°C until processed and tested for virus by plaque assay. All mosquitoes fed on days 3 to 5 postinfection were individually disrupted by sonic energy (13) in 1 mL of BA-1 diluent and centrifuged at -4°C for 15 min at 1,500 x *g*. The supernatants were frozen at -70°C until tested by plaque assay. The three lots of mosquitoes that fed on the sick horse on days 8 and 9 postinfection were processed and tested in three pools, titurated in 1.8 mL of BA-1, and centrifuged for 4 minutes at 20,000 x *g* in a refrigerated microcentrifuge.

Mosquito Saliva Collection

Some of the WNV-infected mosquitoes used for horse challenge were chilled at 4°C for 5 minutes and held on ice. Mosquitoes were immobilized by removing their legs and wings. Individual mosquito proboscises were placed in a capillary tube containing Type "B" immersion oil. Saliva was collected in the oil-filled tube (14). Capillary tubes containing individual mosquito saliva were placed in 1.7-mL centrifuge tubes containing 200 mL of BA-1 diluent and centrifuged (10^5 rpm for 2 min). Serial dilutions were made, and the contents were inoculated into Vero cell culture six-well plates. A double agarose overlay was used, with the second overlay containing neutral red, and plaques were counted on days 4 and 5 postinoculation.

West Nile Equine IgM Capture ELISA

The WNV equine immunoglobulin (Ig)M capture enzyme-linked immunosorbent assay (ELISA) is a microtiter plate format assay designed to detect IgM antibodies to WNV. Unused 96-well plates (Immunolon II; Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with 75 mL of goat anti-horse IgM antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in carbonate buffer (0.015 M $NaCO_3$, 0.035 M $NaHCO_3$, pH 9.6) at a 1:1,000 dilution. For quality control, 36 outside wells of the plate were not used in the test. After the coating buffer was decanted, 200 mL of blocking buffer (phosphated-buffered saline [PBS], 0.05% Tween 20, 5% dry milk) was added to each well, and the wells were incubated at room temperature for at least 30 minutes. After the mixture was washed five times with wash buffer (PBS, 0.05% Tween 20), 50-mL samples of horse serum, diluted 1:400 in wash buffer, were applied; each sample was added to 6 wells. Positive and negative control equine sera were included on each plate, yielding a final capacity of eight test samples per microtiter plate. Diluted serum was incubated for 1 hour at 42°C. After the serum was washed five times, 50 mL of WNV tissue culture antigen (CDC Cat. No. VA2395), diluted 1:300 in wash buffer, was added to half the wells to which diluted serum was added. Fifty mL normal control tissue culture antigen (CDC Cat. No. VB2396), diluted 1:300 in wash buffer, was added to the remaining wells so that each test serum sample had three viral antigen wells and three normal antigen wells. The antigen was incubated overnight at 4°C. After the samples were washed five times, 50 mL of horseradish peroxidase, conjugated with a monoclonal antibody to WNV (6B6C-1 CDC; ADB [Arboviral Branch] reference reagent) and diluted 1:2000 in blocking buffer, was added to each well and incubated for 1 hour at 42°C. After the wells' contents were washed 10 times with wash buffer, 75 mL tetramethylbencidine (TMB) substrate (GibcoBRL, Life Technologies Inc, Gaithersburg, MD) was added to each well. After incubation with the substrate for exactly 10 minutes, the reaction was stopped by adding 50 mL 1N H_2SO_4. The OD of each well was measured at 450 nm, and results were calculated as follows:

The Final A450nm = [A450 nm of test sera on West Nile antigen] – [A450nm of normal sera on West Nile antigen]

Neutralization Assay

Serum samples were tested for virus-neutralizing (Nt) antibodies to WNV (NY99-4132 strain, 1 Vero passage) by the plaque-reduction neutralization test in Vero cell culture. Earlier serum samples were also tested for Nt antibodies to SLEV (TBH-28 strain, passage history unknown). Briefly, diluted serum was heat inactivated at 56°C for 30 minutes and mixed with an equal volume of a virus preparation in BA-1 containing 8% normal human serum, so that the number of infectious virus particles in the final dilution was approximately 100/0.1 mL. A volume of 0.1 mL was then injected onto a Vero cell monolayer and processed as for the plaque assay. Samples were screened by testing once at a final dilution (after mixture with challenge viruses) of 1:10. Any sample that neutralized the challenge virus dose at a level of $\geq$70% was confirmed by testing in duplicate and titrated by serial twofold dilutions. Neutralization at a level of $\geq$90% was considered positive for each dilution. Horses having preexposure sera that neutralized SLEV at $\geq$70% were excluded from the study. Sera from horses 1 to 4 were challenged with approximately 300 PFU of WNV (Horse Brain WN Isolate # BC 787), and sera from horses 9 to 16 had an average of 86 PFU of WNV added.

Plaque Assay

Virus concentration in serum samples, mosquito homogenates, and tissue homogenates was measured by titration in a plaque assay. Briefly, 0.1 mL of sample was added to a monolayer of Vero cells in a six-well cell culture plate (Costar, Corning Incorporated Life Science, Acton, MA) and incubated 1 hour at 37°C in 5% CO_2. Cells were overlaid with 3 mL per well of 0.5% agarose in M-199 medium, supplemented with 350 mg/L of sodium bicarbonate, 29.2 mg/L of L-glutamine, and antibiotics. After 48 hours of additional incubation, a second 3-mL 0.5% agarose overlay, containing 0.004% neutral

red dye, was added for plaque visualization. The plaques were scored on days 3, 4, and 5 of incubation.

Results

In the first infection trial, 12 to 17 infected mosquitoes fed on each of four horses. Virus titrations were done on 10 blood-engorged mosquitoes from each cohort that fed. All of the 40 mosquitoes tested were infected. Virus titers ranged from $10^{6.6}$ to $10^{7.9}$ Vero cell PFU per mosquito and averaged $10^{6.8}$, $10^{7.2}$, $10^{7.3}$, and $10^{7.4}$ PFU per mosquito in the cohorts that fed on horses 1 to 4, respectively. Mosquitoes infected with the WNV strain isolated from a horse were fed on horses 1 and 2, and mosquitoes infected with a strain isolated from a crow were fed on horses 3 and 4 (Table 1). Serum samples (drawn twice a day from day 0 through days 12 or 13) were tested for virus. Horse 3 did not develop a detectable level of viremia, and horse 1 had viremia detectable only in the afternoon of day 3 post-infection titer of $10^{1.3}$ PFU/mL. Horse 2 had peak titers of $10^{1.3}$, $10^{2.2}$ and $10^{2.2}$ PFU/mL on days 3 to 5, respectively. Horse 4 had titers ranging from $10^{1.0}$ PFU/mL in the afternoon of day 2 to $10^{1.3}$ PFU/mL on day 4.

On the basis of these viremia profiles, we decided to feed uninfected mosquitoes on infected horses in the next trial on days 3 to 5 postinfection to determine whether viremia levels were sufficient to infect susceptible mosquitoes. None of these horses developed fever, hematologic abnormalities, or signs of clinical disease. Horses 1, 2, 3, and 4 were euthanized, and necropsies were performed on days 14 and 16 postinfection. Histopathologic lesions indicative of WNV infection were not observed. Virus was not isolated from any tissues other than blood from these animals, and viral antigens were not detected in brain, spinal cord, or other tissues by immunocytochemical tests.

In the second trial, 7 to 14 infected mosquitoes fed on each of the eight horses. Virus titrations were done on five mosquitoes from each cohort, and 100% of the mosquitoes tested were infected. Virus titers ranged from $10^{6.5}$ to $10^{8.0}$ Vero cell PFU per mosquito and averaged $10^{7.5}$,$10^{7.3}$, $10^{7.3}$, $10^{7.3}$, $10^{7.6}$,$10^{7.3}$, $10^{7.7}$, and $10^{7.4}$ PFU per mosquito in the cohorts that fed on horses numbered 9 to 16, respectively. Mosquito saliva was collected from 10 infected mosquitoes used in the second trial, and virus titers ranged from $10^{1.3}$ to $10^{2.5}$ PFU per sample of saliva.

Mosquitoes infected with a WNV strain isolated from a horse were fed on horses numbered 9 to 12 and mosquitoes infected with a strain isolated from a crow were fed on horses numbered 13 to 16 (Table 1). Seven of the eight horses developed detectable levels of viremia, and all virus-positive serum samples were obtained during days 1 to 6 (Table 2). Virus titers ranged from $10^{1.0}$ PFU/mL of serum, the lowest level detectable in our assay, to $10^{3.0}$ PFU/mL (in horse 13 in the p.m. of day 3).

Twenty-four lots of uninfected recipient mosquitoes were fed on seven infected horses in the mornings of days 3 to 5 postinfection. Virus titers in the seven viremic horses ranged from $10^{1.0}$ PFU/mL to $10^{2.7}$ PFU/mL during the times mosquitoes were fed. The feeding success among uninfected mosquitoes was 92%, 81%, and 71% on days 3 to 5, respectively. Following incubation, only live mosquitoes were saved for testing. Survival rates for the mosquitoes that fed on days 3, 4, and 5 were 256 (87%) out of 293, 246 (92%) out of 266, and 150(64%) out of 236, respectively. All 652 mosquitoes that fed during the 3 days and survived the incubation periods were tested individually for the presence of virus and found to be negative. In addition, after horse number 11 developed clinical symptoms compatible with encephalomyelitis three lots of mosquitoes were fed on animal number 11 on day 8 (a.m. and p.m.) and day 9 (a.m.). The mosquitoes were incubated for 10 days and tested for virus infection in three pools of 30, 41, and 44 specimens. All were negative for the presence of virus.

Table 2. Postinfection levels of West Nile viremia in horses, May–July 2000[a]

Day	Viremia levels (Log-10 Vero cell PFU/mL serum)							
	Horse							
	9	10	11	12	13	14	15	16
1 (a.m.)	1.3	-	-	-	-	-	-	-
1 (p.m.)	-	1.0	-	-	-	-	-	-
2 (a.m.)	-	1.3	-	-	-	-	-	-
2 (p.m.)	-	1.0	-	1.0	-	-	-	1.0
3 (a.m.)	2.1	1.5	1.0	-	1.0	-	2.2	-
3 (p.m.)	2.3	1.3	-	-	3.0	-	-	1.9
4 (a.m.)	2.4	1.6	2.5	1.5	1.3	-	1.3	2.1
4 (p.m.)	1.9	1.5	1.9	1.0	1.3	-	-	2.0
5 (a.m.)	1.6	1.5	2.7	1.0	1.3	-	-	2.5
5 (p.m.)	-	1.6	2.5	-	1.3	-	-	2.7
6 (a.m.)	-	1.6	2.1	-	-	-	-	2.3
6 (p.m.)	-	1.6	2.1	-	-	-	-	2.0

[a]Horses 9 to 12 were infected by bites of mosquitoes inoculated 12 days earlier with WN virus strain isolated from a horse; horses 13 to 16 similarly infected with strain isolated from a crow; dash indicates viremia level was too low to be detectable or was absent.

The only horse from the entire study to show clinical signs of disease was horse 11, which became febrile and showed neurologic signs beginning 8 days after infection. This mare progressed to severe clinical disease within 24 hours and was euthanized on day 9. She had a severe encephalomyelitis and relatively high titers of virus ($10^{4.0}$ to $10^{6.8}$ PFU/tissue) in several areas of the brain and spinal cord. Horse 16 was euthanized on day 30 because of a preexisting respiratory condition unrelated to WNV infection. Horses 9, 10, 12, 13, 14, and 15 were monitored for 91 days after infection. None showed signs of disease at any time during that period, nor was WNV isolated from any of the serum samples collected biweekly through day 91. Oral and rectal swabs collected daily from days 0 through 10 were negative for virus.

All 12 horses became infected with WNV after being bitten by infected mosquitoes as evidenced by clinical encephalo-

myelitis in horse 11 and Nt antibody titers in the others. In the first trial, Nt antibody titers ≥1:10 were first detected in horses 1 to 4 on days 8 to 10 postinfection. Titers in the samples drawn on days 12 and 13 were ≥1:320, 1:20, 1:160 and 1:80 for horses 1 to 4, respectively. These horses were euthanized and underwent necropsy between days 14 and 16. In the second trial, the seven horses that remained well developed Nt antibody titers ≥1:10 between days 7 and 11 post infection. Peak titers in seven horses that did not exhibit clinical illness were reached between days 9 through 13. No samples were tested between days 13 and 27, by which time the titer of horse 16 was ≥1:1,280. Titers for horses 9, 10, and 12 to 15 on day 31 postinfection were 1:320, 1:160, 1:160, 1:160, 1:40, and 1:80, respectively.

The IgM capture ELISA results are summarized in Figures 1 and 2. Specific IgM levels in some horses began to rise perceptibly by day 7 postinfection and continued to increase up to day 13. Horses 3 and 14, which did not have detectable levels of viremia, showed weak responses. Horse 11 also had detectable levels of viremia and was euthanized on day 9.

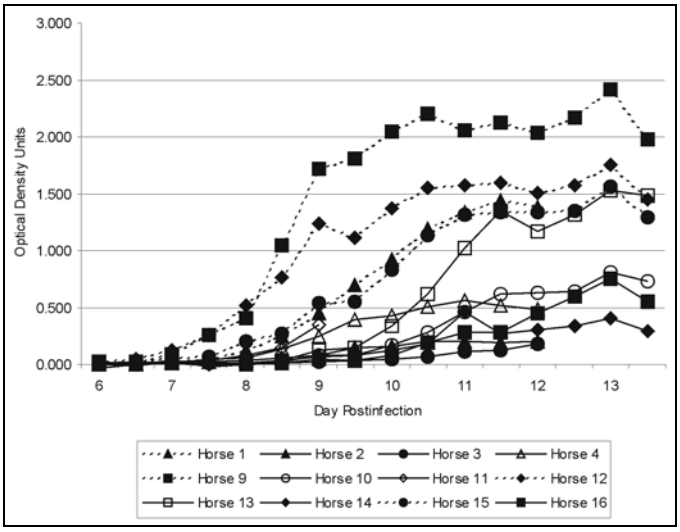

Figure 1. *West Nile virus* (WNV) absorbance attributed to specific WNV Antigen immunoglobulin (Ig) M Interaction in the West Nile Equine IgM Capture enzyme-linked immunosorbent assay (ELISA).

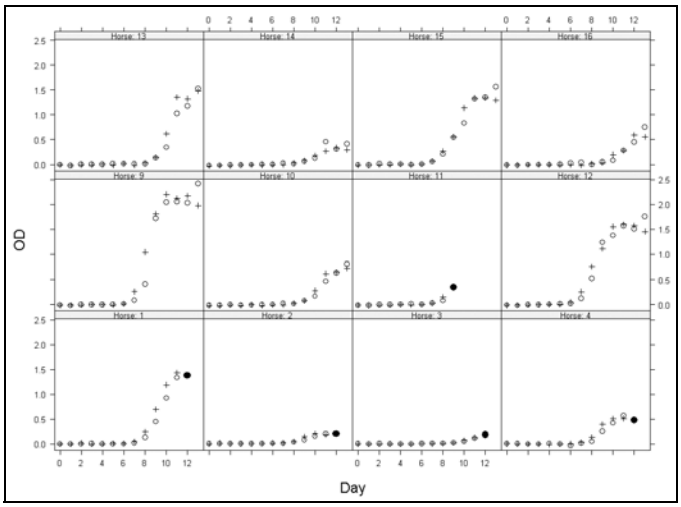

Figure 2. *West Nile virus* (WNV) absorbance attributed to specific WNV Antigen immunoglobulin (Ig) M Interaction in the West Nile Equine IgM Capture enzyme-linked immunosorbent assay (ELISA) on individual horses. The open circle (o) is the a.m. reading, the plus (+) is the p.m. reading, and the filled-in circle is the last reading (all a.m., hence the circle) before the horse was euthanized. Each number corresponds to the horse number.

Discussion

Ae. albopictus mosquitoes were used in these experiments because this species was known to be susceptible to WNV infection per os and by intracoelomic inoculation and to be capable of transmitting the virus by bite (15-17). In addition, information was available on the replication of WNV in intracoelomically inoculated *Ae. albopictus*, incubated under conditions identical to ours, which showed that peak titers were reached by day 5 postinoculation (K. Gottfried, pers. comm.). WNV has been isolated recently from *Ae. albopictus* in New York (18).

The key question addressed in these studies was whether horses develop viremia of sufficient magnitude to serve as amplifying hosts of WNV. The low titers we observed are similar to those seen by Schmidt and Mansoury in Egypt (3). Joubert et al. (19) conducted studies in France; however, they did not report actual titers but rather categories of viremia (weak, moderate, or strong), based on intensity and duration. Recent unpublished work also is consistent with these findings (J. Lubroth, pers. comm.). Our finding of 1 (8.3%) of 12 clinical cases among the experimentally infected horses agrees closely with the results reported for naturally acquired WNV infections among horses in Camargue, France, where the clinical attack rate among approximately 500 free-ranging equines was estimated to be 10% (10).

The highest viremia titer in horses fed on by the recipient mosquitoes was approximately 460 Vero cell PFU/mL in horse 11 during the morning of day 5 postinfection (Table 2). This mare was the only animal in our study to develop clinical encephalomyelitis. Although relatively high titers of WNV were found in several areas of the brain and spinal cord, titers in the horses' blood were not extraordinary. Based on an estimated bloodmeal volume of 5 mL/mosquito, the average virus dose ingested by mosquitoes feeding on this animal was 2.3 PFU. Twenty-two mosquitoes from the cohort that ingested this estimated dose survived to be tested, and all were WNV-negative. We did not feed mosquitoes during the evening of day 3 postinfection when horse 13 was circulating $10^{3.0}$ PFU/mL, or the equivalent of 5 PFU/bloodmeal. Whether this dose, the highest viremia titer recorded in the horses, would have been sufficient to infect our strain of *Ae. albopictus* is unknown. Jupp (20) reported that 41% of a South African strain of *Culex univittatus* became infected after feeding on a viremic chick circulating $10^{2.9}$ suckling-mouse intracerebral lethal-dose$_{50}$ (SMICLD$_{50}$)/mL of WNV. Further, estimates were made of the viremia levels necessary to infect 10% of vector mosquitoes. These 10% infection thresholds, expressed as titers found in viremic chicks, for local strains of *Cx. univittatus, Cx. pipiens, Cx. quinquefasciatus,* and *Cx. theileri* were

$<10^{2.7}$, $10^{2.7}$,$10^{2.7}$ and$<10^{4.1}$ adult-mouse LD_{50}/mL, respectively. The results are somewhat difficult to interpret because not all species were fed on low-titered viremias, and because both adult and infant mice, which differ in their susceptibility to WNV infection, were used for some titrations. Jupp (21) also noted discrepancies in infection thresholds related to the manner of feeding, using blood-virus mixtures fed through membranes or via blood-soaked cotton, as opposed to feeding on viremic animals. Interestingly, these differences were apparent in *Cx. pipiens* and *Cx. quinquefasciatus* but not in the other two species. Results of earlier studies in which data were obtained by artificial feeding techniques should be viewed with caution, at least for the species mentioned. We are unaware of other information on the infection thresholds of species and strains of proven and potential vectors of WNV.

Akhter et al. (17) fed *C. tritaeniorhynchus* and *Cx. quinquefasciatus* on five viremic chicks that were circulating $10^{4.9}$ to $10^{5.3}$ $SMICLD_{50}$ of WNV. All *Cx. tritaeniorhynchus* (n=100) became infected, and 59% to 90% of the *Cx. quinquefasciatus* (n=85) did so. Turell et al. (22) fed a variety of mosquito species from the eastern USA on viremic chicks circulating WNV at titers of approximately $10^{5.2}$ Vero cell PFU/mL and $10^{7.0(\pm\ 0.3)}$ PFU/mL. Based on an estimated bloodmeal volume of 5 mL/mosquito, individual mosquitoes, which fed on these chicks, would have ingested virus doses of about 800 and 40,000 to 160,000 PFU, respectively. Infection rates in mosquito species in these cohorts ranged from 0% to 17% and 0% to 92%. For other arboviruses, a dose-response relationship exists between the titer of the infective meal and the ability of vector mosquitoes to transmit (23). An Israeli strain of the *molestus* biotype of *Cx. pipiens* transmitted WNV to infant mice after feeding on high-titered blood/virus mixtures but not when the titer was $10^{2.8}$/0.3 mL ($10^{4.3}$/mL) (24). Jupp (21) noted that a reduction in the infecting WNV titer from $10^{6.5}$ to $10^{4.3}$ $SMICLD_{50}$ caused a decrease in the transmission rate from 89% to 33% in *Cx. univittatus*. Therefore, if the low-level viremias observed in our study were to prove sufficient to infect mosquito species shown to be more susceptible to per os infection than *Ae. albopictus*, any individuals that did become infected on such low doses of WNV would be less likely to transmit the virus.

The results of the equine IgM-capture ELISA for WNV antibody indicate that this is a simple and efficient method for detecting antibody at about the same time that neutralizing antibody can be detected (Figure 2). The results of the Nt tests are unremarkable and consistent with those from previous studies (3,19).

Our limited work, and that of others cited above, support the conclusion that horses infected with WNV develop viremias of low magnitude and short duration and that infected horses are unlikely to serve as important amplifying hosts for WNV in nature.

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Dr. Bunning is a veterinary epidemiologist in the Division of Vector-borne Infectious Diseases Division, Centers for Disease Control and Prevention. He currently serves as a liaison between the Department of Defense and the Centers for Disease Control and Prevention. His interests include epidemiology and research on the *West Nile virus*.

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Address for correspondence: Mike Bunning, P.O. Box 2087, Fort Collins, Colorado 80522-2089, USA; fax: 970-221-6476; e-mail: mbunning@cdc.gov

Salmonella enterica Serotype Typhimurium DT104 Isolated from Humans, United States, 1985, 1990, and 1995

Efrain M. Ribot, Rachel K. Wierzba, Frederick J. Angulo, and Timothy J. Barrett

First isolated from an ill person in 1985, multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 emerged in the mid-1990s as a strain of *Salmonella* frequently isolated from humans in the United States. We compared the integron content, plasmid profile, and *Xba*I pulsed-field gel electrophoresis (PFGE) patterns of multidrug-resistant *S.* Typhimurium DT104 (MR-DT104) isolated from humans in the United States in 1985, 1990, and 1995. All isolates contained a 60-mDa plasmid and had indistinguishable PFGE and integron profiles, supporting the idea of a clonal relationship between recent and historical isolates. The data suggest that the widespread emergence of MR-DT104 in humans and animals in the 1990s may have been due to the dissemination of a strain already present in the United States rather than the introduction of a new strain.

Each year, bacteria of the genus *Salmonella* infect an estimated 1.4 million persons; these infections result in several hundred deaths in the United States annually (1). One of the most common strains isolated from humans is multidrug-resistant *Salmonella enterica* serotype Typhimurium definitive type 104 (DT104). This strain was first isolated from humans in 1984 in the United Kingdom, where it emerged as a major cause of human illness in the late 1980s (2) before its emergence in the United States and elsewhere in the mid-1990s (3,4). A national sample, in which all state and territorial public health laboratories were asked to forward every 10th *Salmonella* isolate to the Centers for Disease Control and Prevention (CDC) for antimicrobial susceptibility testing, showed that 275 (28%) of 975 *S.* Typhimurium isolates from humans in the United States in 1995 were resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (R-type ACSSuT), the resistance pattern commonly associated with multidrug-resistant DT104 isolates (MR-DT104). In contrast, only 8 (7%) of 108 *S.* Typhimurium isolates from humans in sentinel counties in 1990 and 7 (5%) of 135 in 1985 were R-type ACSSuT. An isolate collected in 1985 probably represents the earliest isolate of MR-DT104 in the United States. After emerging in the mid-1990s, MR-DT104 has remained prevalent in the United States; in 1999, 114 (31%) of 362 human *S.* Typhimurium isolates received by the National Antimicrobial Resistance Monitoring System (NARMS) for Enteric Bacteria were R-type ACSSuT (NARMS 1999 Annual Report; http://www.cdc.gov/ncidod/dbmd/narms). Since Typhimurium was the most common serotype of *Salmonella* in the United States in 1999, causing 25% of the culture-confirmed infections (CDC Salmonella Surveillance, 1999 Annual Summary; http://www.cdc.gov/ncidod/dbmd/phlisdata/salmtab/), MR-DT104 caused an estimated 7% of *Salmonella* infections.

Food-producing animals are reservoirs for nontyphoidal *Salmonella*, and most human *Salmonella* infections in the United States are a consequence of eating food, particularly foods of animal origin, contaminated with *Salmonella* (5). In 1998, 163 (29%) of 557 *S.* Typhimurium isolates from animals received in NARMS were R-type ACSSuT; most of these isolates were collected from cattle and pigs (NARMS 1998 Veterinary Isolates Report; http://www.fda.gov/cvm/fda/mappgs/narms/1998_data/narms_toc.htm). Limited data are available, however, on the prevalence of MR-DT104 in food-producing animals before 1996. A recent report indicates that MR-DT104 became prevalent in cattle in the Pacific Northwest in the mid-1990s (6); 55 (45%) of 123 cattle *S.* Typhimurium isolates from 1995 to 1997 were R-type ACSSuT, compared with 18 (20%) 90 in 1991 to 1994 and 2 (1%) of 163 from 1985 to 1990. Isolates of MR-DT104 from animals and humans were indistinguishable by molecular subtyping techniques (7).

The genetic determinants responsible for the R-type ACSSuT in MR-DT104 are located in the chromosome (2,8). Molecular characterization of *S.* Typhimurium DT104 R-type ACSSuT isolates from Europe and the United States showed that resistance to ampicillin, streptomycin, and sulfonamides is associated with the presence of two class-1 integrons (7-10). First described by Stokes and Hall in 1989 (11), integrons are a group of apparently mobile elements that can contain one or more antimicrobial resistance genes. Integrons represent an important and efficient mechanism by which many bacteria, including *S.* Typhimurium DT104, can acquire resistance to antimicrobial agents. These genetic elements have been found in a wide variety of organisms and are thought to be largely responsible for the dramatic increase in multidrug-resistant bacteria (11-15). Integrons carrying antimicrobial resistance genes have been found in plasmids and transposons (and trans-

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

poson-like elements) and in the chromosomal DNA of some bacteria. The fact that integrons are widely spread among gram-negative bacteria suggests that these genetic elements have evolved into a highly adaptable and very efficient mechanism by which cells can acquire and express antimicrobial resistance genes.

The goal of this study was to compare the integron structure and gene-cassette content, plasmid profiles, and pulsed-field electrophoresis (PFGE) patterns of recent *S.* Typhimurium DT104 (R-type ACSSuT) isolates (1995) with the earliest identified isolate of MR-DT104, collected in 1985, as well as isolates from 1990. The resulting data would suggest whether the emergence of MR-DT104 in the mid-1990s in humans and animals resulted from the dissemination of a strain already present in the United States or from the introduction of a new strain.

Seven *S.* Typhimurium MR-DT104 strains isolated from ill persons in 1985 (one isolate), 1990 (three isolates), and 1995 (three isolates) were characterized by polymerase chain reaction (PCR), PFGE, and plasmid profile analysis. MR-DT104 isolates used in this study (Table) were sent to CDC by local and state health departments and public health laboratories in 1985, 1990, and 1995 as part of national surveys designed to assess the emergence of antimicrobial resistance in *Salmonella* isolates from humans in United States (16,17). These isolates were serotyped by using a modified version of the method described by Ewing (18). Sensitivity testing of chosen isolates (MICs) was conducted by using a panel of 17 antimicrobial agents (amikacin, amoxicillin-clavulanic acid, ampicillin, apramycin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracyline, ticarcillin, and trimethoprim-sulfamethoxazole) on the Sensititre system (TREK Diagnostic Systems, Westlake, OH). Phage typing was done by using the methods of the Laboratory of Enteric Pathogens, Central Public Health Laboratory, Public Health Laboratory Service, United Kingdom (19).

PFGE analysis was carried out as described by Barrett et al. (20), with the following modifications. The DNA in agarose plugs was restricted with 40 U/plug-slice of *Xba*I restriction enzyme (Roche, Indianapolis, IN), following the manufacturer's recommendations. Restriction fragments were separated by PFGE through 1% SeaKem Gold agarose gels (BioWhittaker, Rockland, ME) in 0.5X Tris-borate-EDTA (45 mM Tris borate, 1 mM EDTA, pH 8.3 [TBE]) buffer at 14°C in a CHEF Mapper (Bio-Rad Laboratories, Hercules, CA). Electrophoresis conditions were as follows: initial switch time of 2.16 seconds, final switch time of 63.8 seconds, 6 V/cm, at an angle of 120° for 19 hours. The PFGE profiles (*Xba*I) of these isolates were indistinguishable from each other, with the exception of isolate C5234, which showed an extra band (indicated with an arrow in the Figure) compared with the other isolates. This level of clonality is remarkable considering that some of the isolates were obtained 10 years apart. The predominant PFGE pattern (Figure) was also seen in 21 of 22 additional MR-DT104 R-type ACSSuT isolates from humans in 1996 tested in our laboratory (E. Ribot, unpub. data). The remaining isolate had a PFGE pattern indistinguishable from the pattern of isolate C5234.

Plasmid DNA was isolated by using the QIAfilter plasmid midi kit (Qiagen, Chatsworth, CA), following the manufacturer's instructions. Plasmid profile analysis was done by loading 15- to 20-µL aliquots into the wells of 1% SeaKem Gold agarose gels and performing PFGE. The pulsing conditions were as follows: electrophoresis for 16 hours on a CHEF Mapper or GenePath System (Bio-Rad), with an initial switch time 6.75 seconds and final switch time 21.7 seconds at 6V/cm. The running buffer consisted of 0.5X TBE. The buffer temperature was kept at 14°C during the PFGE run. The plasmid patterns were visualized by ethidium bromide staining. Plasmid profile analysis showed that all *S.* Typhimurium MR-DT104 isolates contained the 60-mDa plasmid commonly seen in this strain (data not shown). Additional, smaller plasmids were observed in some of the isolates (Table). The nature or function of these smaller plasmids is not known.

Multidrug resistance is associated with the presence of two integrons found in the chromosomal DNA of *S.* Typhimurium MR-DT104 R-type ACSSuT isolates (2,8,9). To investigate the possibility that these integrons were also present in the *S.* Typhimurium DT104 R-type ACSSuT isolates from 1985 and 1990, the first MR-DT104 isolates found in our collection, we used PCR to amplify gene-cassettes located between the two

Table. Molecular characterization of multidrug-resistant *Salmonella* Typhimurium DT104 isolates, 1985–1995

Isolate number	Year	R-type	PFGE pattern	Plasmid content	Amplicons	Gene cassette(s)	*flo*-like gene[a]
B1579	1985	ACSSuT	1	60 mDa	1 kb; 1.2 kb	*ant(3")-Ia; psE1*	+
C3591	1990	ACSSuT	1	60 mDa	1 kb; 1.2 kb	*ant(3")-Ia; psE1*	+
C4501	1990	ACSSuT	1	60 mDa;~5 mDa	1 kb; 1.2 kb	*ant(3")-Ia; psE1*	+
C5234	1990	ACSSuT	1b	60 mDa	1 kb; 1.2 kb	*ant(3")-Ia; psE1*	+
G10217	1995	ACSSuT	1	60 mDa; 3 mDa	1 kb; 1.2 kb	*ant(3")-Ia; psE1*	+
G10518	1995	ACSSuT	1	60 mDa	1 kb; 1.2 kb	*ant(3")-Ia; psE1*	+
G10551	1995	ACSSuT	1	60 mDa	1 kb; 1.2 kb	*ant(3")-Ia; psE1*	+

[a]All integrons are located on the chromosome.
A = ampicillin; C = chloramphenicol; S = streptomycin; Su = sulfamethoxazole; T = tetracycline; PFGE = pulsed-field gel electrophoresis.

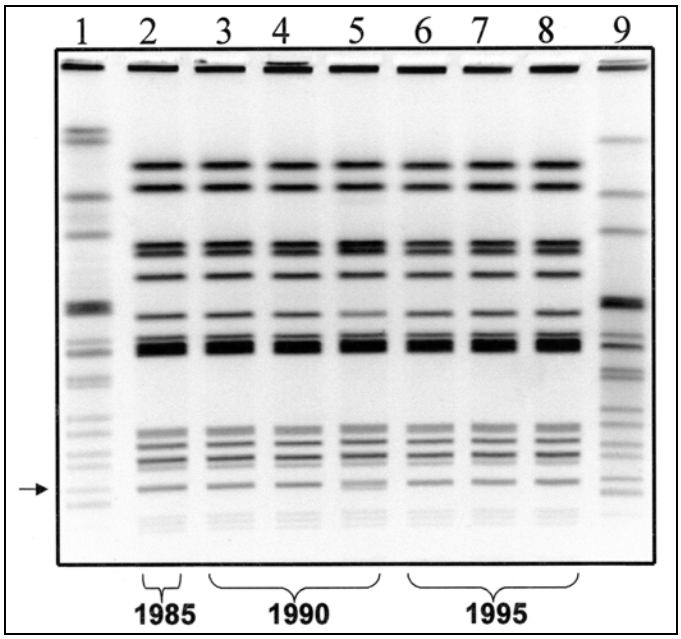

Figure. Gel showing the *Xba*I pulsed-field gel electrophoresis (PFGE) pattern of each isolate. Lane 1 and 9 show the pattern of the *Salmonella* strain used as a size standard. Lane 2 shows the pattern of MR-DT104 strain isolated in 1985. Lanes 3-5 show the patterns of MR-DT104 isolates obtained in 1990. Lanes 6-8 show the PFGE patterns of MR-DT104 isolates obtained in 1995.

conserved regions (5′-CS and 3′-CS) of class-1 integrons. To accomplish this, genomic DNA was isolated by using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN), following the manufacturer's recommendations. PCR amplification was performed on both chromosomal DNA and purified plasmid preparations with synthetic oligonucleotides 5′-CS (5′-GGCATCCAAGCAGCAAG-3′) and 3′-CS (5′-AAGCAGACTTGACCTGA-3′) (12). PCR was also used to amplify the region located between the 5′CS and the sulI (SulI: 5′-TGAAGGTTCGACAGCAC-3′) (12). The cycling conditions consisted of an initial step at 94°C for 10 minutes, followed by 35 cycles at 94°C for 20 seconds, 56°C for 20 seconds, and 72°C for 45 seconds with a final extension step of 10 minutes at 72°C. PCR reactions were performed by using Taq Gold polymerase (PE Biosystems, Foster City, CA) or Taq DNA polymerase (Roche), under the reactions conditions recommended by the manufacturers. A PCR System 2400 (PE Biosystems) was used for all PCR amplifications. The resulting PCR amplicons were loaded onto a 1% SeaKem Gold agarose gel and subjected to electrophoresis at 80 volts for 1 hour and visualized by ethidium bromide staining.

The PCR amplification with the 5′-CS:3′-CS primer set yielded two products, a 1-kb and a 1.2-kb fragment (data not shown). These two fragments represent the gene cassettes contained within two different integrons. The PCR products were purified by using the QIAquick PCR purification kit (Qiagen). No amplicons were obtained from any of the plamid preparations, confirming the chromosomal nature of the antimicrobial resistance. The purified products were sequenced by the dye terminator reaction method described in ABI Prism 377 DNA sequencer (PE Biosystems) instruction manual. DNA sequence analysis was carried out by using the FASTA algorithm of the GCG Wisconsin package.

Computer analysis showed that the 1-kb fragment contained a gene homologous to the ant(3″)-1a gene, a gene that confers resistance to streptomycin and spectinomycin. The 1.2-kb PCR amplicon contained the psE1 gene, which encodes a beta-lactamase that confers resistance to ampicillin. PCR ampification with oligonucleotides specific for the 5′-CS and *sulI* gene yielded a product of the expected size (approximately 1.1 kb larger than the product obtained with the 5′-CS:3′-CS primer set), indicating the presence of *sul*I, which confers resistance to sulfonamides.

We were also interested in determining whether resistance to chloramphenicol was due to the presence of the *flo*-like gene described by Bolton et al. in 1999 (21). Detection of *flo* was carried out by using the oligonucleotide primers, *flo*-1 (5′-AATCACGGGCCACGCTGTATC-3′) and *flo*-2 (5′-CGCCGTCATTCTTCACCTTC-3′) (21). PCR and sequencing data confirmed that all the isolates carried the florfenicol resistance (*flo*-like) gene (data not shown). These findings are consistent with those of earlier reports (7-9,21) and further demonstrate the high degree of clonality among isolates of *S.* Typhimurium MR-DT104 dating back to 1985.

Our data suggest that the strain of MR-DT104 that became prevalent during the mid-1990s had not caused frequent human illness in the United States in 1985 and 1990. Local health officials interviewed the four patients from whom these MR-DT104 isolates were collected in 1985 and 1990; none of them reported traveling outside the United States 30 days before onset of illness. None reported other underlying illnesses, and none reported taking any antibiotics before specimens were collected. These data suggest that domestic transmission of MR-DT104 to humans, perhaps through contaminated food, occurred in 1985 and 1990. Human infection with MR-DT104 did not become prevalent, however, until the mid-1990s. The factors that led to the widespread dissemination of MR-DT104 in humans in the United States in the mid-1990s are unknown.

However, the limited animal data available indicate that MR-DT104 became disseminated in food animals at approximately the same time (6). Factors that contributed to the dissemination of MR-DT104 in animals are poorly understood. Since food animals are the reservoir for most domestically acquired human *Salmonella* infections and transmission from animals to humans occurs through the food supply (5), the rapid dissemination of MR-DT104 among humans in the mid-1990s was likely the consequence of dissemination of MR-DT104 in food animals during the same period. If this hypothesis is correct, it would parallel the experience of the United Kingdom, where MR-DT104 was identified as early as 1984 but did not become epidemic in humans until it was established in cattle in the late 1980s (2).

Molecular evidence supports the suggestion that some of the antimicrobial resistance determinants found in *S.*

Typhimurium DT104 R-type ACSSuT may have emerged, perhaps in Asia, in the early 1980s in other bacteria and were transferred horizontally to DT104 (5). For example, chloramphenicol resistance in MR-DT104 is encoded by a *flo*-like gene that confers resistance to both chloramphenicol and florfenicol (21,22). *flo* was first identified in *Pasteurella piscicida*, the causative agent of pseudotuberculosis, a common disease of marine fish in Asia (23). Florfenicol was evaluated as a therapeutic agent in fish in Asia in the early 1980s (22-26). Kim et al. (27) reported the emergence of florfenicol-resistant strains of *P. piscicida* due to the acquisition of transferable resistance plasmid containing the *flo* gene, in addition to other antimicrobial resistance markers (ampicillin, kanamycin, sulfonamide, and tetracycline). Furthermore, nucleotide sequence analysis of the DNA region containing the florfenicol resistance gene and the two tetracycline genetic determinants showed a 94% similarity to a sequence found in a plasmid from *P. piscicida* (22). Additional evidence supporting the idea of horizontal transfer of multiple antimicrobial resistance determinants comes from a recent study conducted on multidrug resistant *S.* Agona isolates containing a DT104-like antimicrobial resistance gene cluster in their genome (28).

In summary, MR-DT104 isolates with indistinguishable PFGE patterns and carrying the same or highly similar integrons as more recent isolates were present in the United States as early as 1985. These data and the fact that food-producing animals are the reservoirs for most human *Salmonella* infections in the United States suggest that the emergence of MR-DT104 during the mid-1990s probably resulted from the dissemination of MR-DT104 in food-producing animal reservoirs. Laboratory and epidemiologic evidence is insufficient to determine whether this dissemination resulted from spread of a strain already present in the United States or the reintroduction of the strain through importation of contaminated livestock or other means.

Dr. Ribot is the chief of the PulseNet Methods Development and Validation Laboratory, Foodborne and Diarrheal Diseases Laboratory Section, Centers for Disease Control and Prevention. His research interests focus on molecular epidemiology, development of subtyping methods, and identification and characterization of mechanisms of antimicrobial resistance of foodborne pathogens.

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Address for correspondence: Timothy J. Barrett, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop C03, Atlanta, GA 30333, USA; fax: 404-639-3333; e-mail: Tbarrett@cdc.gov

Introduction of *West Nile virus* in the Middle East by Migrating White Storks

Mertyn Malkinson,* Caroline Banet,* Yoram Weisman,* Shimon Pokamunski,† Roni King,‡ Marie-Thérèse Drouet,§ and Vincent Deubel§

West Nile virus (WNV) was isolated in a flock of 1,200 migrating white storks that landed in Eilat, a town in southern Israel, on August 26, 1998. Strong, hot westerly winds had forced the storks to fly under considerable physical stress before reaching the agricultural land surrounding the town. Most of the flock were fledglings, <1 year old, which had hatched in Europe. Thirteen dead or dying storks were collected 2 days after arrival and submitted to the laboratory for examination. Four WNV isolates were obtained from their brains. Out of 11 storks tested six days after arrival, three had WNV-neutralizing antibodies. Comparative analysis of full-length genomic sequences of a stork isolate and a 1999 flamingo isolate from the USA showed 28 nucleotide (nt) (0.25%) and 10 amino acid (0.3%) changes. Sequence analysis of the envelope gene of the stork isolate showed almost complete identity with isolates from Israeli domestic geese in 1998 and 1999 and from a nonmigrating, white-eyed gull in 1999. Since these storks were migrating southwards for the first time and had not flown over Israel, we assume that they had become infected with WNV at some point along their route of migration in Europe.

Since the early 1950s, West Nile fever (WNF) epidemics in human populations of many African, Middle Eastern, and some Mediterranean countries have occurred at approximately 10-year intervals. With the exception of a minor outbreak in France in 1962 (1), however, WNF was considered unimportant to human health in Europe. The situation changed radically in 1996, when an epidemic swept through the city of Bucharest, Romania; nearly 400 human cases of encephalitis occurred and approximately 40 people died (2). Several more cases were reported in 1997 and 1998 (3). Then, July through September 1999, a widespread epidemic of WNF was reported in southern Russia involving approximately 1,000 cases with at least 40 dead (4). Within the 5-year period from 1996 through 2000, WNF was also diagnosed in isolated human patients in the Czech Republic, in 14 horses in Tuscany, Italy, and in 78 horses from the Herault and Gard Provinces, France (5-7). Another European report of a WNF epidemic was received from West Georgia (former Soviet Union) in 1998 (8). In July through October 1999, an outbreak of WNF in humans, horses, and wild and zoo birds was reported in New York (9) and neighboring states. This outbreak was caused by a virus almost identical genetically to one isolated previously from domestic geese in Israel in 1998 (10).

WNF epidemics occur in the late summer and early fall months in temperate regions of Europe when bird migration is at its peak and mosquito populations are greatest. Nevertheless, WNV has been isolated occasionally from actively migrating birds in Europe, emphasizing their importance as carriers of several arboviruses (11). On the other hand, WNV antibodies have been found in wild birds caught in many countries of Europe, Africa, and Asia (12).

The white stork (*Ciconia ciconia*) migrates over the Middle East each fall in numbers estimated at 500,000, (13) but for reasons connected with difficulties in catching them, none of the published serosurveys of wild birds from Europe, Africa, and Asia mention storks. In early September 1998, we received dead storks and serum samples from the town of Eilat in southern Israel. The storks and samples were from a flock of 1,200 birds that had landed in Eilat on August 26, 1998. The storks were weak, having been blown off their usual route of migration through Jordan. The storks' appearance in Eilat was a very rare sight because they normally fly in thermals which take them on a route down the Arava stretch of the Syrio-African Rift Valley where they turn southwest south of the Dead Sea and cross the Sinai Peninsula into Africa (14). The previous recorded sighting of storks in Eilat had been in August and September 1980 (R. Yosef, pers. comm.).

In 1998, unusually strong winds had carried them eastwards, and in an attempt to reach the Sinai, the flock had resorted to powered (flapping) flight. From their bodily conformation, bright golden beaks and legs and wing feathering, most of the flock was classified as juveniles, i.e., hatched in 1998. In this report, we present the results of virologic and serologic studies on this flock and compare the sequences of the stork WNV genome with Israeli isolates from geese (*Anser anser domesticus*) in 1998 and 1999 and a White-eyed Gull (*Larus leucophthalmus*) in 1999. In addition, we include information on the serology of migrants and resident storks in Israel from which blood samples were taken in 1998 through 2000. Based on these data and an evaluation of the routes and seasons of the storks' migration, we suggest that WNV could have been imported into Israel by storks that were infected

* Kimron Veterinary Institute, Beit Dagan, Israel; † Veterinary Services and Animal Health, Beit Dagan, Israel; ‡ Nature and Parks Authority, Jerusalem, Israel; and § Pasteur Institute, Lyon, France

along their fall migratory routes over southeast Europe and the Near East.

Materials and Methods

Storks

Dead and dying storks were collected from the area around the landing site 2 days after arrival of the flock on August 26. They were immediately placed in cold storage until transported to the laboratory on September 4, September 9, and October 8, when the birds were thawed and weighed, and necropsies were performed. A total of 13 frozen storks were received.

White-Eyed Gulls

A small breeding colony of approximately 20 gulls was housed in a closed pen with wire-mesh walls at the Department of Zoology, University of Tel Aviv. In November 1999, several birds were found paralyzed, and two had died.

Isolation of Virus

Brains were removed aseptically from the storks and gulls and homogenized by grinding in borate-buffered saline (pH=7.6). The homogenate was centrifuged at 1,500 rpm for 10 min. Other tissues were not examined. The supernatant was removed and used for intracranial inoculation of suckling mice and infection of Vero cells and yolk sac inoculation of 7-day-old embryonated eggs. Embryonic mortality occurred 3 to 4 days after inoculation and the embryos were colored cherry red but without hemagglutinating activity for chicken erythrocytes in the allantoic fluids. An arbovirus was therefore suspected. Likewise, morbidity of the mice was accompanied by spastic paralysis, a further sign of virally induced encephalitis. In Vero cell culture, a cytopathic effect (CPE) was seen within 3 to 5 days of inoculation.

Serology

Storks were aged either as fledglings (<1 year old) or mature birds by their wing feathering and intensity of the yellow pigment of the beaks and legs, (E. Gorni, pers. comm.). Blood samples were taken from the Eilat flock 6 days after arrival. Other storks from this flock were placed in a nature reserve to recuperate, and blood samples were taken in September and October. In October 1998 and September 1999, storks were caught as they were migrating over Israel, and blood samples were taken. The January 2000 flock consisted of late migrators that were caught while roosting. The blood samples were taken from the White-eyed Gulls 1 week after isolation of WNV.

Sera were diluted twofold commencing at 1:10 in WNV suspension containing 100 50% tissue culture infective dose ($TCID_{50}$)/0.1 mL in microwell plates. The virus-serum mixture was allowed to stand at room temperature for 1 hr, and a suspension of 10^5 Vero cells in 0.1 mL was added to each well. The plates were incubated at 37°C in 5% CO_2/95% air for 4 days. The neutralizing titer of the serum was calculated as the highest dilution with 50% CPE.

Indirect Immunofluorescence Assay (IFA)

When CPE of the infected Vero cell culture was observed, a glass coverslip was placed in the dish receiving the succeeding passage. Three days later the coverslip was removed and the cells fixed with cold acetone. Identification of the cytopathic agent was performed by indirect immunofluorescence. Panels of monoclonal antibodies to *Alphavirus*, and Flaviviruses (Table 1) were reacted with the Vero cells and then incubated with fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulins. The *Flavivirus* monoclonal antibodies included 2B4, 6B8, and 6E12 from South Africa; 5F10, 1C9, and 1B4 from Israel, F7/101 from Oxford, U.K.; and 3H6 and 813 from Townsville, Australia. The *Alphavirus* (*Sindbis virus* [SINV]) antibodies were 30.11 and 30.12 from Oxford and 2F2 from Australia. Counter-staining was performed with Evans blue.

RT-PCR

Brain extracts were performed as described above and the supernatant used to extract RNA with the QIAamp Viral RNA kit (Qiagen, Valencia, CA) and the RNAs resuspended in 60 µL of Viral Lysis Buffer (Qiagen, Valencia, CA) elution buffer according to the manufacturer's protocol. Reverse transcription-polmenase chain reaction (RT-PCR) was performed on a gene fragment of the envelope protein using the primer pair WN132 (5′ GAAAACATCAAGTATGAGG 3′) and WN240 (5′ GAGGTTCTTCAAACTCCAT 3′) (genome positions 1,402 and 1,656), resulting in the synthesis of a 255-bp

Table 1. Monoclonal antibodies used for characterization of goose, stork, and gull isolates study, Israel, 1998 and 1999

MAb	Reactivity	Reference
2B4	WNV-type specific (E gene)	15
6B8	WNV-type specific (E gene)	15
6E12	WNV-type specific except KUN (NS4a gene)	15
5F10	WNV specific	Bat-El Lachmi
1C9	Flaviviruses	Bat-El Lachmi
1B4	Flaviviruses	Bat-El Lachmi
F7/101	West Nile	16
6D12	Flaviviruses except *Edge Hill* and *Dengue virus*	17
3H6	*Murray Valley encephalitis virus* (*Flavivirus*)	17
813	*Yellow fever virus* (*Flavivirus*)	18
30.11	SINV	19
30.12	SINV	19
2F2	SINV	[a]

WNV=*West Nile virus*; SINV=*Sindbis virus*.
[a]TropBio, Townsville

product (20). The resulting DNA fragment was visualized on 1.5% agarose gel stained with ethidium bromide.

Gene Sequence Analysis

WNV RNA was extracted from the second passage of Vero cell-infected culture fluid after isolation in a suckling mouse brain, using the QIAamp Viral RNA kit (Qiagen). Viral RNA was extracted from supernatant fluid according to the manufacturer's protocol and the RNA resuspended in a final volume of 100 µLof RNase-free water. Six overlapping double-stranded cDNA templates were generated by using WNV-specific primer pairs provided by the Centers for Disease Control and Prevention (9). Additional primers with sequences designed from the sequence of WN-NY99 were used to amplify about 100 nucleotides from the far 3′ and 5′ extremities (Table 2). The envelope protein genes from WN viruses isolated in 1998 and 1999 from geese and in 1999 from a White-eyed Gull were amplified using primer pairs designed in the corresponding strands.

Amplified cDNAs were purified by ion-exchange chromatography and precipitated with 2v of isopropanol. Both strands of the purified cDNAs were sequenced by using the Taq Dye Deoxy Terminator Cycle sequencing kit (Perkin Elmer Corp./ Applied Biosystem, Norwalk, CN) using primers spaced about 400 bases apart on the genome (9). Cycle sequencing was performed by combining about 0.2 pmols of purified cDNA and 30 pmols of primer and following the manufacturer's protocol. Sequences were aligned with the Clustal W alignment (21).

Results

Virus Isolation from Storks and Gulls

A total of four Flaviviruses were isolated from storks in Vero cells—one from a pool of three brains from the first group of dead storks collected on August 28, 1998; one isolate from one of six individual brains; and two isolates from four brains also collected on August 28. The three pooled stork brains from the first group were also injected into three litters of baby mice, causing paralysis. As noted previously, all the storks had been collected 2 days after landing and were frozen immediately. The four isolates were confirmed as WNV by indirect immunofluorescent antibody (IFA) using monoclonal antibodies and by RT-PCR. No *Alphavirus* reactivity was detected in any of the brains by IFA (Table 3). One WNV was isolated from the brain of one of the White-eyed Gulls found dead in November 1999 in Tel Aviv (data not shown).

Table 2. Oligonucleotide primers used for framing the entire *West Nile virus* (WNV) genome and the envelope (E) gene, IS98-ST1

Localization in the WNV genome	N° nucleotide at the 5′ end	Sequence
5′-end	1	AGTAGTTCGCCTGTGTGAGCTGA-CAAAC
3′-end	10,934 (c)	AGATCCTGTGTTCTCGCACCAC-CAGCCAC
M-gene	889	GGATGGATGCTWGGKAGCAAC
NS1-gene	2,557 (c)	CCATCCAAGCCTCCACATC

(c)=complementary sequence; W=(A+T); K=(G+T).

RT-PCR

All three homogenates of the mouse brains inoculated with the brains of the first group of storks were RT-PCR positive. Two of the six brains of the second group and all four of the third group were RT-PCR positive. Thus, a total of one pool of 3 brains and 6 individual brains from 10 storks tested were RT-PCR positive (Table 3).

Sequence Analysis

The full-length RNA genome of the WNV isolate from one of the stork-1998 (IS98-ST1) (GenBank accession No. AF 481864) has been sequenced. With the exception of about 30 nucleotides at each end of the genome used as primers for cDNA amplification (Table 2), the complete nucleotide sequence of the viral RNA has been determined (data not shown). The WNV genome arrangement is similar to those published for WNV-Nigeria, WNV-NY99, and HNY1999 (9, 22, 23). The genome is 11,029 nucleotides in length and contains one long open reading frame of 10,302 nt starting at nt 97. Nucleotide sequence comparison of IS98-ST1 with WNV-NY99 (9) showed 28 mutations in the coding region (99.75% similarity). Of the mutations, five were transversions, and the rest occurred at the third codon. Ten amino acid changes were found. The change at position 51 (A51 to V) in WNV-NY99 E protein was not found in the HNY1999 sequence. Other mutations were observed in NS1 (N17 to S), NS2A (R165 to G), NS2B (G82 to D and E83 to G), NS3 (P496 to L and E521 to D), and NS5 (S54 to P, N280 to K, and A372 to V).

The envelope gene sequence of IS98-ST1 was compared to those from goose98 (GenBank accession No. AY033388) and goose99 (GenBank accession No. AY033391) and gull99 (GenBank accession No. AY033390) and included HNY 99 as the consensus sequence (Figure 1). The comparison showed 3 nt changes, of which one was unique for IS98-ST1 strain (position 1,179), one common to goose99 and gull99 (position 729) and one shared by gull99 and IS98-ST1 (position 3). The 501 amino acids of the E genes of the four strains (IS98-ST1, goose98, goose99, and gull99) were identical (data not shown).

Serology

Table 4 presents the WNV neutralization titers of groups of storks according to the month and year of the blood samples, the storks' age, and geographic location. In 1998 sera were collected from the Eilat flock on September 2, and from eight older storks in a wildlife sanctuary during September and October. Late migrating storks were also captured in northern Israel during their autumn migration in October, and 10 birds from various bird sanctuaries were also sampled. Between January and May 1999, all five storks resident in northern Israel had WNV antibodies. With the fall migration, blood samples

Table 3. Summary of viral isolations and polymerase chain reaction (PCR) examinations of the 13 storks that arrived in Eilat, Israel, August 26, 1998

Date of collection (1998)	Date of laboratory examination	No. of storks examined	No. of WNV isolates (Vero cells)	No. of brains PCR positive
August 28	Sept 4	3[a]	1[a]	3[b]
August 28	Sept 9	6	1	2
August 28	Oct 8	4	2	4
Total		13	4	9

[a] Pooled sample.
[b] From three litters of suckling mice inoculated with the pool of three stork brains.
WNV=*West Nile virus*

were taken from a group of six storks, consisting of four fledglings and two older birds. One stork in each age group had WNV antibodies.

Storks examined through June 30, 2000, consisted of two groups, one was a flock of overwintering adults that had migrated in September 1999 and the others were four fledglings hatched in April 2000 from parents that had overwintered and bred on the Golan Heights. Antibodies were detected in 9 of the 12 adults but in none of the young birds.

Thus, a total of 65 stork sera were examined of which 19 were from birds <1 year old. In the 19 birds <1 year, neutralizing antibodies were found in 4 of them (21.1%), whereas in the older group of 46 storks, 33 (71.7%) were seropositive.

Of the 11 White-eyed Gull sera examined 1 week after the isolation of WNV, 8 were seropositive, 6 had titers > 1:1280, one of 1:640, and one of 1:80, and three were < 1:10.

Discussion

In this report we describe the isolation of WNV and detection of virus activity by RT-PCR in the brains of storks that were grounded while migrating southward on a route that took them along the eastern edge of the Syrian-African Rift Valley, Jordan. Strong winds in the last days of August 1998 carried the flock off course, but by active flying the birds had landed in Eilat. The relatively high number of isolates and PCR-positive brains may be due to a compounded effect of the stress imposed on the flock by migration and the strenuous physical efforts exerted by the birds in flying back on course (24,25). Recoveries of SINV and WNV from white storks caught in southern Sinai during the fall of 1998 through spring 1999 as they migrated towards Africa have been reported recently (26). Viruses were isolated from 13 of 25 birds; of these 12 were SINV and one was WNV.

We were able to gauge the age of the serum samples according to whether the stork had hatched in the year it was caught (fledgling bird) or in a previous year. With very few exceptions all the fledglings, and notably the Eilat flock, had hatched in Europe in the spring of the same year and had yet to complete a full migratory cycle by overwintering in Africa. Thus, these birds could have been exposed to WNV in Europe, either near the nesting sites or along the route of migration, especially along the River Danube and its tributaries. The ornithologic and epidemiologic features of the Danube Delta have recently been described by Savage et al. (27) in relation to the 1996 epidemic in Bucharest.

The stork summer migration from northern European countries follows the Danube to reach the Black Sea and is in the reverse direction to the storks' breeding sites in central and northern Europe during the spring migration (14). Since none of the fledglings were leg-banded, we cannot identify where infection could likely have occurred. It is unlikely that infection occurred in Eilat because the injured and dead birds were collected within 2 days of landing there. In contrast, seropositive older birds were most likely exposed either to a disease-endemic environment, as is found in most African countries where they overwinter (28), or in Europe, where they breed. Savage et al. (27) has recently shown that serum samples from domestic and wild fowl collected in the vicinity of Bucharest during the 1996 WNV epidemic had neutralizing antibody to

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          1                                                    50
Goose 98  ********** ********** ********** ********** **********
Goose 99  ********** ********** ********** ********** **********
Gull 99   **t******* ********** ********** ********** **********
IS98-ST1  **t******* ********** ********** ********** **********
WN-NY 99  TTCAACTGCC TTGGAATGAG CAACAGAGAC TTCTTGGAAG GAGTGTCTGG

          701                                                  750
Goose 98  ********** ********** ********** ********** **********
Goose 99  ********** ********** ********a* ********** **********
Gull 99   ********** ********** ********a* ********** **********
IS98-ST1  ********** ********** ********** ********** **********
WN-NY 99  GGAACAGAGA GACGTTAATG GAGTTTGAGG AACCACACGC CACGAAGCAG

          1151                                                1200
Goose 98  ********** ********** ********** ********** **********
Goose 99  ********** ********** ********** ********** **********
Gull 99   ********** ********** ********** ********** **********
IS98-ST1  ********** ********** ********t* ********** **********
WN-NY 99  TAGTGGTGGG CAGAGGAGAA CAACAGATCA ATCACCATTG GCACAAGTCT
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Figure. Abbreviated alignment of E gene sequences of Israeli isolates Goose 98, Goose 99, White-eyed gull 99, and IS98-ST1 with the consensus sequence of WN-NY99. The nucleotide numbers correspond to their location in the envelope gene.

Table 4. *West Nile virus* neutralizing antibodies of storks caught at various sites, Israel, 1998–2000

Date of collection					
Year	Month	Location	Age	No. pos/ No. test	SN[a] titer
1998	Sept 1	Eilat I	<1 year	3/11	2x40, 1x>1280
	Sept 9	Eilat II	Adult	3/3	320, 640, 1280
	Oct 8	Eilat III	Adult	5/6	20->1280
	Oct 12	Kfar Rupin (migrating)	Adult	4/8	2x80, 2x640
	Oct 20	N. Israel (Resident)	Adult	6/10	1x40, 4x80,1x160
1999	Jan 10	N. Israel	Adult	2/2	80, 160
	Apr 10	(Resident)		1/1	80
	May 12			2/2	160, 320
	Sept 5	Kfar Rupin	<1 year	1/4	40
		(migrating)	Adult	1/2	80
2000	Jan 3	Neve Eitan (migrating)	Adult	9/12	4x80, 4x160, 1x320
	Jun 15	Golan Heights (resident breeders)	3 months	0/4	<10
		Total	<1 year	4/19	(21.1%)
			Adults	33/46	(71.7%)
				37/65	(56.9%)

[a]SN=serum neutralization

WNV. Thirty (41%) of 73 domestic birds, including 13 ducks and 1 goose, were positive, although the actual titers were not stated.

With the appearance of the flock in Eilat in August, the fall migration was proceeding over Israel at the same time. In September a WNV outbreak affecting young geese was recorded on farms throughout Israel. Mortality was as high as 40% in some flocks (10). The marked susceptibility of domestic geese to WNV had not been reported previously in Israel or elsewhere, although goose management had not changed over the years with flocks traditionally reared in open farmyards. The emergence of the goose as an incidental host therefore appears to be related to a change in the WNV genome possibly related to what had occurred in Bucharest in 1996. A strong homology in the nucleotide sequence was observed between the goose-1998/1999, gull-1999, and stork-1998 isolates. Comparative analysis of a short E gene fragment of several WNV strains from Romania 96, Kenya 98, and Russia 99, has yielded a high level of identity (96%) among the strains (9,28-30). This suggests that the WNV epizootic observed in Israel between 1997 and 2000 may be attributable to viruses that were circulating in eastern Europe or elsewhere in the Near East since 1996. On the other hand, the identification in 2000 of a second genotype circulating in Israeli human and avian populations clouds the molecular epidemiologic picture (31, Banet et al., manuscript in preparation). Based on a comparison of a 1,278-bp sequence of the E gene and a 1,648-bp fragment spanning the preM, M, and the 5′ terminus of the E gene, this second genotype resembled even more closely (99% homology) the Romanian 96, Kenyan 98, and Volgograd 99 strains. Therefore, a different migrating flock or species might have introduced this variant.

The observation that IS98-ST1 is also highly virulent for zoo and wild birds is mirrored by reports from the northeastern United States, where many species of wild birds succumbed to infection (32,33). A unique feature of the Israeli isolate was its marked pathogenicity for flocks of young geese between 3 and 12 weeks of age, causing considerable mortality, whereas the New York isolates targeted mainly the crow (34). Despite the phylogenetic similarity of these two isolates, no plausible explanation has been offered for the unique appearance of WNF in North America in 1999. Attempts to link transatlantic migration of birds, mainly from southern and western Europe, with the dispersion of WNV have identified several candidate species that migrate across the Atlantic Ocean (35). In this respect the role of water birds, especially members of the gull family, should also be considered as carriers of flaviviruses.

The emergence of the epidemic in Romania in 1996 was a unique epidemiologic event that heralded the renewed spread of WNV in Europe after more than 30 years of silence. The virus' dispersion through the Near East and beyond follows a pattern that is best explained by bird migration.

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Biographical Sketch

Dr. Malkinson works in the Division of Avian Diseases, Kimron Veterinary Institute on research related to avian viruses.

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Address for correspondence: M. Malkinson, Kimron Veterinary Institute, Beit Dagan, P.O. Box 12, Israel 50250; fax: 972-3-9681739; e-mail: martinm@moag.gov.il

Outcomes of Treated Human Granulocytic Ehrlichiosis Cases

Alan H. Ramsey,*† Edward A. Belongia,‡ Craig M. Gale,‡ and Jeffrey P. Davis†

We conducted a case-control study in Wisconsin to determine whether some patients have long-term adverse health outcomes after antibiotic treatment for human granulocytic ehrlichiosis (HGE). A standardized health status questionnaire was administered to patients and controls matched by age group and sex. Consenting patients provided blood samples for serologic testing. Among the 85 previously treated patients, the median interval since onset of illness was 24 months. Compared with 102 controls, patients were more likely to report recurrent or continuous fevers, chills, fatigue, and sweats. Patients had lower health status scores than controls for bodily pain and health relative to 1 year earlier, but there was no significant difference in physical functioning, role limitations, general health, or vitality measures. The HGE antibody titer remained elevated in one patient; two had elevated aspartate aminotransferase levels. HGE may cause a postinfectious syndrome characterized by constitutional symptoms without functional disability or serologic evidence of persistent infection.

Human granulocytic ehrlichiosis (HGE) is a recently identified tickborne infectious disease caused by a bacterium species of the genus *Ehrlichia* that preferentially infects granular leukocytes (1). HGE was first described in the United States in 1994 in residents of Wisconsin and Minnesota (2). During 1986 through 1997, 449 HGE cases were identified in the United States, mostly in the Northeast and Upper Midwest, despite limited reporting requirements (3). The primary vector of HGE is *Ixodes scapularis* (4), commonly known as the deer tick, which is also the vector of Lyme disease.

Onset of clinical signs and symptoms of acute HGE typically follow a 5- to 10-day incubation period. The acute illness is nonspecific and often includes fever, chills, headache, and myalgia. Abnormal laboratory findings may include leukopenia, thrombocytopenia, and mildly elevated liver enzymes (5). Seventeen percent to 56% of patients with HGE are hospitalized, and the case-fatality rate may be 0.7%-4.9% (3,5). Patients treated with doxycycline usually defervesce within 24 to 48 hours (5).

The acute phase of HGE is well characterized. The potential for persistent infection has been suggested but has not been evaluated. We conducted an exploratory case-control study of patients previously treated for HGE to assess health status, symptoms, and changes in serologic status.

Methods

Case Definition

The Centers for Disease Control and Prevention (CDC) HGE case definition was used. A confirmed case of HGE was defined as any acute febrile illness with laboratory confirmation consisting of 1) a fourfold or greater change in antibody titer to *Ehrlichia equi* by immunofluorescence antibody (IFA) test, 2) amplification of specific ehrlichial DNA sequences by polymerase chain reaction (PCR), or 3) demonstration of bacterial microcolonies (morulae) in leukocytes together with a single elevated titer. A probable case was defined as an acute febrile illness with a single elevated antibody titer $\geq$1:64 or presence of morulae (6).

Patients

Both confirmed and probable cases of HGE were included in the case-control study. Although HGE officially became a notifiable disease in Wisconsin on April 1, 2000, we identified 111 cases of HGE in northwestern Wisconsin through specific surveillance activities from May 1996 to December 1998 (7). These included 86 (77%) confirmed and 25 (23%) probable cases. The 1996 cases were detected by laboratory-based surveillance within the Marshfield Clinic system, a network that provides health care to persons in northern and northwestern Wisconsin. The 1997 and 1998 cases were detected through active surveillance in a 13-county region in northwestern Wisconsin.

We selected 225 controls from a pool of approximately 880,000 living Wisconsin residents who had received medical care from the Marshfield Clinic regional network. Controls were randomly selected from the same zip codes as patients and frequency matched on age group ($\leq$19, 20-39, and 10-year intervals for persons $\geq$40 years old) and gender. A structured questionnaire on health status and symptoms was administered by telephone to patients and controls. The survey consisted of items from six domains of the Medical Outcomes Trust 36-Item Short Form Health Survey (SF-36) (8), including questions about physical function, role limitations due to physical health problems, bodily pain, general health, vitality (energy vs. fatigue), and health relative to 1 year earlier. In each domain, a higher score indicated a better health state. In addition, participants were asked if they had experienced the following symptoms continuously or repeatedly in the previous year: fever, fatigue, shaking chills, sweats, muscle aches,

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Wisconsin Division of Public Health, Madison, Wisconsin, USA; and ‡ Marshfield Medical Research Foundation, Marshfield, Wisconsin, USA

headache, joint pains, muscle weakness, nausea, cough, shortness of breath, diarrhea, vomiting, poor appetite, or confusion. The choice of signs and symptoms was based on known clinical manifestations of acute HGE (2,5,7).

Appropriate informed consent was obtained, and clinical research was conducted in accordance with guidelines for human experimentation as specified by the U.S. Department of Health and Human Services and the Marshfield Medical Research Foundation.

Diagnostic Methods

Case-patients were asked to provide blood samples for HGE serologic testing and measurement of aspartate aminotransferase (AST). We performed polyvalent IFA on sera using *E. equi* substrate (ProtaTek International, St. Paul, MN) and fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin (Kallestad Diagnostics, Chaska, MN) diluted to 1:100. *E. equi* is closely related or identical to the agent that causes HGE (1,9).

Statistical Analysis

Data were analyzed by using univariate logistic regression models with calculation of odds ratios (OR) and 95% confidence intervals (CI) by using SAS software (Version 6.12, SAS Institute, Cary, NC); p values $\leq$0.05 were considered statistically significant, and all are two-sided.

Results

We enrolled 85 (77%) of 111 persons with previously treated HGE (patients) and 102 (45%) of 225 controls. Of the 111 patients, we were unable to contact 13 (12%); 8 (7%) refused participation; 4 (4%) had since died of causes unrelated to HGE; and 1 (1%) did not complete the survey. Among the 225 controls, we were unable to contact 56 (25%); 61 (27%) declined to participate; and 6 (3%) surveys were incomplete.

There were 73 (86%) confirmed and 12 (14%) probable cases of HGE. Among confirmed cases, 32 (44%) were confirmed by serology alone; 13 (18%) by serology and polymerase chain reaction (PCR); 10 (14%) by PCR alone; 9 (12%) by serology, PCR, and presence of intracytoplasmic morulae; 6 (8%) by serology and presence of morulae; and 3 (4%) by PCR and morulae. Of the 12 probable cases, 9 (75%) had a single IFA serologic titer $\geq$64, and 3 (25%) had intracytoplasmic morulae identified in blood. The median age of patients was 58 years (range 6-88 years), and the median age of controls was 57 years (range 6-88 years); 66% of patients and 73% of controls were male. Data on coexisting conditions were available for all 102 controls and 39 (53%) of 73 confirmed cases. Comparison of the 39 cases and 102 controls demonstrated no significant difference in the prevalence of cancer (p=0.36), stroke (p=0.49), heart disease (p=0.89), or diabetes (p=0.65).

For HGE patients, the median interval from illness onset to telephone interview was 24 months (range 10-40 months). Twelve patients had onset of HGE in 1996, 35 in 1997, and 38 in 1998. The illness was severe enough in 32 (38%) patients to require hospitalization. All 85 enrolled patients received some form of antibiotic treatment for acute illness. Of the 59 patients for whom we have sufficient information, 53 (90%) were prescribed doxycycline for $\geq$7 days. The median interval from illness onset to initiation of treatment was 7 days. Nineteen (22%) enrolled patients had evidence of Lyme disease during the acute illness, either the characteristic rash, erythema migrans (14 patients), or seroconversion to *Borrelia burgdorferi* (7 patients). Six (8%) of 76 enrolled patients tested also seroconverted to *Babesia microti* after the acute illness.

Compared with controls, patients were more likely to report the following constitutional symptoms either continuously or repeatedly during the previous year: fevers, chills, sweats, and fatigue (Table 1). Patients had lower SF-36 health status scores for bodily pain (p=0.03) and health compared with 1 year earlier (p=0.02), but no differences existed for physical function, role limitations due to physical health, general health, or vitality (Table 2). When probable cases were excluded from analysis, confirmed patients were also more likely to report fevers (OR 4.1, 95% CI 1.0-15.9), chills (OR 3.8, 95% CI 1.3-11.4), sweats (OR 2.8, 95% CI 1.4-5.8), and fatigue (OR 1.7, 95% CI 1.0-3.1) during the previous year. Confirmed cases also had lower SF-36 scores for relative health (p=0.02), but no significant differences existed for bodily pain, physical function, role limitations due to physical health, general health, or vitality. When asked about their health status relative to before infection, 39% of patients believed their current health was "somewhat worse" or "much worse."

The presence of continuous or recurrent constitutional symptoms and the duration of acute illness were not correlated. Patients who were hospitalized or who started antibiotics more than 14 days after onset of illness were no more likely

Table 1. Recurrent or continuous symptoms experienced during the preceding 12 months by human granulocytic ehrlichiosis patients and controls, Wisconsin, 1999

Symptom	Patients (N=85) number (%)	Controls (N=102) number (%)	OR	95% CI
Fevers	12 (14.1)	3 (2.9)	5.4	1.7-24.4
Shaking chills	16 (18.8)	5 (4.9)	4.5	1.7-14.3
Sweats	31 (36.5)	16 (15.7)	3.1	1.6-6.3
Fatigue	47 (55.3)	38 (37.3)	2.1	1.2-3.7
Confusion	16 (18.8)	12 (11.8)	1.7	0.8-4.0
Muscle weakness	27 (31.8)	30 (29.4)	1.1	0.6-2.1
Headache	21 (24.7)	13 (12.8)	1.1	0.7-1.9
Muscle aches	40 (47.1)	44 (43.1)	1.0	0.7-1.5
Joint pains	50 (58.8)	54 (52.9)	0.9	0.6-1.2
Nausea	14 (16.5)	15 (14.7)	0.8	0.5-1.4

OR = odds ratio; 95% CI = 95% confidence interval.

Table 2. Mean scores and standard errors (SE) in six domains of the Medical Outcomes Study 36-Item Short-Form Health Survey in patients and controls[a]

Domain	Patients mean (SE)	Controls mean (SE)	p
Physical function	77 (2.3)	74 (2.8)	.83
Physical health-related role limitations	68 (4.1)	74 (3.7)	.16
Bodily pain	62 (2.9)	69 (2.5)	.03
General health	59 (1.4)	59 (1.4)	.64
Vitality	57 (2.4)	58 (2.1)	.46
Relative health	45 (2.4)	52 (1.8)	.03

[a]Optimal score in each category is 100.

to experience recurrent symptoms than those who received antibiotic treatment within 14 days. Similarly, patients with 1) a preexisting chronic illness, 2) intragranulocytic morulae in an acute-phase blood smear, 3) laboratory evidence of concurrent Lyme disease or babesiosis, 4) anemia, or 5) a high acute- or convalescent-phase reciprocal HGE IFA antibody titer ($\geq$512) were no more likely than the other patients to experience one or more of the recurrent or continuous symptoms.

Serum specimens were submitted for serologic testing by 70 (82%) of 85 patients. The HGE IFA antibody titer remained elevated ($\geq$1:64) in one (1.4%) of 70 specimens tested. This patient had a very high titer after acute infection (1:2,048), which remained elevated (1:256) 1 year later. He experienced continuous or recurrent fatigue, vomiting, and headaches. Two (2.9%) of 69 patients had elevated (>100 U/L) serum AST levels; one complained of continuous or recurrent chills, sweats, and fatigue.

Discussion

Our results demonstrate that some patients with treated HGE may experience more fevers, chills, sweats, and fatigue than controls 1-3 years after onset of illness. Some patients also experience more bodily pain and have a poorer perception of their health compared with 1 year ago than controls, but they do not have any functional disability. Except for bodily pain, these findings persisted when only confirmed cases were included in the analysis. We found no serologic evidence to suggest the occurrence of persistent ehrlichial infection. These symptoms may therefore be attributed to a postinfectious syndrome rather than persistent or recurrent infection.

Few previous studies have evaluated the long-term serologic profile of treated HGE. Results of one study of treated HGE demonstrated that antibody titers remained elevated 11 to 14 months after onset in 5 of 10 patients tested (10). In another study of HGE patients, not all of whom were treated, Bakken et al. detected *E. equi* antibodies in 11 (46%) of 24 patients at 12 months, 4 (44%) of 9 at 18 months, and 2 patients (denominator not reported) at 30 months (5). The same researchers found that sera from 71 (15%) of 475 asymptomatic residents of northwestern Wisconsin contained antibodies to *E. equi* (11). In our study, only 1 of 70 patients had a persistently elevated antibody titer; however, specimens were collected more than 1 year after acute onset in approximately 95% of patients, and the geographic distribution of patients differed from that of the Bakken study.

Animal studies and case reports have suggested the possibility of chronic or recurrent HGE. Experimental ehrlichiosis infections in dogs and horses have demonstrated the presence of *E. canis* and *E. equi*, respectively, in tissues up to 2 months after treatment with doxycycline (12,13). An HGE patient from Wisconsin was one of the first anecdotal reports to suggest this possibility in humans. In that case, serologic evidence of a chronic ehrlichial infection or reinfection was identified 3 years after treatment for Lyme disease and HGE coinfection (14). Another study from Connecticut included one patient who had a specimen positive by PCR 7 weeks after treatment, indicating a possible persistent ehrlichial infection. In that study, PCR was used to demonstrate the presence of ehrlichial DNA in the blood of some patients who were seronegative (15). Dumler and Bakken detected HGE agent DNA by PCR 2 to 30 days after illness onset in four patients; two of them had no HGE antibody detectable at the time (16). Compared with the serology testing used in this study, PCR may be a more sensitive assay in detecting late ehrlichial infection (17).

Our study used current national case definitions for HGE (6). These criteria allow for positive PCR alone as laboratory confirmation. Ten (14%) of the 73 confirmed HGE cases were confirmed by positive PCR alone. However, a recent report by the American Society of Microbiology's Task Force on Consensus Approach for Ehrlichiosis (CAFÉ) considers positive PCR alone (without other laboratory support) to represent probable laboratory evidence of HGE (18). If we had applied CAFÉ criteria to our study, we would have had 63 (74%) confirmed and 22 (26%) probable cases of HGE.

The primary outcome measures in this study were based on self-reported symptoms. Without laboratory confirmation of persistent infection, recall bias should be considered as a possible explanation for the findings. Because of the severity of their past illness and because this was not a blinded study, HGE patients may have been more aware of their constitutional symptoms and had better recall of them than controls. Selection of controls is another potential source of bias if they were less likely to have coexisting chronic diseases compared with cases. However, we found no statistical difference in the prevalence of cancer, stroke, heart disease, or diabetes between cases and controls.

Previous studies of various infectious diseases have suggested that convalescence from illness is significantly dependent on the emotional state of the patient. In those studies, as in ours, fatigue was often a persistent symptom (19-21). In a study of recovery from influenza, Imboden noted that "delayed recovery following acute self-limited illness occurs in persons who respond to psychological tests in patterns characteristic of depression-prone patients" (19). We did not collect baseline psychological data, but the subset of HGE patients with

recurrent or persistent constitutional symptoms may have been psychologically predisposed to a protracted convalescence.

In summary, we found that a subset of patients with HGE have persistent constitutional symptoms 1-3 years after treatment, without functional disability. Further research is needed to determine whether these symptoms are related to the previous ehrlichial infection or other causes. In the absence of serologic evidence of persistent infection, we believe these symptoms are most likely due to a postinfectious syndrome. Further studies that use PCR testing of convalescent-phase samples would be helpful to exclude the remote possibility of persistent *Ehrlichia* infection.

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Dr. Ramsey is currently a resident in the University of Wisconsin Family Medicine Residency Program. His research interests are broad and include infectious disease epidemiology and international health.

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Address for correspondence: Alan H. Ramsey, UW Department of Family Medicine; 777 South Mills Street, Madison, WI 53715-1896, USA; fax: 608-263-5813; e-mail: aramsey@belville.fammed.wisc.edu

Laboratory Reporting of *Staphylococcus aureus* with Reduced Susceptibility to Vancomycin in United States Department of Veterans Affairs Facilities[1]

Stephen M. Kralovic,*†‡ Linda H. Danko,*† and Gary A. Roselle*†‡

A national survey was sent to all appropriate Veterans Health Administration (VA) medical facilities asking about the ability to test for *Staphylococcus aureus* with reduced susceptibility to vancomycin (SARV) (MICs ≥4 μg/mL). For those with this ability, a request was made for the number of patients having SARV isolated during a 1-year period. Nineteen patients from eight sites across the country had isolation of SARV. Of these, MicroScan (Dade Behring, Inc, MicroScan Division, West Sacramento, CA) technology was used for 17 patients, Vitek (Hazelwood, MO) was used for 1 of the remaining 2 patients, and E-test (AB Biodisk North America, Inc, Piscataway, NJ) for the other. All patients with this organism had microbiology testing done onsite in the reporting VA facility's College of American Pathologists-approved laboratory. For comparison, similar data were obtained for a 1-year period 2 years prior to the current survey; seven patients from four sites were verified to have a SARV. Between the two survey periods the reported cases of SARV increased 170%, indicating a need for continued surveillance and potentially a need to initiate a collection of isolates for further analysis.

Emerging microbial resistance is a substantial threat to health (1). With the discovery of methicillin-resistant *Staphylococcus aureus* (MRSA) that also had intermediate resistance to vancomycin in 1996 in Japan, more intense scrutiny has been given to identifying resistance and reduced susceptibility in staphylococcal species (2-4). Even before the Japanese isolate was identified, in vitro evidence that vancomycin-resistant enterococci (VRE) could transfer resistance to staphylococci led to concern for spread of vancomycin resistance to the staphylococci (5-7).

In 1995, the Centers for Disease Control and Prevention (CDC) recommended microbiology laboratories be vigilant for the occurrence of vancomycin resistance in staphylococci along with confirmatory testing and reporting such resistance to public health authorities (5). Further, recommendations in 1997 called for vigilance for reduced susceptibility to vancomycin (MIC ≥4 μg/mL) rather than just vancomycin resistance (MIC ≥32 μg/mL) (8,9). These recommendations included awareness of the significance of isolates with reduced susceptibility, confirmatory testing of suspect isolates, retesting staphylococci isolated from patients who have failed to respond to vancomycin therapy, and notification of public health authorities. The National Committee for Clinical Laboratory Standards (NCCLS) has set *S. aureus* breakpoints for vancomycin at ≤4 μg/mL is interpreted as susceptible, 8-16 μg/mL is intermediate and ≥32 μg/mL is resistant (10). Despite the fact that an MIC = 4 μg/mL is defined as susceptible by NCCLS standards, it is considered to be at the borderline of resistance (11). In particular, *S. aureus* strains that are methicillin or oxacillin resistant and that have an MIC to vancomycin of ≥4 μg/mL should be suspected for decreased susceptibility to vancomycin and should be considered for additional testing strategies because of the possible subpopulation heterogeneity of *S. aureus* isolates with these MIC results (11,12).

Recent studies from CDC indicate that proper identification of antibiotic resistance may be difficult despite adequate capacity for testing (13). A selected survey of laboratories participating in CDC surveillance (Active Bacterial Core Surveillance and Emerging Infections Programs Network) indicates that these issues may occur despite active participation in CDC activities (13). A more recent study involving the worldwide WHONET users suggested that these difficulties in identification of antibiotic resistance might be even greater (14). These studies indicate that real-world application of recommended standards into typical day-to-day functioning does not mimic the functioning and results seen in tightly controlled study situations.

*Department of Veterans Affairs Central Office, Washington, D.C., USA; †Cincinnati Veterans Affairs Medical Center, Cincinnati, Ohio, USA; and ‡University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

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Through its annual survey for federal fiscal year (FY) 1999 (October 1, 1998 through September 30, 1999), the Infectious Diseases Program Office of the United States Department of Veterans Affairs (VA) undertook a national assessment of the VA health-care system surveillance for SARV. In addition to identifying cases with vancomycin (glycopeptide)-resistant or -intermediate isolates, we sought to identify those cases with the potential for decreased susceptibility to vancomycin. Therefore, we have chosen to use the designation of SARV to encompass all of these. During FY 1999, the VA health-care system served a population of >3.6 million persons in its 172 medical centers and >600 outpatient clinics; it had approximately 600,000 inpatient discharges and >35 million outpatient visits during that same period.

Materials and Methods

Annually since 1990, the Infectious Diseases Program Office for VA Central Office has distributed an Infectious Diseases/Infection Control survey requesting data on several topics to all VA medical center reporting sites across the country. The process for the survey begins with the distribution of the annual survey instrument (questionnaire) to each VA medical center reporting site; this is delivered to the administrator responsible for the facility. Subsequently, a 2-week period is established for receipt of responses to the survey. Responses are made by electronic entry into a central database by each site. Each site notes a point of contact for subsequent data verification. After the 2-week period, the ability to access the database for entry is closed to the medical center reporting sites.

Administrative review by the Infectious Diseases Program Office identifies medical centers that have omitted data. The point of contact is queried (either by telephone or e-mail) as to the nature of the omission. Concomitant with the review of data for omission, a preliminary analysis of the submitted data is undertaken to assess for accuracy of other reported data and consistency of reporting with previously submitted data from the medical center. There are several questions in the Infectious Diseases/Infection Control annual survey that serve as controls for analysis. If there is concern that the submitted data may be inaccurate, the point of contact for the site is also queried to verify these data.

Beginning in 1998 (for FY 1997 data), the survey included two questions regarding SARV. The following questions were asked, 1) Does your facility do or obtain testing to identify reduced susceptibility to vancomycin (MIC $\geq$4 μg/mL) for *Staphylococcus aureus*? Yes or no? 2) If yes, report the number of patients (not cultures) with *Staphylococcus aureus* with reduced susceptibility to vancomycin (MIC $\geq$4 μg/mL).

Any site reporting presence of a patient with SARV was contacted by the Infectious Diseases Program Office to verify accuracy of the report for both FY 1997 and 1999 data. During the contact by the Infectious Diseases Program Office (January 2001) for FY 1999 data, additional information was requested from those sites that reported and verified patients with SARV. This additional information included verification that the isolate was indeed *S. aureus*, identification of the susceptibility testing methods, source of the specimen, inpatient or outpatient status at the time of specimen acquisition, and MIC to vancomycin. Query was also made regarding confirmatory testing of vancomycin susceptibility of the patient isolate, susceptibility testing to other antimicrobial agents, and current availability of the isolate.

Results

For FY 1997, there was 100% response to the survey instrument (146 reporting sites), although not all questions were completed. Initially 11 sites reported 284 patients with SARV. After contact and verification of the survey results by the Infectious Diseases Program Office with these sites, seven patients were reported to have SARV from four of the sites. Rationale for discounting initially reported cases after verification included misinterpretation of the question to be requesting information on VRE or misinterpretation of the question to mean MRSA.

For FY 1999, there was 99% response to the survey instrument (142 of 143 reporting sites), though not all questions were initially completed. With regard to the ability of the reporting facility to do or obtain testing to identify SARV, 142 reporting sites answered this question, with 123 (86%) of the sites responding "yes." Of the 123 sites reporting yes, initially 13 sites reported 195 patients with SARV. After contact and verification by the Infectious Diseases Program Office, the number of verified, reported cases was revised to eight sites reporting 19 patients with SARV. Reasons for change of reported numbers to verified numbers included misinterpretation of the question to mean MRSA as well as one isolate with a difficult determination by the original MIC method used (reporting an MIC $\geq$16 μg/mL) but with confirmatory testing defining an MIC = 1.5 μg/mL. Microbiology testing was noted to be done onsite in a CAP-approved laboratory for all reported and verified cases.

The specimen sources for these isolates were five from tissue or wounds, five from a urinary source, four from sputum, two from abdominal or peritoneal sources, and one each from blood, eye, and synovial fluid. Initial susceptibility testing showed 17 used MicroScan technologies (Dade Behring, Inc, MicroScan Division, West Sacramento, CA) and one each of bioMerieux Vitek (Hazelwood, MO), and E-test (AB Biodisk North America, Inc, Piscataway, NJ) (Table 1). Confirmatory testing was done on only 2 of the 19 reported cases, using E-test and MicroScan technology (Table 1). One isolate was sent to CDC for confirmation. However, as noted above, confirmatory testing had also been done on at least one occasion to refute presence of SARV. Sixteen of the isolates were reported to have an MIC = 4 μg/mL, one was reported to have an MIC = 8 μg/mL (noted to be an intermediate sensitivity interpretation), one was reported at $\geq$16 μg/mL, while one was reported at $\geq$32 μg/mL; these last two isolates were interpreted as being resistant. Six of these specimens were obtained from patients

Table 1. Information from reported isolates of *Staphylococcus aureus* with reduced susceptibility to vancomycin from United States Veterans Health Administration medical facilities, FY[a] 1999

Case	Inpt/Outpt/ NH[b]	Specimen	Method[c] (Instrumentation/panel)	MIC/ susceptibility[d]	Confirmation of susceptibility	Confirmation methodology
1	Outpt	Ear tissue	MicroScan Walkaway version 22.01/Gram Pos Combo Panel 10	> 16 µg/mL/R	Yes	MicroScan Walkaway
2	Inpt/SICU[e]	ABD[f] and VP[g] shunt	MicroScan Walkaway version 22.06/Pos Combo 12	= 4 µg/mL/S	No	--
3	Inpt	Sputum	MicroScan Walkaway version 22.26/Pos Combo 14	= 4 µg/mL/S	No	--
4	Outpt	Urine	MicroScan Walkaway version 22.06/Pos Combo 12	= 4 µg/mL/S	No	--
5	Outpt	Leg	MicroScan Walkaway/Pos Combo Panel 10	= 4 µg/mL/S	No	--
6	Outpt	Eye	MicroScan Walkaway/Pos Combo Panel 10	= 8 µg/mL/(I)	No	--
7	Inpt	Peritoneal	MicroScan Walkaway/Pos Combo Panel 10	= 4 µg/mL/S	No	--
8	Inpt	Urine	MicroScan Walkaway/Pos Combo Panel 10	= 4 µg/mL/S	No	--
9	Outpt	Wound	MicroScan AutoScan/Pos Combo 11	= 4 µg/mL/S	No	--
10	NH	Sputum	MicroScan AutoScan/Pos Combo 11	= 4 µg/mL/S	No	--
11	NH	Sputum	MicroScan AutoScan/Pos Combo 11	= 4 µg/mL/S	No	--
12	NH	Urine	MicroScan AutoScan/Pos Combo 11	= 4 µg/mL/S	No	--
13	NH	Urine	MicroScan AutoScan/Pos Combo 11	= 4 µg/mL/S	No	--
14	NH	Wound	MicroScan AutoScan/Pos Combo 11	= 4 µg/mL/S	No	--
15	Inpt	Foot wound	E-test; VCN[h] screen plate	= 4 µg/mL/S	Yes	Sent to CDC[i]; E-test
16	Outpt	Synovial fluid	MicroScan Walkaway/Gram Pos Combo Panel 10	= 4 µg/mL/S	No	--
17	Inpt/MICU[j]	Sputum	MicroScan Walkaway/Gram Pos Combo Panel 10	= 4 µg/mL/S	No	--
18	Inpt/ICU[k]	Blood	MicroScan AutoScan version 22.01/Pos Combo Panel 10	= 4 µg/mL/S	No	--
19	Inpt/MICU	Urine	BioMerieux Vitek VTK-R version 07.01/GPS-102	≥ 32 µg/mL/R	Unable to determine	--

[a]FY=Federal fiscal year.
[b]Inpt=inpatient; Outpt=outpatient; NH=nursing home.
[c] bioMerieux Vitek (Hazelwood, MO), E-test (AB Biodisk North America, Inc., Piscataway, NJ), MicroScan (Dade Behring Inc., MicroScan Division, West Sacramento, CA). Where data were available, software version of technology provided. All MicroScan methods used conventional 24-hour incubation susceptibility panels.
[d] Susceptibility interpretation at the reporting site (S=sensitive, I=intermediate, R=resistant).
[e] SICU=surgical intensive care unit.
[f]ABD=abdominal.
[g]VP=Ventriculo-peritoneal.
[h]VCN=vancomycin.
[i]CDC=Centers for Diseases Control and Prevention.
[j]MICU=medical intensive care unit.
[k]ICU=intensive care unit.

during an outpatient encounter, while seven were obtained while patients were on inpatient status, and five were from patients in a nursing home. Only one of the 19 case isolates had been stored and is available for further analysis.

Reported susceptibility testing to other antimicrobial agents are noted in Tables 2 and 3 where data were available; not all isolates had susceptibility testing done against all antimicrobial agents reported. Beta-lactamase activity was present for 14 of the 16 isolates. For penicillin-type antibiotics, 12 of the 19 isolates had oxacillin resistance. There was also a relatively high degree of resistance to the cephalosporins tested (data not shown) and six of eight isolates were resistant to imipenem. Antibiotic susceptibility testing against other agents used to treat gram-positive infections showed varying degrees of resistance, with 4 of 15 isolates resistant to rifampin, 14 of 18 resistant to erythromycin, 12 of 17 resistant to clindamycin, 4 of 17 resistant to tetracycline, only 1 of the 19 isolates resistant to trimethoprim/sulfamethoxazole, and none of 4 isolates resistant to chloramphenicol. For the quinolones and other agents, 5 of 16 were resistant to ciprofloxacin, 4 of 5 were resistant to levafloxacin, 4 of 7 were resistant to ofloxacin, and 3 of 16 were resistant to gentamicin.

Discussion

In this survey, patients were reported to have SARV in the VA system for both FYs 1997 and 1999. Two VA sites reported cases of SARV in both surveys. Comparison of the two surveys indicates an increase of 170% in the number of cases reported in 1999 compared with 1997. This finding is in contradistinction to information reported by MRL Pharmaceutical Services, where none of 3,797 *S. aureus* isolates had reduced susceptibility to vancomycin in 1999 (15). Even

Table 2. Susceptibility to selected gram-positive agents of isolates of reported *Staphylococcus aureus* with reduced susceptibility to vancomycin from United States Veterans Health Administration medical facilities, FY 1999[a]

Case	Beta-lac-tamase	MIC/sensitivity status							
		oxacillin	imipenem	rifampin	TMP/SMX	erythromycin	TCN	clindamycin	chloramphenicol
1	negative	/R			/S	/R	/S	/R	
2	positive	<0.5 μg/mL	<4 μg/mL	<1 μg/mL	<2 μg/mL	=0.5 μg/mL	<2 μg/mL	<0.25 μg/mL	
3	positive	>2 μg/mL	>8 μg/mL/R	<1 μg/mL	<2 μg/mL	>4 μg/mL/R	<2 μg/mL	>2 μg/mL/R	
4	positive	>4 μg/mL	>8 μg/mL/R	<1 μg/mL	<2 μg/mL	>4 μg/mL/R	<2 μg/mL	>2 μg/mL/R	
5	positive	<0.5 μg/mL/S	>8 μg/mL/R	>2 μg/mL/R	≤2/38 μg/mL/S		>8 μg/mL/R	>2 μg/mL/R	
6	positive	>2 μg/mL/R	2 μg/mL/R	≥1 μg/mL/S	≤2/38 μg/mL/S	>4 μg.mL/R	≤2 μg/mL/S		8 μg/mL/S
7	positive	>2 μg/mL/R	>8 μg/mL/R	>2 μg/mL/R	>2/38 μg/mL/R	>4 μg/mL/R	>8 μg/mL/R		16 μg/mL/I
8	positive	>2 μg/mL/R	>8 μg/mL/R	≥1 μg/mL/S	≤2/38 μg/mL/S	>4 μg/mL/R	≤2 μg/mL/S	>2 μg/mL/R	16 μg/mL/I
9	positive	>2 μg/mL/R		<1 μg/mL/S	<2 μg/mL/S	>4 μg/mL/R	<2 μg/mL/S	>2 μ/mL/R	
10	negative	<0.5 μg/mL/S		<1 μg/mL/S	<2 μg/mL/S	<0.25 μg/mL/S	<2 μg/mL/S	=0.5 μg/mL/S	
11	positive	<0.5 μg/mL/S		<1 μg/mL/S	<2 μg/mL/S	>4 μg/mL/R	<2 μg/mL/S	<0.25 μg/mL/S	
12	positive	>2 μg/mL/R		<1 μg/mL/S	<2 μg/mL/S	>4 μg/mL/R	<2 μg/mL/S	>2 μ/mL/R	
13	positive	<0.5 μg/mL/S		<1 μg/mL/S	<2 μg/mL/S	>4 μg/mL/R	>8 μg/mL/R	>2 μg/mL/R	
14	positive	>2 μg/mL/R		>2 μg/mL/R	<2 μg/mL/S	>4 μg/mL/R	<2 μg/mL/S	>2 μg/mL/R	
15		/R			<2 μg/mL/S	/R	<2 μg/mL/S	<2 μg/mL/S	8 μg/mL/S
16		1 μg/mL/S			<2/38 μg/mL/S	0.5 μg/mL/S		>2 μg/mL/R	
17		>4 μg/mL/R			<2/38 μg/mL/S	>4 μg/mL/R		>2 μg/mL/R	
18	positive	<0.05 μg/mL	<4 μg/mL	<1 μg/mL	≤2 μg/mL	<0.25 μg/mL	≤2 μg/mL	0.5 μg/mL	
19	positive	≥8 μg/mL/R		≥4 μg/mL/R	≤16 μg/mL/S	≥8 μg/mL/R	>16 μg/mL/R	≥8 μg/mL/R	
Total[b]	14/ 16=pos	12/19 =R	6/8=R	4/15=R	1/19=R	14/18=R	4/17=R	12/17=R	0/4=R

[a]FY=Federal fiscal year; S=susceptible, I=intermediate and R=resistant based on laboratory interpretative criteria; TMP/SMX=trimethoprin/sulfamethoxazole; TCN=tetracycline
[b]The authors took the liberty of placing interpretation on some reported MIC values that did not have an interpretation of S, I, or R on information provided from the facility.

though different methods of data accrual were used, both surveys rely on NCCLS-based susceptibility criteria (10). Despite the fact that presence of SARV appears to be a low-incidence occurrence at this time, the reason for different occurrences of SARV between the VA and this other national data set (15) is not readily apparent from the data present. The difference of findings, even though both use NCCLS-based susceptibility criteria, may be based on the fact that the MRL study used one consistent microbroth dilution method for susceptibility testing whereas our population-based reporting survey encompasses numerous susceptibility testing methods (MicroScan, Vitek, E-test, screening plates) more analogous to real-world application of technologies. Tenover et al. (11) demonstrated different methods of susceptibility testing (e.g., MicroScan Rapid panels and disk diffusion) have been shown to be unreliable in detecting *S. aureus* strains with reduced susceptibility of vancomycin; none of the VA laboratories reporting SARV used those methods considered to be unreliable (Table 1). Sampling size may be a factor, as we do not have the total number of isolates tested nationwide in the VA, but the total number likely exceeds the number of isolates in the MRL study. Different populations sampled or the recent CDC studies indicating difficulty in delimiting antibiotic resistance (13,14) might all contribute to this difference as well.

Because of limitations in our survey methods, we are unable to supply information on the total number of *S. aureus* isolates (or persons with *S. aureus* isolated) within the VA system nationwide, from which to determine prevalence estimates for comparative purposes to other studies (16,17). Data from Wilcox et al. and Aucken et al. indicate that about 15% of isolates in the United Kingdom had a vancomycin MIC of 4 μg/mL on initial testing (16,17). These two reports also indicate a low prevalence of vancomycin (glycopeptide)-intermediate or -resistant isolates upon confirmation. The reports also lend support to the finding that susceptibility testing of *S. aureus* to vancomycin by disk diffusion, which is commonly used in the United Kingdom, is not as reliable as other methods of testing for reduced susceptibility of *S. aureus* to vancomycin (11).

For each of the 2 years surveyed, more sites initially reported patients with SARV than were present after verification by the Infectious Diseases Program Office. The most common reason noted for the inaccurately reported data was

Table 3. Reported susceptibility to quinolones and other agents of reported isolates of *Staphylococcus aureus* with reduced susceptibility to vancomycin from United States Veterans Health Administration medical facilities, FY 1999[a]

	MIC/sensitivity status					
Case	ciprofloxacin	levofloxacin	ofloxacin	norfloxacin	gentamicin	furodantin
1	/S					
2	<1 µg/mL			<4 µg/mL	<1 µg/mL	<32 µg/mL
3	>2 µg/mL/R	>4 µg/mL/R		>8 µg/mL	<1 µg/mL	<32 µg/mL
4	>2 µg/mL/R			>8 µg/mL	<1 µg/mL	<32 µg/mL
5	≤1 µg/mL/S	4 µg/mL/I			≤1 µg/mL/S	
6	>2 µg/mL/R	>4 µg/mL/R			8 µg/mL/I	
7	>2 µg/mL/R	>4 µg/mL/R			>8 µg/mL/R	
8	>2 µg/mL/R	>4 µg/mL/R			>8 µg/mL/R	
9	>2 µg/mL/R		>4 µg/mL/R		<1 µg/mL/S	<32 µg/mL
10	=2 µg/mL/I		<2 µg/mL/S		<1 µg/mL/S	=64 µg/mL
11	>2 µg/mL/R		>4 µg/mL/R		<1 µg/mL/S	<32 µg/mL
12	>2 µg/mL/R		>4 µg/mL/R		<1 µg/mL/S	<32 µg/mL/S
13	>2 µg/mL/R		>4 µg/mL/R		<1 µg/mL/S	<32 µg/mL/S
14	<1 µg/mL/S		<2 µg/mL/S		<1 µg/mL/S	<32 µg/mL
15					/R	
16						
17						
18	<1 µg/mL				<1 µg/mL	
19	≤0.5 µg/mL/S		≤1 µg/mL/S		≤2 µg/mL/S	
Total	9/16=R	4/5=R	4/7=R		3/16=R	

[a]FY=Federal fiscal year; S=susceptible, I=intermediate and R=resistant based on laboratory interpretative criteria.

misinterpretation of the question to mean MRSA, indicating that despite a simply worded question giving specific definitions, data validation is important. Validation is especially important for low-incidence diseases, for which a few misreported cases may significantly alter the final outcome.

Despite CDC recommendations on confirmatory testing of suspect isolates, repeat susceptibility testing is not being performed consistently for all isolates. If confirmatory testing is being performed, it is not being recorded; therefore, it is not reported in this retrospective review. NCCLS does not indicate the need for repeat testing (10). Further, from the survey we were not able to determine if confirmatory testing of the isolate to indeed be *S. aureus* was occurring. However, each site did note that it was confident of the organism identification. Perhaps of greater importance is the recognition that despite CDC recommendations for confirmatory testing to be done for isolates of staphylococci with MICs ≥4 µg/mL to vancomycin, the Infectious Diseases Program Office was informed by several of the sites reporting SARV that an MIC of 4 µg/mL was interpreted as susceptible by NCCLS criteria (10); therefore, it was not necessary to confirm this result. The lack of confirmatory testing indicates poor recognition for the significance of *S. aureus* having the potential for reduced susceptibility to vancomycin. Also, only one site (of eight sites in FY 1999) reported contacting a public health authority about a SARV isolate (i.e., sent to CDC for confirmatory testing), again indicating a lack of recognition of important Public Health Service recommendations (8). No other reports indicate the extent of adherence to such national recommendations about SARV. Even though this lack of recognition and reporting is not the same as the capacity to detect antimicrobial resistance, it is analogous to CDC findings that confirmation of susceptibility for suspected SARV was as low as 39% (13).

Upon review of other antimicrobial susceptibilities of the 19 SARV cases from FY 1999, a high degree of resistance to other agents was found; however, a third of isolates were noted to be susceptible to oxacillin or methicillin. Therefore, not all reported cases of SARV were also MRSA. Non-*aureus* staphylococcal species were not reviewed in this survey. Some reports indicate that coagulase-negative staphylococci also have reduced susceptibility to vancomycin (18).

Given the limitation to two basic questions imposed by the Infectious Diseases/Infection Control annual survey methods, the full extent and characterization of *S. aureus* with reduced susceptibility to vancomycin in the VA cannot be accurately assessed. However, this method has indicated that SARV exists in the VA health-care system, the occurrence of which has increased between the 2 years reviewed. This study also

identified the importance of data validation as evidenced by misinterpretation of clearly stated and defined questions. With the presence of this organism and its apparent increase in occurrence, continued surveillance is indicated. A more thorough analysis of the extent and characteristics of this organism in the VA system would be beneficial to both VA and public health in general; this analysis might include patient characteristics, antibiotic use in patients before acquisition of SARV, and collection and storage of isolates for further laboratory analysis. Along with further characterization of the epidemiology of this organism, increasing awareness as to the significance of SARV is indicated.

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Dr. Kralovic is medical epidemiologist for the Emerging Pathogens Initiative (EPI) of the Veterans Health Administration's Central Office Infectious Diseases Program Office. He is also assistant professor of medicine in the Division of Infectious Diseases and assistant professor of environmental health in the Division of Epidemiology and Biostatistics, University of Cincinnati College of Medicine. His research pursuits are in infectious diseases epidemiology in healthcare systems, including traditional hospital infection control, with special interest in mycological epidemiology.

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Address for correspondence: Stephen M. Kralovic, Infectious Diseases Program Office, c/o Medical Service, 111, Cincinnati VA Medical Center, 3200 Vine Street, Cincinnati, OH 45220, USA; fax: 513-475-6399; e-mail: stephen.kralovic@med.va.gov

Genetic and Antigenic Analysis of the First A/New Caledonia/20/99-like H1N1 Influenza Isolates Reported in the Americas

Luke T. Daum,* Linda C. Canas,* Catherine B. Smith,† Alexander Klimov,† William Huff,* William Barnes,* and Kenton L. Lohman*

From February through May of 1999, 13 cases of *Influenza A virus* (FLUAV), type H1N1 were reported at a Department of Defense influenza surveillance sentinel site in Lima, Peru. Genetic and antigenic analysis by hemagglutination inhibition and direct nucleotide sequencing of the HA1 region of the hemagglutinin gene were performed on two isolates, A/Peru/1641/99 and A/Peru/1798/99. Both isolates were distinct from the Bayern/7/95-like viruses circulating in the Americas and closely related to a Beijing/262/95-like variant, A/New Caledonia/20/99. With the exception of travel-related cases, the detection of these isolates represents the first appearance of New Caledonia/20/99-like viruses in the Americas. Since the characterization of these Peru isolates, a number of New Caledonia/20/99-like viruses have been reported worldwide. For the 2000/01 and 2001/02 influenza seasons, the World Health Organization (WHO) has recommended the inclusion of A/New Caledonia/20/99 as the H1N1 vaccine component for both the southern and northern hemispheres.

Influenza epidemics occur in most parts of the world and typically arise every 2 to 3 years, causing approximately 20,000 deaths above the yearly mortality baseline (1). The incidence of recurring epidemics is primarily attributed to the high frequency of mutational changes in the hemagglutinin (HA) and neuraminidase (NA) major surface glycoproteins. The hemagglutinin protein, responsible for viral uptake into host epithelial cells, is also the antigen targeted by antibodies. The HA1 subunit of the H1N1 hemagglutinin protein consists of the globular head and contains five major antibody binding sites: Sa, Sb, Ca_1, Ca_2, and Cb (2-5). Mutational changes (antigenic drift) at these four sites are thought to be driven by selective antibody pressure, which can produce novel strains capable of evading the immunologic response. Monitoring the antigenic drift of viruses in global populations is essential for optimal selection of component strains for the annually updated trivalent influenza vaccine.

Since 1976, the United States Air Force (USAF) has contributed to influenza surveillance through a collaborative partnership with the Centers for Disease Control and Prevention (CDC) and World Heath Organization (WHO) (6-7). In 1997, the USAF expanded surveillance to include US Navy and Army installations and became the Department of Defense Global Emerging Infections System (DOD-GEIS) (6). The DOD-GEIS influenza surveillance network receives samples from training bases, ports of entry, and many military sentinel sites in the United States, Pacific Rim, and Europe, and throughout Central and South America.

Since 1995, the predominant *Influenza A* viruses, type H1N1 isolated in North and South America were closely related to A/Bayern/7/95, while viruses belonging to another genetic and antigenic lineage of H1N1 viruses, A/Beijing/262/95, have remained geographically restricted to Asia (8-10). During spring 1999, Beijing/262/95-like variants, containing a characteristic deletion mutation at amino acid 134 of the HA gene, were isolated from 13 persons at a DOD-GEIS influenza surveillance site in Lima, Peru. These antigenically distinct H1N1 viruses were closely related to A/New Caledonia/20/99, a recently characterized variant from the Beijing/262/95 lineage. Before these Peru isolates, no circulation of amino acid-134 deletion mutants in the Americas was documented.

Materials and Methods

Sources of Specimens

Influenza throat swab specimens were collected in accordance with the case criteria outlined in Canas et al. (6). The criteria included patients with a fever >37.8°C accompanied by cough or sore throat. Throat swabs were taken within 72 hours of the onset of symptoms, placed in viral transport media (MicroTest, M4) (VTM), and delivered via commercial carrier to the clinical laboratory at the Epidemiological Surveillance Division, Brooks Air Force Base. The two influenza isolates used in this study were randomly selected from 13 positive patient samples isolated from February to April 1999 from a Department of Defense sentinel site in Lima, Peru.

*Brooks Air Force Base, San Antonio, Texas, USA; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Viral Detection

All influenza viruses examined in this study were passaged one time through Primary Rhesus Monkey Kidney (PMK) tissue culture (Viro-Med, Minneapolis, MN; BioWhittaker, Walkersville, MD). A total of 500 ml of VTM was injected onto a shell vial containing a coverslip with PMK tissue culture. Cultures were tested for viral infection using the centrifugation-enhanced shell-vial technique that includes a cell monolayer grown on a coverslip contained in a shell vial. Specimens are inoculated onto the cell monolayer, subjected to a low speed centrifugation for 60 minutes, incubated at 35°C for 48 hours to 72 hours and then screened with monoclonal antibodies (Chemicon, Inc., Temecula, CA) to detect the presence of *Influenza A or B viruses*. Aliquots of all specimens used in this study for the purposes of genetic analysis were stored at -80°C until use.

Antigenic and Genetic Typing

For antigenic analysis, aliquots of each isolate were sent to CDC for characterization in hemagglutiniation inhibition (HI) tests with postinfection ferret antisera as previously described (11).

For genetic typing, approximately 1 mL of VTM was used to infect PMK tissue culture (Viro-Med, Minneapolis, MN; BioWhittaker, Walkersville, MD) and incubated at 37°C for 3 to 5 days. RNA was extracted from a 300 µl aliquot of tissue culture fluid using the High Pure Viral RNA Kit (Boehringer-Mannheim, Indianapolis, IN). Influenza RNA was amplified into double stranded DNA by reverse transcription and polymerase chain reaction (RT-PCR) using the Titan One Step RT-PCR Kit (Boehringer-Mannheim). An 1,187 bp cDNA fragment consisting of the HA1 coding region of influenza A hemagglutinin was amplified using forward primer F6-(5'-AAGCAGGGGAAAATAAAA-3', mRNA) and reverse primer R1193-(5'-GTAATCCCGTTAATGGCA-3', vRNA sense). The RT-PCR reaction was performed by using a Perkin Elmer 2400 thermocycler (Forest City, CA). The PCR product was purified using the QIAquick spin column purification (Qiagen, Valencia, CA). Purified HA1 amplicon was cycle sequenced by using the Big Dye Terminator Cycle Sequencing Kit (PE Biosystems) run on a 377-XL automated DNA sequencer according to manufacturer recommendations (PE Biosystems). Four internal oligonucleotide sequencing primers (Table 1) were used to sequence the entire HA1 amplicon in both the forward and reverse directions. Multiple sequence alignments, protein translation, and phylogenetic analysis were performed by using the software package Lasergene (version 3.18) (DNASTAR, Madison, WI). The nucleotide and amino acid sequences for A/Peru/1621/99 and A/Peru/1798/99 are available from Genbank under the accession numbers AF268313 and AF268312, respectively. Additional nucleotide and amino acid sequences characterized by Brooks Air Force Base and depicted in phylogenetic analysis are available from Genbank under the accession numbers AY029287-AY029292.

Table 1. Characterization of Influenza A H1N1 (HA1) Sequencing Primers

Primer/nt position	Sequence	% GC[b]	Tm[a](°C)
H1F-272	5′-AATCATGGTCCTACATTG-3′	38.9	57
H1F-734	5′-ACTACTACTGGACTCTGC-3′	50	58
H1R-365	5′-TTCCTCATACTCGGCGAA-3′	50	58
H1R-1110	5′-CCATCCATCTATCATTCC-3′	44.4	55

[a]Tm= 81.5 + 16.6 * log [Na+] + 41 * (#G + #C)/length–500/length
Where [Na+]= 0.1M.
[b]GC= (Guanosine (G) + Cytidine (C))/ (Adenosine (A)+ Thymidine (T)+ Guanosine (G) + Cytidine (C)) x 100

Results

Antigenic Analysis

Antigenic characterization by HI, using postinfection ferret antisera, was performed with reference antigens representing two globally cocirculating *Influenza A virus*, type H1N1 antigenic/genetic lineages (A/Beijing/262/95, which includes the variant A/New Caledonia/20/99; and A/Bayern/07/95, which includes A/Johannesburg/82/96). Both Peru isolates were antigenically related to the A/Beijing/262/95 rather than to the A/Bayern/07/95 lineage (Table 2). Both Peru isolates were antigenically close to the new variant, A/New Caledonia/20/99, that recently evolved from the A/Beijing/262/95 virus. H1 titers of the Peru isolates were within a two-fold difference from the homologous titer demonstrated by A/New Caledonia/20/99 (160 vs. 320), while they were fourfold lower than the A/Beijing/262/95 homologous titer (160 vs. 640).

Genetic Analysis

The complete nucleotide and deduced amino acid sequences of the HA1 of the two Peru isolates were compared with those of the previous vaccine strain, A/Beijing/262/95, and the current vaccine strain, A/New Caledonia/20/99. Based on nucleotide alignments, A/Peru/1621/99 and A/Peru/1798/99 share identical HA1 sequences and are genetically closer (97.9%) to A/New Caledonia/20/99 (97.9%) than they are to

Table 2. Hemagglutination Inhibition Reactions of Influenza A(H1N1) Viruses[a] Reference *Ferret Antisera*

Reference Antigens	Bei/262	NC/20	Bay/07	Joh/82
A/Beijing/262/95	640	320	20	20
A/New Caledonia/20/99	160	320	10	<10
A/Bayern/07/95	40	10	1280	1280
A/Johannesburg/82/96	40	10	1280	1280
Test Antigens				
A/Peru/1621/99	160	160	<10	<10
A/Peru/1798/99	160	160	<10	<10

[a]Test viruses are considered antigenically similar to the reference strain if the H1 titer is equal or two times lower than the homologous titer of the reference virus. If the difference in H1 titers is 4-fold or higher, the test viruses are considered to be antigenically different from the reference strain.

the A/Bayern/07/95 (94.8%) or A/Beijing/262/95 (96.8%) reference strains.

A comparison of the amino acid residues of the Peru isolates with A/New Caledonia/20/99 and A/Beijing/262/95 is shown in Figure 1. The Peru isolates contained only 8 amino acid differences compared with A/New Caledonia/20/99 and 11 amino acid differences compared with A/Beijing/262/95. A substitution at residue 156(G156E) shows a neutral to acidic amino acid change similar to those observed in some other Beijing/262-like viruses. Amino acid substitutions at positions 190(N190D) and 194(L194I) are also characteristic of the Beijing/262/95 lineage. Substitutions at position -1 (signal sequence), 9, 82, 186, and 218 represent novel changes not present in A/New Caledonia/20/99 or the majority of characterized Beijing/262-like viruses.

A phylogenetic analysis (Figure 2) comparing the HA1 nucleotide sequence of the Peru isolates with H1N1 viruses isolated after 1990 was performed with the software package MegAlign (1993-1998) (DNASTAR, Madison, WI), using the Jotun-Hein Method (12). Based on the topography, the Peru isolates and other A/New Caledonia/20/99-like viruses isolated by DOD-GIES during the 1999-2000 influenza season appear to have evolved from Beijing/262/95-like viruses into a distinct A/New Caledonia/20/99 sublineage.

Discussion

A/Beijing/262/95-like viruses first appeared in China during the 1994-95 influenza season and then spread throughout Asia over the next 5 years (8-10). These viruses have a characteristic deletion mutation in the HA molecule at residue 134. In geographic areas outside Asia, Bayern/7/95-like viruses have predominated during the 1996-97, 1997-98, and 1998-99 seasons (8-10). In May and June 1999, A/New Caledonia/20/99, an antigenic variant of Beijing/262/95, was first reported in New Caledonia in the Southern Pacific (13). Shortly thereafter, A/New Caledonia/20/99-like viruses were detected throughout South Africa and Asia. Isolates A/Peru/1621/99 and A/Peru/1798/99 were collected during spring 1999. These isolates were important because they were the first Beijing/262/95-like viruses in the Americas with the signature HA1 deletion mutation at residue 134. However, the Peru isolates were antigenically distinct from Beijing/262-like viruses and antigenically similar to A/New Caledonia/20/99 (Table 2).

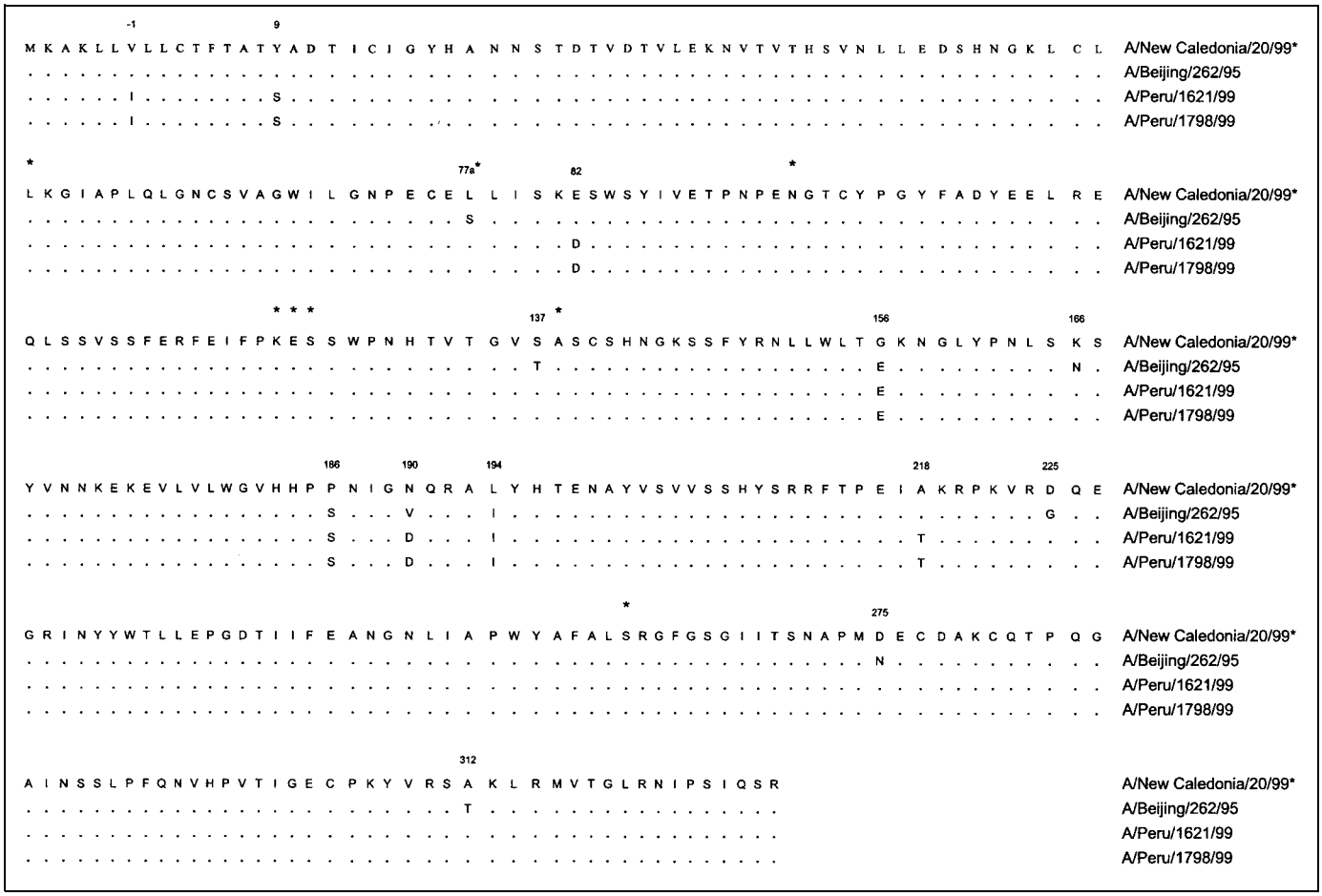

Figure 1. The amino acid sequence of the HA1 region of the hemagglutinin of the Peru isolates and A/Beijing/262/95 compared with the 2000/01 and 2001/02 vaccine strain, A/New Caledonia/20/99. Amino acid numbering corresponds to H3 subtype (19), with additional amino acids residues present in the H1 subtype sequence indicated by an asterisk. Residue substitutions are shown. A dot (.) indicates amino acid homology to A/New Caledonia/20/99.

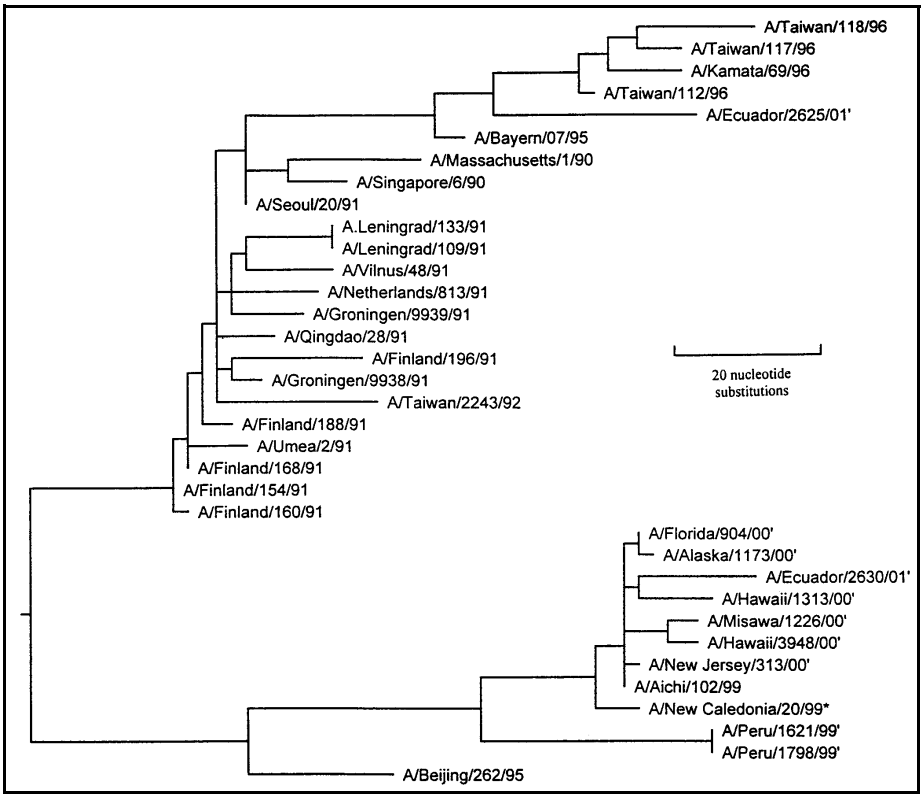

Figure 2. An unrooted phylogenetic analysis of the HA1 gene nucleotide sequence of influenza A H1N1 viruses isolated since 1990. Peru isolates 1621 and 1798 are within the Beijing/262/95 lineage but are more similar to A/New Caledonia/20/99. A number of isolates characterized by Brooks Air Force Base from 1999 to 2001 were also A/NC/20/99-like. The tree was generated by using the Jotun-Hein algorithm (6) in MegAlign software (version 3.18). Horizontal lines are proportional to the number of substitutions between branch points. Asterisk (*) denotes 2000/01 vaccine strain; ([1]) denotes isolates characterized by Brooks Air Force Base. Brooks Air Force Base isolates are available from Genbank under accession numbers AF268312, AF268313 and AY029287-AY029292.

Sequence comparisons further supported the results of the antigenic analysis. Eleven amino acid differences in the HA1 protein were observed between the Peru isolates and A/Beijing/262/95, the vaccine strain for the 1998-99 and 1999-2000 influenza seasons. Four of these 11 substitutions were located in 3 antigenic sites and could potentially alter antibody-binding properties of the viruses. Wilson and Cox proposed that a drift variant of epidemiologic importance usually contains four or more amino acid substitutions located in two or more of the antigenic sites on the HA1 protein (14). Substitutions at residues 218(A218T) and 225(G225D) were present in the Ca antigenic site located approximately halfway down the side of the HA1 globular head (3,15). Two other substitutions, 82(E82D) and 166(N166K), were observed within antigenic sites Cb and Sa, located at the base and tip of the HA1 globular head, respectively (3,15).

Genetic comparisons between Peru isolates and A/New Caledonia/20/99 show a difference of only eight amino acids (Figure 1). Three of these eight residues, 156(E), 186(S), and 194(I), are present in the A/Beijing/262/95 vaccine strain. The substitution at residue 190(D) is also typical for many Beijing/262/95-like viruses, although not A/Beijing/262/95 itself (16). The remaining four substitutions, at residues -1(L), 9(S), 82(D), and 218(T), are unique compared with most recently characterized New Caledonia/20/99 or A/Beijing/262/95-like viruses.

The phylogenetic analysis of *Influenza A viruses* from 1990 to 2001 confirmed the coexistence of the two major H1N1 lineages (Figure 2). The Beijing/262/95-like lineage is easily distinguished from the Bayern/07/95-like viruses by a characteristic deletion at residue 134. With the exception of one Bayern/07/95-like isolate, all the viruses genetically characterized by DOD-GEIS since these Peru isolates were detected contain the residue 134-deletion mutation and appear to be evolving from A/Beijing/262/95 into a distinct sublineage (Figure 2). The A/Peru/1621/99 and A/Peru/1798/99 isolates are depicted as a distinct branch in the phylogeny of New Caledonia-like viruses.

Since these Peru strains were first identified in 1999, DOD-GEIS and CDC have characterized similar New Caledonia/20/99-like viruses isolated from throughout North and South America during the 1999/2000 influenza season. The sudden widespread appearance of these viruses prompted WHO to recommend the change to A/New Caledonia/20/99 as the 2000/01 H1N1 influenza vaccine component (13). This recommendation was timely because the 1999/2000 vaccine containing the A/Beijing/262/95 H1N1 component induced a cross-reactive response to A/Bayern/7/95-like viruses but induced lower titers of antibodies to A/New Caledonia/20/99-like strains (17). Therefore, a change in the H1N1 component was necessary to ensure that the vaccine would provide effective immunization against emerging A/New Caledonia/20/99-like viruses.

During the 2000/01 influenza season, New Caledonia-like viruses continued to predominate over the H1N1 Bayern-like lineage and were associated with outbreaks in many countries (13, 18). During this season, DOD-GEIS characterized 83 influenza A H1N1 viruses. Of these, 80 (96%) were antigenically related to A/New Caledonia/20/99; three (4%) were Bayern/7/95-like. Our antigenic data are consistent with findings that approximately 84% of H1N1 viruses characterized by CDC during the 2000/2001 influenza season were similar to A/New Caledonia/20/99 (13, 18). The continued persistence of New Caledonia-like viruses in the global population encouraged WHO to maintain A/New Caledonia/20/99 as the H1N1 component for the current 2001/2002 influenza season (20).

Studies based on amino acid analyses of viruses from previous years to predict the evolution of future H1N1 epidemic

strains would be advantageous as a surveillance tool and could contribute to the vaccine selection process. Using a retrospective approach, Bush et al. determined that specific codons in the HA1 domain of the hemagglutinin gene of human influenza subtype H3 are under positive selection to mutate (21-22). Certain amino acids in the HA1 of human H1N1 viruses mutate at positively selected codons, giving rise to new viral lineages. The characterization of these Peru variants emphasizes the need to be vigilant in examining sublineage amino acid changes that may be indicators for the emergence of future strains.

The effectiveness of an annually determined trivalent influenza vaccine depends on choosing component strains that offer optimal immunity from the numerous variants in global circulation. In addition, timing is critical because the vaccine strains are selected months before the onset of influenza season to ensure the production of adequate amounts of vaccine by drug manufacturers.

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Luke T. Daum is a Molecular Biologist with the Department of Defense at Brooks Air Force Base, San Antonio, TX. He received a B.S. from the University of Michigan, Ann Arbor, and a M.S. from Central Michigan University. His current research involves the molecular surveillance of influenza in the Department of Defense and the development of rapid PCR-based methods for detecting emerging viruses and bacteria.

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Address for correspondence: Kenton L. Lohman, Molecular Epidemiology Branch, 2601 West Gate Road, Suite 114, Brooks AFB, San Antonio, Texas 78235-5241, USA; fax: 210-536-2638; e-mail: kenton.Lohman@brooks.af.mil

European Bat *Lyssavirus* Infection in Spanish Bat Populations

Jordi Serra-Cobo,* Blanca Amengual,† Carlos Abellán,‡ and Hervé Bourhy†

From 1992 to 2000, 976 sera, 27 blood pellets, and 91 brains were obtained from 14 bat species in 37 localities in Spain. Specific anti-*European bat lyssavirus 1* (EBL1)-neutralizing antibodies have been detected in *Myotis myotis, Miniopterus schreibersii, Tadarida teniotis,* and *Rhinolophus ferrumequinum* in the region of Aragon and the Balearic Islands. Positive results were also obtained by nested reverse transcription-polymerase chain reaction on brain, blood pellet, lung, heart, tongue, and esophagus-larynx-pharynx of *M. myotis*, *Myotis nattereri, R. ferrumequinum,* and *M. schreibersii*. Determination of nucleotide sequence confirmed the presence of EBL1 RNA in the different tissues. In one colony, the prevalence of seropositive bats over time corresponded to an asymmetrical curve, with a sudden initial increase peaking at 60% of the bats, followed by a gradual decline. Banded seropositive bats were recovered during several years, indicating that EBL1 infection in these bats was nonlethal. At least one of this species (*M. schreibersii*) is migratory and thus could be partially responsible for the dissemination of EBL1 on both shores of the Mediterranean Sea.

Rabies is a worldwide zoonosis due to *Lyssavirus* infection; multiple host species act as reservoirs. This disease infects the central nervous system of humans and other mammals. Bats are no exception, as proved by the 630 positive cases detected in Europe from 1977 to 2000 (1,2). Recent molecular studies have shown genetic differentiation in lyssaviruses that cause rabies among European bats, leading to a classification into two new genotypes, 5 and 6, which correspond to *European bat lyssavirus 1* (EBL1) and EBL2, respectively (3,4). As a result of a recent molecular study, two new lineages within genotype 5 have been identified—EBL1a and EBL1b; the latter is potentially of African origin, which suggests south-to-north transmission (5). However, despite molecular advances and many European cases verified to date, knowledge of the prevalence and epidemiology of EBL is limited. Of the 30 insectivorous bat species present in Europe, approximately 95% of cases occur in the species *Eptesicus serotinus* (2). This species, which is nonmigratory, cannot be linked to all the different foci of positive cases in Europe (6). In Spain, the first case of bat lyssaviruses was recorded in 1987 in Valencia. Sixteen more cases were reported in *E. serotinus* (7). The distribution of positive cases in Spain is indicated in Figure 1.

Recently, clinically silent rabies infection has been reported in zoo bats (*Rousettus aegyptiacus)* in Denmark and the Netherlands (8). This observation, together with the results of an experimental challenge, suggests that this frugivorous bat species of African origin can survive EBL1 infection or inoculation (9). Silent infection has also been described in the American bat (*Tadarida brasiliensis mexicana)* (10,11) and suggests an alternative viral strategy for *Lyssavirus* infection of European insectivorous bats compared with the terminal infection commonly associated with rabies infection.

To investigate these observations, a 9-year study was undertaken in Spain to locate and determine the colonies and species of bats carrying EBL or *Lyssavirus* antibodies, monitor the prevalence of seropositive bats, and characterize circulating lyssaviruses.

Material and Methods

Selection of Bat Colonies and Banding

The study area consisted mainly of the Spanish Autonomous Regions of Aragon, Balearic Islands, Catalonia, and

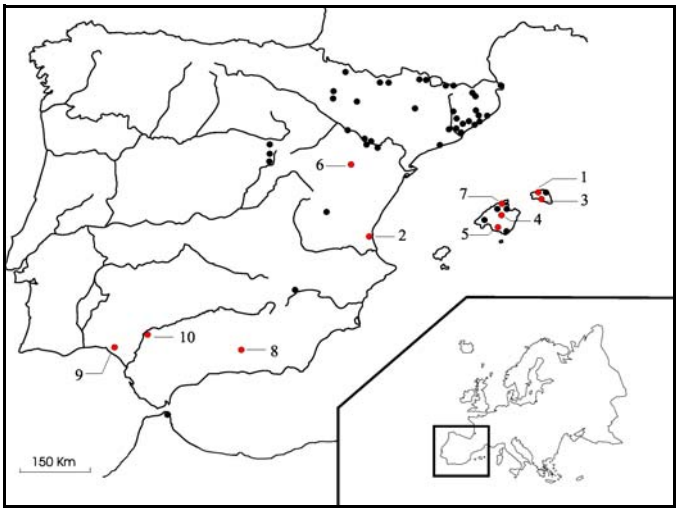

Figure 1. Map showing the localities in Spain where bats have been analyzed. 1. Ciutadella; 2. El Saler; 3. Ferreries; 4. Inca; 5. Llucmajor; 6. Oliete; 7. Pollença; 8. Granada; 9. Huelva; 10. Sevilla. Points in red indicate colonies where positive results were obtained according to our study (Localities Nos. 1, 3, 4, 5, 6, and 7) and previous studies (Localities Nos. 2, 8, 9, and 10) (7).

*Universitat de Barcelona, Barcelona, Spain; †Institut Pasteur, Paris, France; and ‡Ministerio de Sanidad y Consumo, Madrid, Spain

Valencia (Figure 1) (12-15). The region of Ceuta (North Africa, near the Straits of Gibraltar) was also studied because of its proximity to Europe. Bat colonies were selected according to the following criteria: colony behavior (anthropophilic, migratory, gregarious) and proximity of the colonies to urban areas. The Valencia bat colony was widely sampled because the first case of bat *Lyssavirus* in Spain was reported there (7). Colonies exhibiting positive sera were more intensively explored during the years after the first detection. From 1996 to 2000, bats from the locations Nos. 4, 5, and 7 were banded in the forearm to facilitate monitoring of their movements between colonies (16).

Blood Sampling

To draw blood, we set the bat face upward with a stretched wing. The patagium was wiped clean and locally disinfected with a sanitary towel soaked in 96% alcohol to prevent infections. Immediately afterwards, a small puncture was made next to the radius proximal epiphysis. Blood was collected in an Eppendorf vial by using a Pasteur pipette. The amount of blood sampled varied from 0.2 mL to 0.5 mL, according to the size of the animal. A sterilized absorbent hemostatic sponge impregnated with gelatin was administered to prevent bleeding and facilitate healing. Pressure was applied to the wound with a sanitary towel for 30 seconds. The bats were given 10% glucose water to drink to prevent dehydration and provide rapidly assimilated compounds for energy. Once bleeding ceased, the bat was released. Vials containing blood were stored at 4ºC for a few hours. Samples were centrifuged for 20 minutes at 5,000 rpm, and the serum was extracted with a pipette. Serum samples and blood pellets were stored at –20°C.

Detection of EBL Antibodies

The technique used for the detection of EBL antibodies is an adaptation of the Rapid Fluorescent Focus Inhibition Test (17). A constant dose of a previously titrated, cell culture-adapted EBL1 challenge virus 8918FRA (5) was incubated with threefold dilutions of the sera to be titrated. After incubation of the serum/virus mixtures, a suspension of BSR (a clone of BHK-21) cells was added. After 24 hours' incubation, the cell monolayer was acetone-fixed and stained with a fluorescent anti-nucleocapsid antibody (Bio-Rad, Marnes-la-Coquette, France) to detect the presence of non-neutralized virus (fluorescent foci). Titers are presented as an arithmetic mean of two independent repetitions. Serum samples with antibody titers <27 are considered negative for EBL1-neutralizing antibodies. The percentages of seropositive bats and the years in which bats were analyzed (from 1996 to 2000) were correlated, and regression curves were obtained. To confirm the specificity of the reaction, the same test was performed on selected sera by using the challenge virus strain (CVS) (17) and 9007FIN EBL2 challenge viruses (5).

Brain Sampling

Brain samples were obtained from dead bats, submitted by citizens. Dead bats found in the studied refuges were also gathered. The bats found dead from 1994 to 1996 were analyzed by direct immunofluorescence technique (17,18). The bats found dead from 1997 to 2000 were analyzed by nested reverse transcription-polymerase chain reaction (RT-PCR) (9). To eliminate cross-contamination at necropsy, sterilized instruments were used.

Detection of EBL Antigens

The standard fluorescent antibody test (FAT) was performed on brain tissue specimens of the bats by using the polyclonal fluorescein isothiocyanate-labeled rabbit anti-rabies nucleocapsid immunoglobulin G, as described by the manufacturer (Bio-Rad). Brain smears obtained from noninfected and CVS-infected mice were incorporated as controls in each FAT test run.

Detection of EBL1 RNA

Total RNA was extracted from tissue samples (50 mg -100 mg) by using the TRIzol method (Invitrogen, Groningen, the Netherlands), purified with chloroform and precipitated with iso-propanol (Merck, Darmstadt, Germany). After being washed with 70% ethanol, the RNA pellet was dried, resuspended in a volume of 50 mL bidistilled water and stored at –20ºC. cDNA synthesis of the genomic and antigenomic sense of the EBL1a nucleoprotein RNA was performed by annealing, at 70ºC for 3 minutes, 2 mL of total RNA extract with 15 pmol of primers N60 (5'-TCCATAATCAGCTGGTCTCG-3', positions 98-117, relative to rabies genome) (19) and N41, as described previously (5).

Amplification of 5 mL of the cDNA template was performed in a final volume of 50 mL containing 1x magnesium-free PCR buffer (Invitrogen), 5 mM deoxynucleoside triphosphate (NTP) mix (containing 1.25 mM each of dATP, dCTP, dGTP, and dTTP), 5 mM magnesium chloride (Invitrogen), 2 U *Taq* DNA polymerase (Invitrogen), and 30 pmol of primers N60 and N41. The amplification was performed on a GeneAmp PCR System 9700 Thermal cycler. The program started with one denaturation step at 94ºC for 5 minutes, followed by 30 cycles of 94ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 40 sec. The amplification was finalized by an ultimate elongation step at 72ºC for 5 min. The primary amplification products were stored at –20ºC. For nested RT-PCR, the amplified product was diluted 10 times in distilled water. Then the second amplification was performed as described above with the following modifications: 30 pmol of primers N62 and N63 (N62: 5'-AAACCAAGCATCACTCTCGG-3', position 181-200; N63: 5'-ACTAGTCCAATCTTCCGGGC-3', position 342-323 relative to the *Rabies virus* genome) (19) were used, and the elongation steps were performed at 72ºC for 30 sec. Aliquots (5 µL) of nRT-PCR products were analyzed by horizontal agarose (1.5%) gel electrophoresis. Gels were stained with 1 µg/mL ethidium bromide and photographed under UV light.

Extraction of RNA was performed in a level-2 biosafety laboratory. Then we prepared the template and RT-PCR mix

and added DNA to the mix with aerosol-resistant tips in two different rooms. We also performed nRT-PCR on tissue RNA, omitting reverse transcriptase. Positive (isolate no. 2002FRA) and negative (H_2O) controls were incorporated into each of the following steps: total RNA extraction, cDNA synthesis, and each of the two steps of the amplification program. To avoid false-positive results, usual precautions for PCR were strictly followed in the laboratory (20,21).

The threshold of detection of the nRT-PCR method was determined by preparing 10-fold dilutions of a pretitrated suspension of Strain 8918FRA (4) in TRIzol (GIBCO-BRL). Total RNA extraction, cDNA synthesis, and the RT-PCR procedures were performed as described above.

Sequencing of amplified products was performed by using the primers N62 and N63 and an Applied Biosystems 373A sequencer (Foster City, CA), according to the Applied Biosystems protocol. Multiple sequence alignments were generated with the Clustal W 1.60 program (22).

Results

Presence of EBL1 Antibodies in Six Bat Colonies

We describe here a very efficient technique of blood collection, which is more humane than collection by cardiac puncture (23,24). The bats recaptured 1 week after the blood extraction did not show any trace of a scar. Furthermore, our technique is easier than collection by puncture of the uropatagium or the propatagium cardiac veins (25). To eliminate any false- or doubtful positive reactions in seroneutralization, the threshold of positivity (titer=27) was chosen higher than the one adopted by other authors (24). Two independent repetitions of the seroneutralization also reinforced the accuracy of our results.

Throughout the 9-year study, 976 sera obtained from 14 bat species in 37 different locations were analyzed (Table 1); 76 (7.8%) were positive (Table 2). *Lyssavirus* antibodies were detected in four bat species (*Myotis myotis, Miniopterus schreibersii, Tadarida teniotis,* and *Rhinolophus ferrumequinum*). Sixteen positive sera and 5 negative sera against EBL1 (genotype 5 of lyssaviruses) were further tested against standard strains of genotypes 1 (CVS), and 6 (EBL2). These sera were obtained from the four EBL1-seropositive bat species and from another bat species that remained negative (*R. euryale*). None of them reacted positively against CVS and EBL2, confirming the specificity of the positive reactions against EBL1 obtained in these species (Table 3).

The highest percentages of seropositive bats, 22.7% and 20.8%, were observed in the Balearic Islands in the locations of Inca (No. 4) and Llucmajor (No. 5), respectively (Table 2). From spring to autumn, location No. 4 shelters a plurispecific colony of approximately 1,000 bats belonging to the following species: *M. myotis* (25% of seropositives), *M. schreibersii*, *R. ferrumequinum*, *M. capaccinii,* and *M. nattereri*. At the beginning of summer, *M. myotis, M. nattereri,* and *M. schreibersii* species form breeding pairs. Location No. 5 shelters a summer-breeding colony of approximately 500 bats of the species *M. myotis* (22.5% of seropositives), *M. schreibersii* (7.1% of seropositives), and *M. capaccinii*. In both sites the most abundant species is *M. myotis*.

Seropositive bats were also found in four other locations, Nos. 1, 3, 6, and 7. Location No. 1 (5.5% of seropositive *R. ferrumequinum*) shelters a breeding colony of *R. ferrumequinum*.

Table 1. Number of bat samples analyzed per species, 1992–2000[a]

Species	1992	1993	1994	1995	1996	1997	1998	1999	2000	Total
R. ferrumequinum		8				9/3		11/1	30/3	58/7
R. euryale		6			10					16
R. hipposideros				16		0/1				16/1
P. pipistrellus	61	64	75	18	13/5	0/16	0/14	3/15		234/50
P. kulhii				1						1
E. serotinus	21		44	33/1	1					99/1
M. myotis		1		63	65/2	44	29/2	58/8	35/3	295/15
M. blythi		20	1	2						23
M. nattereri		1					0/1		0/1	1/2
M. capaccinii						3				3
M. emarginatus				9		7/2				16/2
P. austriacus			3	6	2/4			1		12/4
Mi. schreibersii	8	18			14	8	9/2	70/6	41/1	168/9
T. teniotis					22	12				34
Total	90	118	123	148/1	127/11	83/22	38/19	143/30	106/8	976/91

[a]Where fractions (x/y) are shown, the numerator (x) corresponds to the number of sera analyzed and the denominator (y) to the number of brains analyzed.
E = *Eptesicus*; M = *Myotis*; Mi=*Miniopterus* P = *Plecotus*; R = *Rhinolophus*; T = *Tadarida*.

Table 2. Positive serologic results in bat populations, the Spanish Autonomous Regions of Balearic Islands and Aragon, 1995–2000

Location and coordinates	Variables analyzed	Years					
		1995	1996	1997	1998	1999	2000
No. 1	A/B[a]	-	-	1/5	-	0/11	1/20
	X±SD[b]	-	-	515	-	-	34
39°58'N,3°58'E	Species[c]	Rf	Rf	Rf	Rf	Rf	Rf
No. 3	A/B[a]					1/34	0/31
	X±SD[b]					215	
39°58'N,3°59'E	Species[c]	Ms	Ms	Ms	Ms	Ms	Ms
No. 4	A/B[a]	1/30	16/27	11/27	7/22	3/30	3/29
	X±SD[b]	90	348±237	191±225	718±657	78±27	58±42
39°44'N,2°58'E	Range		49–908	29-783	79-1677	47-95	29–107
	Species[c]	Mm	Mm	Mm	Mm	Mm	Mm
No. 5	A/B[a]	7/21	7/32	3/17 0/6	3/7 1/8	5/28	0/6
	X±SD[b]	122±45	207±159	218±136	412±454 8,508	106±61	
39°25'N,2°55'E	Range	83-195	53-442	129-374	87-930	29-176	
	Species[c]	Mm	Mm	Mm Ms	Mm Ms	Mm	Mm
No. 6	A/B[a]		0/22	2/12			
	X±SD[b]			243±284			
41°01'N,0°39'W	Range			420-444			
	Species[c]	Tt	Tt	Tt	Tt	Tt	Tt
No. 7	A/B[a]		2/14			2/19	
	X±SD[b]		93±68			35±6	
39°50'N,3°00'E	Range		45-141			31-40	
	Species[c]	Ms	Ms	Ms	Ms	Ms	Ms

[a]A = no. bats positive, B = no. bats analyzed.
[b]X = seroneutralization average; SD = standard deviation.
[c]Species analyzed: Rf = *Rhinolophus ferrumequinum*; Ms = *Miniopterus schreibersii*; Mm = *Myotis myotis*; Tt = *Tadarida teniotis*.

In spring, the colony also includes some *M. schreibersii*. Location No. 3 (2.9% of seropositive *M. schreibersii*) is a hibernation refuge for approximately 2,200 *M. schreibersii;* some *M. capaccinii* are also present. Location No. 6 (5.8% of seropositive *T. teniotis*) is a big sinkhole with a resident bat colony belonging to the following species: *T. teniotis*, *M. blythii*, *M. daubentonii*, *Pipistrellus pipistrellus*, *Pipistrellus kuhlii*, *Hypsugo savii*, *E. serotinus*, *Plecotus austriacus,* and *Barbastella barbastellus* (26). Location No. 7 (12% of seropositive *M. schreibersii*) shelters a colony of *M. schreibersii*, *M. capaccinii,* and *M. myotis*.

Evolution of the Percentage of Seropositive Bats in Colonies Nos. 4 and 5

In Location 4, the percentage of seropositive bats rose from 3.3% in 1995 to 59.3% in 1996 (Table 2). Then it decreased significantly ($Y=-15.6X + 31{,}196.5$, $r=-0.989$, $p<0.05$) until 1999, when it reached 10%. This percentage remained stable in 2000. The percentage of seropositive bats remained stable in Location No. 5 from 1995 to 2000.

Exchange of Animals Between Colonies and Survival of Seropositive Bats

During the period 1996-2000, 355 and 87 *M. myotis* were banded in Locations Nos. 4 and 5, respectively (Table 4). Recapture of the banded *M. myotis* allowed us to prove a few exchange of bats between the colonies. Two percent of *M. myotis* banded in Location No. 5 moved to Location No. 4 (the refuges are about 35 km apart). During the same period, 13 and 33 *M. schreibersii* were banded in Locations Nos. 5 and 7, respectively. One of the 33 *M. schreibersii* moved to Location No. 5 (the refuges are approximately 47 km apart); another moved to Location No. 4 (a distance of 11 km) (Figure 1).

Banding also allowed us to follow the seroneutralization titer of some bats during the study period. The serum of a *M. schreibersii* captured in Location No. 7 in 1996 was negative;

Table 3. Specificity of results from serologic studies of bat populations, Spanish Autonomous Regions of Balearic Islands and Aragon, 1995–2000[a]

Location	Species	CVS	EBL1	EBL2
No. 1	*Rhinolophus ferrumequinum*	0	51	ND
		0	<27	ND
No. 4	*Myotis myotis*	0	588	<27
		0	222	<27
		0	350	<27
		0	246	<27
		0	709	<27
		0	<27	ND
		0	537	<27
		0	95	<27
No. 5	*M. myotis*	0	53	<27
		ND	421	<27
		0	97	<27
		0	188	<27
No. 6	*Tadarida teniotis*	0	42	ND
		0	444	ND
		0	<27	ND
No. 7	*Miniopterus schreibersii*	0	45	<27
		0	141	<27
		0	<27	<27
	Rhinolophus euryale	0	<27	<27

[a]ND = not done; CVS = challenge virus strain; EBL1 = *European bat lyssavirus 1*.

another serologic sample obtained from the same bat 2 years later in Location No. 5 yielded a titer of 8,508. During spring 2000, 12 *M. myotis* previously banded and analyzed were recaptured in Location No. 4. Four (33%) of them had already been shown to be seropositive in preceding years: two in summer 1997 (titers 29 and 145, respectively), one in summer 1998 (titer 303), and one in summer 1999 (titer 95). This indicates that some seropositive bats may survive at least 3 years after *Lyssavirus* infection.

Detection and Characterization of EBL1 RNA in Bats

During 1995 through 1996, 12 brain samples were only analyzed by FAT. After 1996, the brain samples (n=79) were also analyzed by nested RT-PCR (Table 1). All brains (n=91) analyzed by FAT were negative. In contrast, brains of 1 *M. myotis*, 1 *M. nattereri,* and 1 *M. schreibersii* (No. 140) of Location No. 4 and 1 *R. ferrumequinum* (No. 128) of Location No. 1 (all collected in 2000) were positive by nested RT-PCR. Four animals (*M. schreibersii* [No. 140] and *R. ferrumequinum* [No. 128], whose brains were positive by nested RT-PCR, and two *R. ferrumequinum* [No. 123 and No. 135], whose brains were negative) were completely necropsied. Various organs and tissues (medulla, liver, kidney, spleen, heart, tongue, esophagus-larynx-pharynx, and lung) were collected and subjected to nRT-PCR. Esophagus-larynx-pharynx and lung of bat No. 135 and tongue, lung, and heart of bat No. 128 were positive (Figure 2).

Table 4. No. of recaptured and analyzed bats in Locations 4, 5, and 7, Spain, 1996–2000

Species[a]	BB[b]	BA[c]	BR[d]	BRD[e]	ATT[f]
Mm	442	221	25	2	4
Ms	46	46	0	2	1

[a]Species studied: Mm = *Myotis myotis*; Ms = *Miniopterus schreibersii*.
[b]BB = No.of bats banded.
[c]BA = No. of bats banded and analyzed.
[d]BR = No. of bats banded and recaptured in the same location.
[e]BRD = No. of bats banded and recaptured in different localities.
[f]ATT = No. of bats analyzed twice at interval of ≥1 year.

Twenty-seven blood pellets of bats collected in 2000 were also analyzed by nRT-PCR. These samples were obtained from 8 *R. ferrumequinum* (location No. 1), 1 *R. ferrumequinum* (Location No. 3), 1 *M. myotis* (Location No. 5), 14 *M. myotis* (Location No. 4), and 3 *M. schreibersii* (Location No. 4). The blood pellets of three *M. myotis* from Location No. 4 were found positive by nRT-PCR. None of the blood samples showing positive RT-PCR results on the pellet were found positive by seroneutralization.

The threshold of detection of the nRT-PCR for the amplification of the EBL1a genomic and antigenomic RNAs of the N gene was 5 x 10^{-2} fluorescent forming units of EBL1a/mL. In all these experiments, negative controls performed individually for each step (extraction, RT, primary, and secondary PCR) were negative. Furthermore, nRT-PCR performed on positive tissues without previous reverse transcription gave negative results, demonstrating the absence of complementary DNA contamination.

Nucleotide (nt) sequences were determined by using the positive nRT-PCR products obtained from the four brains and from one blood sample. These 122-nt long sequences of the nucleoprotein gene were strictly similar to the sequence of two EBL1b Spanish isolates (94285SPA and 9483 SPA) described previously (5), except that the sequence obtained from the positive blood pellet exhibited a T→A mutation in position 145 of the coding region of the nucleoprotein gene. Four mutations distinguished the sequence of the positive control corresponding to a French bat (No. 2002FRA) from the different sequences obtained from Spanish bats (not shown). This further confirms the specificity of the products amplified from the Spanish bat samples.

Discussion

This is the first report of the presence of EBL1-specific neutralizing antibodies in four European insectivorous bat species (*M. myotis, M. schreibersii, T. teniotis,* and *R. ferrumequinum*). These findings lead to the following observations on the circulation and possible bat species involved in the dispersion of EBL1 in southern Europe. First, the identification of EBL1 antibodies in 24% of the *M. myotis* analyzed in Locations No. 4 and No. 5 in 1995 through 2000 (n=276) indicates that bats of this genus are infected with EBL1. Second, the distribution of *T. teniotis* and *M. schreibersii* in southern Europe and northern Africa (13,27) could contribute to the dispersion of EBL1 in southern Europe and is concordant with the possible African origin of EBL1, as suggested by Amengual et al. (5).

Although the seasonal movements of *T. teniotis* are scarcely known, the quick, straight flight of this species

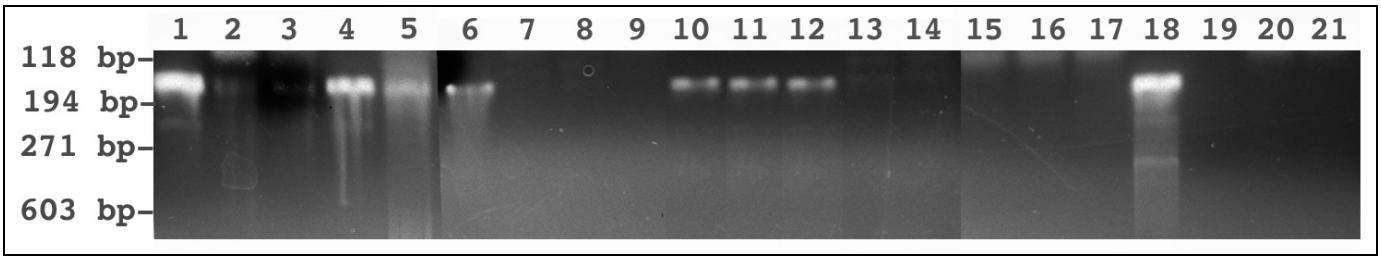

Figure 2. Detection of *European bat Lyssavirus 1* RNA in bats by nested reverse transcription-polymerase chain reaction (PCR). Lanes: 1, brain of *Miniopterus schreibersii* No. 140; 2- 5, medulla, tongue, esophagus-larynx-pharynx, and lung of *Rhinolophus ferrumequinum* No. 135, respectively; 6-14, brain, medulla, esophagus-larynx-pharynx, liver, lung, heart, tongue, spleen, and kidney of *R. ferrumequinum* No. 128, respectively; 15, negative control of RNA extraction of bat No. 135; 16, negative control of RNA extraction of bat No. 128; 17, negative control of RNA extraction of bat No. 140; 18, positive control; 19, negative control of first PCR; 20, negative control of second PCR.

suggests that such movements are long, as is the case with the American bat (*T. brasiliensis mexicana),* which is capable of performing annual migrations of more than 1,000 km. Since *M. schreibersii* makes seasonal migrations (some of them >350 km) (16), this species could also be one of the dispersion vectors of the disease in southern Europe, where it abounds. *M. schreibersii* dwells in five out of the six sites where seropositive bats have been found. In three of them, *M. schreibersii* forms mixed colonies with *M. myotis*, in one it shelters next to *R. ferrumequinum,* and in the fifth it shelters alone. *M. schreibersii* and *M. myotis* have direct physical contact in the mixed colonies. However, it is unlikely that *Pipistrellus nathusii* is a dispersion vector of the lyssaviruses in Spain, as Brosset (6) suggests, since this is a very rare bat in the Iberian Peninsula.

The results obtained in 1995-2000 in Location No. 4 show that the evolution in the number of seropositive bats after a *Lyssavirus* infection corresponded to an asymmetrical curve, with a sudden initial increase reaching more than 60% of the colony and a gradual decline over subsequent years (24)—unless a new episode took place (Figure 3). Because of the gregarious behavior of this species, a quick increase and a high seropositive percentage (almost 60% in this location) after a *Lyssavirus* episode are not unusual. The intimate contact that always exists among bats must facilitate viral transmission and antibody development. A high seropositive percentage also occurs in colonies of *T. brasiliensis mexicana*, where percentages >80% have been observed (10,11). The transmission of lyssaviruses between bats from mixed colonies could take place through breathing or biting but is currently not documented.

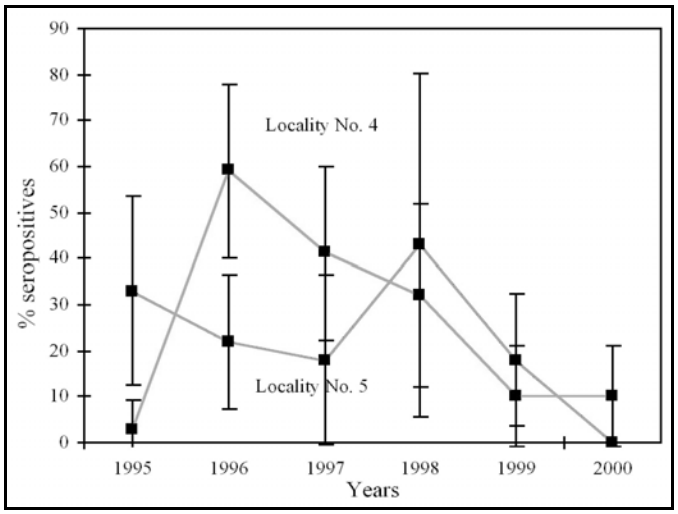

Figure 3. Incidence of seropositive bats observed in *Myotis myotis* colonies, Spanish Locations No. 4 and No. 5, 1995–2000 (95% confidence intervals shown).

The low prevalence (0 of 91, <1.1%) of active infection as determined by FAT is concordant with previous results obtained in America, which show a prevalence of active rabies infection in bats between 0.1 and 2.9% (10,28,29). However, we report the first detection of EBL1 RNA by nRT-PCR in several tissues (brain, blood pellet, lung, heart, tongue, and esophagus-larynx-pharynx) of four *M. myotis*, one *M. nattereri*, one *M. schreibersii,* and two *R. ferrumequinum*. These isolates show the existence of a low or nonproductive infection in these species, although some small remnant of RNA remaining in a clinically normal bat as a result of an earlier nonlethal exposure to a *Lyssavirus* is also possible. This low amount of viral DNA present in the tissues underscores the need to use nRT-PCR as a very sensitive technique for epidemiologic studies of EBL1 in bat populations. Rønsholt et al. (8) also comment on the difficulty of detecting *Lyssavirus* infection by immunofluorescence in bats when a clinically silent infection exists.

EBL1 are known to actively infect the brain, lung, and tongue of *E. serotinus* (3). However, this is the first report that EBL1 RNA can be detected in various organs and tissues in the absence of active infection, as demonstrated by negative results obtained by FAT. Most of these bats were dead when collected but were kept in conditions that allowed the classic diagnosis by FAT to be performed properly. These negative FAT results indicate that these bats died of causes other than their low productive *Lyssavirus* infection. The recapture of seropositive bats over several years also shows that some of these bats survived EBL1 infection. The detection of EBL1b sequences in the blood pellet of bats (3/27) is also a new finding. This technique would be an easy test for screening positive bats. However, further studies are needed to establish the interest and sensitivity of this sample.

The sensitivity of the different European bat species to EBL infection probably varies according to the animal and virus species involved. Therefore, we have summarized in

Table 5. Bat species positive for *Lyssavirus,* Europe, 1954–2000[a]

Family	Species	Lyssavirus [b]	Antibodies [c]
Vespertilionidae	*Eptesicus serotinus*	EBL1a & b	EBL1
	Pipistrellus pipistrellus	NC	ND
	Pipistrellus nathusii	NC	ND
	Vespertilio murinus	EBL1a	ND
	Myotis dasycneme	EBL2a	ND
	Myotis daubentonii	EBL2a & b	ND
	Myotis myotis	EBL1b	EBL1
	Myotis nattereri	EBL1b	ND
	Nyctalus noctula	NC	ND
	Miniopterus schreibersii	EBL1b	EBL1
Molossidae	*Tadarida teniotis*	NC	EBL1
Rhinolophidae	*Rhinolophus ferrumequinum*	EBL1b	EBL1

[a]The additional information was obtained from Kappeler (29), Pérez-Jordá et al. (24), Amengual et al. (5), Bulletin épidemiologique mensuel de la rage en France (30), and Muller (2).
[b]NC = not characterized.
[c]ND = not done.

Table 5 (2,5,24,30,31) the bat species in which either *Lyssavirus* or antibodies against *Lyssavirus* have been detected. Further studies are needed to determine which of the European bat species are the reservoir of EBL infection and if different species act as sentinels for the presence of the virus in the colony.

The presence of EBL1 RNA and immunity to EBL1 in several wild bat colonies also has important implications for bat management and public health. The probability of humans' having contact with these colonies should be reduced and controlled. In our study, most bat colonies were found in sites that are frequently visited by speleologists, tourists, and bat-lovers. As a consequence of our findings, the entry to these caves is now controlled and limited during the periods when bats are present (in spring, summer, and autumn for Location No. 4). Entry is limited by horizontal bars that allow the bats to fly across them but prevent access to people without obscuring the view.

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Jordi Serra-Cobo is a member of the Quality Research Team (Biology of Vertebrates, 96-SGR0072) of the Universitat de Barcelona and a contracted doctor by the Instituto Pirenaico de Ecología (CSIC). His areas of expertise are vertebrates, population ecology, and bat lyssaviruses. Since 1990 he has been working in the research of Spanish bat lyssaviruses for the Ministerio de Sanidad y Consumo and the Conselleria de Sanitat of the Balearic Autonomous Government.

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Address for correspondence: Jordi Serra-Cobo, Departament de Biologia Animal, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal, 645, 08028 Barcelona, Spain; fax: 34-93-403-57-40; e-mail: bamengua@pie.xtec.es

Invasive Group A Streptococcal Infections, Israel

Allon E. Moses,* Sara Goldberg,† Zinaida Korenman,‡ Miriam Ravins,† Emanuel Hanski,† Mervyn Shapiro,* and the Israeli Group for the Study of Infections Caused by *Streptococcus pyogenes*[1]

We conducted a prospective, nationwide, population-based study of invasive group A streptococcal infections in Israel. We identified 409 patients (median age 27 years; range <1-92), for an annual incidence of 3.7/100,000 (11/100,000 in Jerusalem). The mortality rate was 5%. Bacteremia occurred in 125 cases (31%). The most common illnesses were soft-tissue infection (63%) and primary bacteremia (14%). Thirty percent of patients had no identifiable risk factors for infection. Eighty-seven percent of pharyngeal carriers had the same serotype as the index patient. M types included M3 (25%), M28 (10%), and M-nontypable (33%). A marked paucity of M1 serotype (1.2%) was detected. The results highlighted concentrated pockets of invasive disease in the Jewish orthodox community (annual incidence 16/100,000).

Group A streptococcus (GAS) causes human disease ranging from noninvasive infections such as pharyngitis or impetigo to life-threatening conditions such as bacteremia, necrotizing fasciitis (NF), and toxic-shock syndrome (TSS). Invasive GAS infections are thought to result from entry of bacteria through the skin, although often the site of entry cannot be determined. Since the mid-1980s, retrospective reviews of invasive GAS disease in different geographic areas have described an increase in deaths from these infections (1-4). These studies have also emphasized the changing nature of the population affected and have shown that young, healthy persons often have severe infections (4,5). This increased severity of invasive GAS infections has produced an augmented search for new virulence factors and host determinants that may amplify the potential of this organism for producing disease. Since GAS vaccines are being developed by several groups (6,7), baseline incidence data on severe GAS infections are needed. Information regarding the geographic distribution of M types will assist in directing vaccine development to prevalent strains. Prospective population-based studies provide an assessment of the true incidence of severe infection and are thus the preferred method for studying the epidemiology of disease. Few such studies of severe GAS infections have been performed (5,8-11); no previous studies have encompassed the epidemiology of an entire country.

We report the clinical characteristics of patients and bacterial attributes of GAS isolates from a 2-year, nationwide, prospective, population-based study to determine the incidence of invasive GAS diseases in Israel. In the greater Jerusalem area, we conducted an in-depth study to determine the prevalence of carriage of GAS in household contacts of index patients with invasive disease.

*Hadassah University Medical Center, Jerusalem, Israel; †Hebrew University Medical School, Jerusalem, Israel; and ‡Ministry of Health Streptococcal Reference Laboratory, Jerusalem, Israel

Methods

We studied invasive GAS infections in Israel from January 1997 through December 1998. Collaboration between the study center in Jerusalem and 24 of the 25 acute-care hospitals in Israel was coordinated with the infectious diseases consultant or the director of the microbiology laboratory at each hospital. These hospitals serve approximately 95% of the Israeli population.

We provided each hospital with kits for transporting bacterial isolates and a questionnaire requesting the following demographic and clinical data for each case: age, sex, infection site, presence of hypotension, and signs of organ damage, including renal failure, adult respiratory distress syndrome, disseminated intravascular coagulation, or mental changes.

In a 1995 census from the Israeli Central Bureau of Statistics, the population of Israel was 5,548,000; 81% were Jews. Of the total population, 698,000 were children ≤5 years of age, 990,000 were 6-15 years of age, 2,550,000 were 16-45 years old, and 1,310,000 were persons >45 years of age. The median age of the population in Israel was 27.4 years (23.2 in the greater Jerusalem area).

In the greater Jerusalem area, the total population was 602,100; 421,200 were Jews, and 180,900 were Arabs. Among

[1]Israeli Group for the Study of Infections Caused by *Streptococcus pyogenes*: V. Agmon, A. Mates, Ministry of Health Streptococcal Reference Laboratory; E. Ambun, Barzilai Hospital, Ashkelon; E. Azam, French Hospital, Nazareth; S. Berger, Y. Igra, Ichilov Medical Center, Tel Aviv; I. Boldur, A. Itzhaki, Assaf Haroffe Hospital, Zriffin; N. Chashan, R. Finkelstein, Rambam Hospital, Haifa; Y. Glick, Laniado Hospital, Natanya; R. Gutman, M. Dan, Wolfson Medical Center, Holon; E. Halperin, Bikur Cholim Hospital, Jerusalem; M. Kaupstein, D. Chasin, Hillel Yaffe Hospital, Hadera; N. Keller, Sheba Medical Center, Tel Hashomer; R. Kolodney, R. Raz, Haemek Hospital, Afula; A. Lefler, Naharia Hospital; D. Lev, M. Efros, Carmel Hospital, Haifa; A. Linben, Poryia Hospital, Tiberias; M. Maayan, M. Chovers, Meir Hospital, Kfar Saba; A. Miskin, Z. Landau, Kaplan Hospital, Rehovot; I. Moskovitz, Hasharon Hospital, Petah Tiqva; G. Rahav, D. Engelstein, Hadassah Medical Center, Jerusalem; Z. Samra, A. Bushara, Rabin Medical Center, Petah Tiqva; I. Sarugo, I. Potesman, Bnei Zion Hospital, Haifa; Y. Schlezinger, B. Rodensky, D. Atias, Share Zedek Hospital, Jerusalem; M. Shechter, Ziv Hospital, Zafat; and P. Yagupsky, Soroka Medical Center, Beer Sheva

the Jewish population, 120,000 were Orthodox Jews, with an average of 5.5 members per family, compared with 2.9 in the nonorthodox Jewish population. Using age-adjusted rates for *Human herpesvirus 3 (HHV-3)* infection (12), we estimated that 4,100 cases of varicella occurred annually in children <10 years of age in the Jerusalem cohort.

Invasive disease was defined as the isolation of GAS from a normally sterile site such as blood, joint, or wound infection or from deep tissue retrieved during surgery. Isolates from the throat, ear, and eye were excluded. TSS was defined according to conventional criteria (13).

A study nurse (S.G) interviewed household contacts (defined as persons who lived in the same household as the index patient) within 3 days of obtaining a positive culture from an index patient who had been admitted to a hospital (one of three medical centers) serving the greater Jerusalem area. Information was obtained about antibiotic treatment and the presence of a recent throat or skin infection. Pharyngeal cultures were obtained from all household contacts.

Hospital laboratories identified the isolate as GAS by using a commercial latex agglutination kit. We sent all isolates to the Israel Ministry of Health Streptococcal Reference Laboratory for M- and T-typing and confirmatory GAS antigen typing. The presence of the genes encoding the two exotoxins A and C (*spe*A, *spe*C) was assessed by polymerase chain reaction (PCR) (14).

Statistical analysis was done by using the chi-square test for differences in proportions. A p value of <0.05 was regarded as significant.

Results

Clinical and Epidemiologic Characteristics

During the 2-year study period, 24 medical centers in Israel submitted 423 specimens. Of these, 14 isolates were not included in the study: 6 were found not to be GAS, and 8 were isolates from an eye or ear infection. Thus, 409 isolates were available for further study. The audit of case reporting showed a sensitivity that ranged from 30% to 70% among different institutions. However, for patients with bacteremia the average sensitivity of case reporting was 75%.

The annual incidence of invasive GAS infection in Israel was 3.7 per 100,000 population (Table 1). The median age was 27 years (range 1 week to 92 years). Incidence was highest in children ≤5 years and adults >45 years of age (Table 1). For bacteremia and TSS, the annual incidences in the national cohort (Table 1) were 1.1/100,000 and 0.25/100,000, respectively, with the highest incidence in persons ≥45 years of age (p<0.001). The case-fatality rate of the national cohort could not be assessed because deaths were underreported.

The disease entities associated with invasive GAS infection are summarized in Table 2. Of the 409 cases, 125 (31%) had bacteremia, which was considered to be primary (14%) if no source of infection was identified. The median age of bacteremic patients was 48 years, considerably higher than the median age (27 years, p<0.001) of all participants. Of 42 cases with cellulitis, 59% also had bacteremia. Of 14 cases of necrotizing fasciitis, 4 were bacteremic, and GAS was isolated from deep fascia in 10 others. Fourteen patients had pneumonia: in 5 patients GAS was isolated from blood, in 2 from pleural fluid, in 1 from lung tissue, and in the other 6 from sputum. Of 10 patients with burns, 7 were from one burn unit. Three of these seven comprised a single nosocomial outbreak.

In the national cohort, of 28 cases defined as TSS, 16 (57%) were male. In 20 (71%) of the TSS patients, GAS was isolated from blood cultures; in 10 this bacteremia was primary. Six had concurrent NF, two were children with chickenpox, and two had cellulitis. Only one patient was known to be positive for HIV infection.

Characteristics of Bacterial Isolates

Serologic typing was performed for 401 isolates (98%). Of these, 33% were M-nontypable. The typable strains belonged to 23 different M serotypes. The most common strain was M3, constituting 25% of all isolates. The next most common serotypes were M28 (10%), M2 (5%), M62 (4%), M41 (3%), and M12 (3%). M1 was isolated from only five cases (1.2%). Four isolates gave a positive serologic reaction with two M serotypes.

Among the M-nontypable isolates, 21 different T serotypes were identified (data not shown). The most prevalent were T28 (11%), T12 (9%), T11 (8%), and T3/13/B3264 (8%); 28% were T-nontypable. T serotypes were different from those

Table 1. The incidence of diseases from the national cohort, the Jerusalem cohort, the cohort of patients with bloodstream isolates, and the toxic-shock syndrome cohort, by age group, 1997–1998

Age group (years)	National cohort: no. of patients	Annual incidence[a]	Jerusalem: no. of patients	Annual incidence	Bacteremia: no. of patients	Annual incidence	TSS: no. of patients	Annual incidence
<5	88	6.3	38	19	26	1.86	5	0.36
6-15	53	2.68	16	6.8	11	0.56		
16-45	116	2.27	41	8	24	0.47	8	0.16
>45	109	4.16	38	15	62	2.37	15	0.57
Unknown	43				2			
Total	409	3.69	133	11	125	1.13	28	0.25
Median age (yr)	27		24		48		49	

[a]Annual incidence = cases per 100,000 population. TSS = Toxic-shock syndrome.

Table 2. Clinical characteristics of invasive GAS infections: comparison of the national cohort to the Jerusalem cohort, 1997–1998

Disease	National cohort	%	Jerusalem cohort	%
Soft tissue infection[a]	272	67	88	66
Primary bacteremia	57	14	11	8.3
Pneumonia	14	3.4	4	3
Postpartum	10	2.4	6	4.5
Arthritis	8	2	1	< 1%
Lymphadenitis	6	1.5	2	1.5
Chickenpox	5	1.2	5	3.8
Meningitis	5	1.2	2	1.5
Peritonitis	5	1.2	4	3
PID	5	1.2	1	< 1%
Osteomyelitis	4	< 1%	2	1.5
Others	14	3.4	5	3.8
Unknown	4	< 1%		
Total	409	100	133	100

[a]Soft tissue infection includes 35 patients with abscesses, 14 with necrotizing fasciitis, and 10 with burns. PID = pelvic inflammatory disease.

usually associated with M1. The M serotype distribution was similar in the national and Jerusalem cohorts.

Four hundred and one isolates were tested for the presence of *spe*A or *spe*C by PCR. Ten percent were positive for *spe*A in both cohorts. Thirty-two percent and 37% were positive for *spe*C in the national and Jerusalem cohorts, respectively. Of the 101 M3 strains, 21% were positive for *speA*.

Jerusalem Cohort

Of 409 patients who could be evaluated, 133 were from the Jerusalem area. In Jerusalem, the audit of case reporting showed a sensitivity of 94%. The median age was 24 years. The annual incidence of disease was 11/100,000 (Table 1). In this cohort the highest annual incidence was in the orthodox Jewish community (16/100,000; Table 3). The incidence differed by age group, with the groups ≤5 years (19/100,000) and >45 years (15/100,000) having the highest incidence ($p < 0.01$). Males accounted for 65%. In the Jerusalem group there were 86% Jews and 14% Arabs (Table 3).

The overall case-fatality rate in the Jerusalem area was 5% (7/133) but was 14% (6/44) among patients with bacteremia. Four of the seven patients who died in Jerusalem were >65 years of age.

All five cases of chickenpox-related GAS infection were in the Jerusalem cohort, for an estimated attack rate of 61/100,000 cases per year. One child died of the infection. All were children <4 years of age from orthodox Jewish families, but no epidemiologic association could be demonstrated since strains belonged to a variety of M serotypes.

In the Jerusalem cohort of 133 cases, we assessed the presence of underlying medical conditions that may predispose to GAS infection. Forty patients (30%) had no underlying disease. Nineteen percent had an acutely infected skin lesion. Twelve percent had a chronic skin condition, which was acutely infected with GAS. Ten percent of patients had diabetes mellitus, and nine percent had various forms of cancer. Nine pregnant women (7%) were infected before or shortly after delivery. Five children had chickenpox.

In the Jerusalem cohort, pharyngeal cultures were obtained from 302 contacts of 60 index patients. Relatives of 73 index patients were not studied because of lack of informed consent (22), nonavailability due to early discharge (19), or absence of family contacts (32). The mean number of family contacts per index patient was 5 (range 1-9). Twenty-eight index patients were associated with 61 household carriers of GAS, 75% of whom were ≤15 years old. Only one contact (a child with pharyngitis) was symptomatic. The gender distribution of the carrier cohort was similar to that of the general population. Comparison of the M serotypes of the carriers and their respective index patients disclosed identity in 87% of cases.

Discussion

Our prospective population-based study is a first nationwide survey of the incidence of invasive GAS. The annual incidence of invasive GAS infections in Israel (3.7/100,000) is similar to that reported from Pima County, Arizona (4), and Atlanta, Georgia (9), but is considerably higher than that reported for Ontario, Canada (1.5/100,000) (8). The annual incidence in the Jerusalem cohort was three times higher (11/100,000) than that of the national cohort, reflecting, at least in part, a greater accuracy of reporting, achieved by frequent contact of the study nurse with the three medical centers. The relatively large number of orthodox Jews living in the Jerusalem area may also have contributed to the higher incidence of infection in this city. Unlike Ontario or Connecticut (8,11), reporting invasive GAS infections in Israel is not mandatory, contributing to the relatively low reporting accuracy. Thus, the true incidence of invasive GAS disease in Israel may be closer to that of the Jerusalem cohort, which is substantially higher than that reported by other population-based studies. We made routine telephone calls to the hospital contacts to encourage participation and confirm that cases were being reported. Audits of one third of the large and small hospital laboratories were conducted twice during the study period to evaluate the proportion of cases actually reported. Since some of the Arab

Table 3. Religious distribution of patients in the Jerusalem cohort, 1997–1998

Religion[a]	Number (n=133)	%	Annual incidence[b]
Jewish	113	85	13
Orthodox Jewish	39/113	26	16
Moslem/Christian Arabs	18	14	5
Other	2	1	

[a]The Orthodox Jewish cohort is included in the Jewish cohort.
[b]Annual incidence = cases per 100,000 population.

population in Jerusalem uses the East Jerusalem Arab hospitals, which were not included in the study, infections occurring in a small proportion of Jerusalem Arab patients may have been missed.

Risk factors for GAS infection were not studied in the national cohort. In the Jerusalem cohort, 30% were previously healthy patients without evident risk factors. This finding is consistent with those of other studies, supporting the notion that underlying illnesses appear to play an important role in the occurrence of invasive GAS disease (4,8,9,15-17). Previous skin lesions, diabetes mellitus, and cancer were the most common conditions predisposing to GAS infection. Alcoholism and AIDS are relatively rare in Israel (18) and were not found to be risk factors for our patients.

In Israel the incidence of invasive streptococcal disease in children was higher than reported previously (9), consistent with our earlier finding that children with GAS bacteremia in Jerusalem were younger than those reported by others (19). In Jerusalem the incidence of GAS infection in the ≤5-year age group was higher than previously reported (19/100,000). This incidence remained elevated (14/100,000) even after five cases of an infection secondary to chickenpox were excluded from analysis.

In Jerusalem, 40% of children (≤15 years of age) with invasive GAS infections were from the orthodox Jewish community. The overall incidence for this group was 16/100,000 population and was probably even higher in children ≤15 years of age, although data for age distribution were not available for this group. In this community, families are large and the relatively crowded living conditions may facilitate the spread of streptococci (20). Recently, a higher pharyngeal carriage rate (odds ratio 5.0; 95% confidence interval 2.1-11.9) of GAS was reported for an orthodox Jewish community in London (21). In Jerusalem, the incidence of GAS infection was also much higher in the group >45 years of age (Table 1). Thus, the incidence of severe GAS infections reported in the Jerusalem area is much higher than previously reported. The mortality rate (5%) in the Jerusalem cohort was lower than that reported by Davies et al. (8) and Zurawski et al. (9) but is similar to the rate reported for bacteremic patients at the Hadassah Medical Center in Jerusalem (22).

Both bacteremia and TSS occurred at a significantly older age ($P<0.0001$) (median age 48 and 49 years, respectively) than the median age of the general population in Israel (Table 1). The annual rate of TSS in our study was similar to that reported by Davies et al. (0.2/100,000), who also found that severe disease occurred preferentially in older patients. NF was relatively rare in Israel (annual incidence 0.1/100,000), accounting for 3.4% of all patients and 4.5% of the Jerusalem cohort patients. This was similar to the percentage reported by Zurawski (3%) but considerably less than that reported by Davies et al. (13%). Zurawski et al. (9) suggested that the low incidence of NF might have been due to ascertainment bias, engendered by the laboratory-based study methods, which may have missed cases of NF without concomitant bacteremia. However, in Jerusalem the close association between the study team and their clinical and laboratory counterparts in all three medical centers makes such an ascertainment bias unlikely. The differences between population-based studies may be due to microbiologic attributes of the strains involved or other unknown factors.

Nosocomially acquired invasive streptococcal infections were relatively rare. Postpartum infections accounted for 2.4% and 4.5% of the national and Jerusalem cohorts, respectively. Although this infection can be hospital acquired (22, 23), GAS may also arise from the patient's own bacterial flora (8). Seven of the 10 burn-related infections occurred in one hospital, and three cases belonged to a single serotype (M3, *spe*C+). Such an outbreak has been reported to occur by transfer of GAS from medical personnel to patients (24-29).

Our data support the assumption that chickenpox is a risk factor for invasive GAS disease (8,9,30-33). In our cohort, all children with chickenpox were from orthodox Jewish families. Nevertheless, cases were caused by diverse M serotypes and occurred in several different city neighborhoods without any epidemiologic link between them.

The most striking microbiologic characteristic of the GAS isolates of both the national and Jerusalem cohorts is the paucity of the M1 serotype. This finding contrasts with many previous reports, which described the M1 serotype as the prominent isolate from patients with invasive GAS infection, particularly those with NF or TSS (34-40). Our M-nontypable strains had 21 distinct T serotypes that differed from those usually associated with M1 strains. Thus, *emm* typing would be unlikely to categorize these nontypable isolates as M type 1.

The association between M1 and the production of exotoxin A is well established (37) but not universal (41). The prevalence of *spe*A in our cohort (10%) was lower than that found by Zurawski (34%) (9) and Kiska, who observed that 98% of M1 and M3 outbreak strains were *speA* positive (5). Half our *spe*A-positive cases were serotype M3, but few were associated with TSS and NF. We concur with Davies (8) that factors other than *spe*A play a role in TSS pathogenesis. These findings and the paucity of the M1 serotype among our isolates suggest that no single invasive clone is responsible for severe disease (20) and that strains attain their virulence through means other than *spe*A. The relative prevalence of *spe*C in our study was similar to that found by others (8).

Serotype M3, the most frequent M type in our cohort and a relatively common isolate in other studies of invasive GAS (41,42), seems to have replaced M1 as the leading cause of invasive streptococcal disease (8). In a retrospective analysis of GAS bacteremia in Jerusalem over a 6-year period (1987-1992), none of the 41 isolates available were serotype M3, 11 (27%) were nontypable, 4 were M12, and 2 were M1 (22). Thus, an unexplained increase in the rate of invasive M3 strains in Jerusalem has occurred. Nevertheless, this increase has not been accompanied by a change in the absolute number of bacteremic streptococcal cases per year, and the mortality rate has remained constant. Therefore, we cannot conclude that infections with M3 result in more cases of bacteremia or

are more virulent. Only 20% of our M3 strains harbored the *spe*A gene, compared with 100% of the strains from Japan (41). In Israel, 67% of 21,517 GAS isolates (mostly from pharyngeal swabs taken over a 10-year period) were M-nontypable, and 99% were T-typable (43). The most prevalent M serotypes were M12 (17%) and M1 (6%), and the most prevalent T type was 3/13/B3264 (20%). Ten years later, Yagupsky et al. found 90% strains (10/13 cases) of GAS isolated from children with bacteremia to be M-nontypable (44).

The M28 serotype, accounting for 10% of our cases, was reported to be a common serotype in invasive GAS diseases by some investigators (8) but not by others (9). We also had a relatively high percentage of M-nontypable isolates. This finding is in contrast to those who have been able to serologically type >90% of isolates (41) but is similar to findings of surveys in which 60% or 80% of isolates were nontypable (9,45). *emm*-typing (46) may clarify the actual M type of those strains.

In the Jerusalem cohort, we found a particularly high prevalence of patients with family members who had GAS in their pharynx. As reported previously (47), the M types of almost all isolates (87%) from household contacts were identical to those found in the index patients. We chose to administer preventive antimicrobial therapy to positive contacts, although this practice is still controversial (48). None of the contacts had invasive disease.

As in other studies (47), we found that asymptomatic carriers were mostly young children. Whether the index patients were infected from an asymptomatic carrier or vice versa is impossible to determine. The reasons for one person remaining an asymptomatic carrier while another has a severe, sometimes lethal infection have not been clarified. Whether there are any genetic differences between the index patient/contact pair of bacterial isolates or whether varying virulence genes of GAS are expressed under different clinical conditions remains to be determined. Further studies of GAS epidemiology and pathogenesis are required to determine the reasons for acquiring severe invasive GAS diseases in specific hosts. This knowledge will allow a more accurate definition of the risk factors for these infections and may lead to development of effective intervention strategies.

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Dr. Moses is a senior physician in the Department of Clinical Microbiology and Infectious Diseases and a senior lecturer in Clinical Microbiology at the Hadassah-Hebrew University Medical Center. His research interests include the pathogenesis and epidemiology of Group A streptococcal infections.

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Address for correspondence: Allon E. Moses, Hadassah Medical Center, P.O. Box 12000, Jerusalem 91120, Israel; fax: 9722-6758915; e-mail: MOSESA@MD2.HUJI.AC.IL

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Mapping Lyme Disease Incidence for Diagnostic and Preventive Decisions, Maryland

Christina Frank,* Alan D. Fix,* César A. Peña,† and G. Thomas Strickland*

To support diagnostic and preventive decision making, we analyzed incidence of Lyme disease in Maryland on the zip code level. Areas of high incidence were identified on the Upper Eastern Shore of the Chesapeake Bay and in counties north and east of Baltimore City. These latter foci, especially, are not visible when mapping Lyme disease on the county level.

Lyme disease (LD) is a multisystem infectious and inflammatory disease resulting from infection with the spirochete *Borrelia burgdorferi*. It is by far the most commonly reported vector-borne disease in the United States and is transmitted by the bite of infected *Ixodes scapularis* ticks (1). In the United States, areas at high risk for the disease focally occur in temperate wooded habitats sustaining *B. burgdorferi*'s small mammalian hosts, predominantly the white-footed mouse (*Peromyscus leucopus),* as well as the preferred mating place for adult ticks, on the white-tailed deer (*Odocoilus virginianus*) (2).

We have reported the incidence of LD for Maryland by county (3). Glass and colleagues developed a detailed LD risk map of Baltimore County by using environmental risk factors within a geographic information system (GIS) (4). The objective of our report is to show areas of high incidence of LD on a level more detailed than the standard reporting by county. The next most detailed geographic boundary system for which population data are available is the zip code level. It provides intermediate detail between counties and census blocks. The zip code also allows incidence calculations based on census population figures and, by relying on postal address, uses a feature of geographic reference very commonly available to state health departments n Maryland and elsewhere. We believe this level of detail in mapping the focal distribution of LD will improve decision-making regarding diagnoses, personal and community interventions, and cost-effective use of vaccine.

The Study

Included in our report were all cases meeting the national surveillance case definition for LD (5) reported to the Maryland Department of Health and Mental Hygiene (DHMH) with a known date of onset from 1993 through 1998 and a residential zip code mailing address.

All cases were referenced to zip code of residence. Demographic data from the 1990 census are publicly available for the zip code level (6). In 1990, population figures for Maryland zip codes ranged from 39 to 56,594 (median 3,042). To obtain larger units of population for more stable estimates of incidence, small zip codes were combined with the next smallest neighboring zip codes until the aggregated zip code area (AZCA) reached the size of >600 residents or more. Annual average incidence per zip code or AZCA was calculated as the average number of cases from 1993 through 1998 per 100,000 population. One hundred six small-population zip codes were combined with others to give 50 AZCAs, ranging in size from 616 to 51,683 (median 1,791). Most AZCAs were located in western Maryland (non disease-endemic area) and the Eastern Shore (highly endemic area). The 1990 census did not contain population data for 15 zip codes, so their incidence could not be calculated.

Analysis and data management were performed with EpiInfo version 6.04 and Microsoft EXCEL version 7.0; maps were created with ArcInfo (ESRI, Redlands, CA).

A total of 2,399 cases reported to the DHMH with a known date of onset from 1993 through 1998 met the national surveillance case definition for LD. This report includes the 2,371 (99%) patients for whom mailing addresses were available. Cases were reported from 344 (80%) of 431 zip codes. Only 6 of the 33 zip codes from western Maryland (Garrett, Allegheny, Washington, and Frederick Counties) reported cases of LD during the study period.

Two areas of high incidence are evident: the Upper Eastern Shore (Cecil, Kent, Queen Anne's, Caroline, and Talbot Counties), and focal areas north and east of Baltimore City in Baltimore and Harford Counties (Figure). This latter area is part of an arc of increased incidence, from Montgomery County in the south, extending northeast through Howard, southeastern Carroll, Baltimore, and Harford Counties into Cecil County. This arc parallels the "fall line," the topographic boundary where the Coastal Plains meet the Piedmont and land elevations begin to rise towards the Appalachian Mountains.

In Baltimore County, the area with the highest incidence extends along the vegetational corridors bordering the Gunpowder Falls river system and associated reservoirs. In Harford County, a similar but less confined linear pattern follows the runs of Broad Creek and Deer Creek. On the Upper Eastern Shore high incidence is more uniformly observed, without any clear topographic pattern. The scattered zip codes on the Lower Eastern and Lower Western Shores that show higher incidence than surrounding areas are all AZCAs with rather small populations and resultant wide confidence intervals for their incidence estimates.

Conclusions

The detailed mapping of LD in Maryland identifies an area of high LD incidence north of Baltimore City that is not be

*University of Maryland Baltimore, Baltimore, MD, USA; and †Maryland Department of Health and Mental Hygiene, Baltimore, MD, USA.

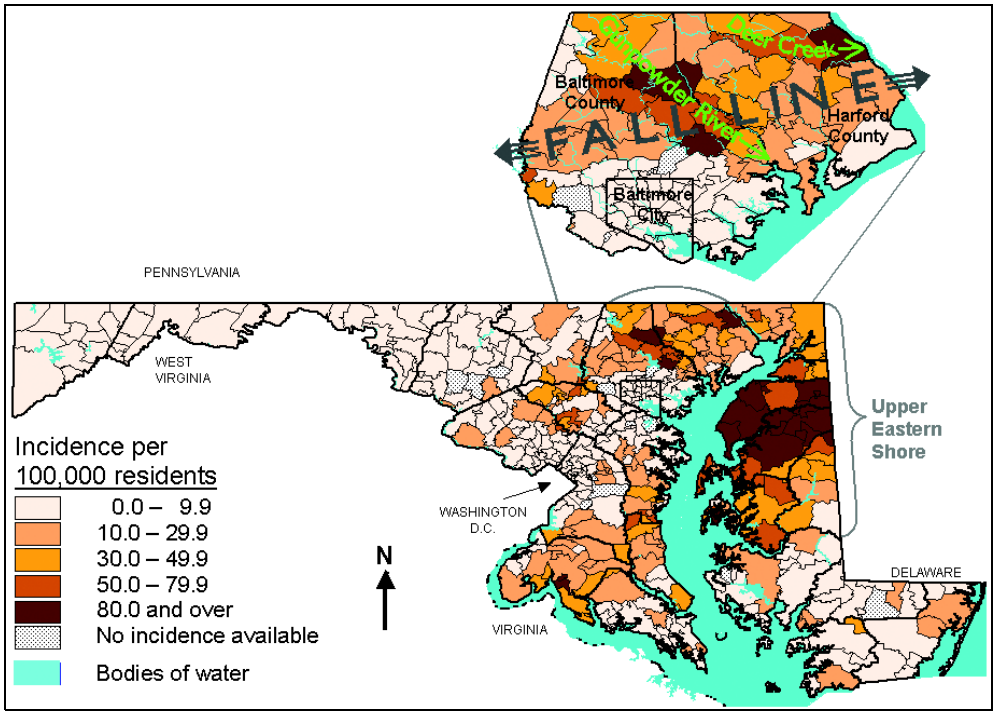

Figure. Average annual incidence of Lyme disease by zip code, Maryland, 1993–1998.

apparent when mapping on the county level (3). When analyzed by counties, focal high incidence along Gunpowder River and Deer Creek is diluted by adjacent areas of lower incidence, especially the northern inner suburbs of Baltimore City with their comparatively urban environment. These foci are aligned along the larger rivers and creeks in an environment that is ideal for transmission of the disease. Within the floodplain and on valley slopes of the rivers descending from the Piedmont lie corridors of forest and brush, cutting through rural and suburban areas. Farms, estates, individual houses, and housing developments lie within and adjoin these ideal tick habitats. Local outdoor recreation is widespread. The extent of the high-risk area in Baltimore County is congruent with Glass's detailed GIS results (4).

In contrast, Maryland's Upper Eastern Shore, a rural area situated entirely in the Coastal Plain with an ideal tick habitat, has uniformly high LD incidence. However, southern Maryland (north of the Potomac River) and the Lower Eastern Shore have a low incidence of LD. This correlates with limited ecologic data showing much lower *B. burgdorferi* infection rates in *I. scapularis* in southern Maryland and the Lower Eastern Shore than in the Upper Eastern Shore (7,8). Almost no LD was reported in western Maryland from Frederick County westward; this virtual absence is consistent with the low prevalence of *I. scapularis* and low *B. burgdorferi* infection rates in this tick species, despite an abundance of rodents and deer in this mountainous region (7,8).

Spot-mapping of LD cases is useful for tracking LD transmission but can be misleading about incidence because population density is not taken into consideration. On a spot map, based on the absolute number of cases, a sparsely populated area with high LD incidence may be indistinguishable from another area with high population density and low incidence. Incidence figures for counties containing both highly urban and highly rural areas are likely not representative of the rural areas because of the concentration of population in the urban part. Health-care providers must appreciate the fact that, for instance, more cases of LD are reported from Baltimore County (population ~1 million) than from Cecil County (population 20,000), even though the county-level incidence of LD is much higher in Cecil County.

Characterization of LD incidence on the zip code level is feasible using data collected routinely by local health departments. Zip code level data provide more detailed information than county level data and require less data and effort than GIS risk modeling based on vegetation parameters and tick distribution (4, 9-11). Although LD risk-mapping based on prevalence of infection in ticks would be the most accurate method (12), tick data are often unavailable, out-of-date, costly, and difficult to collect. A potential limitation of our report is that incidence has been referenced to residential addresses, whereas patients may have been infected elsewhere. However, residence in an LD-endemic area is a well-recognized risk factor for infection (12). Many studies have reported that patients with LD usually believe they were infected at their homes, places of work, or some nearby recreational site (13-17). A calculated entomologic risk index showed a strong positive relation with the geographic LD case rate in Rhode Island (18). Most (58%) of our study participants who remembered a tick bite believed it occurred at or near their place of residence; an additional 21% were bitten during recreation and 9% at work. Patients in high incidence areas were more likely to report a tick bite near their home than were those living in more urban areas or in western Maryland. Referencing LD cases to their residence is a useful proxy for the actual place patients acquired a tick bite.

Knowledge of focal LD risk distilled from mapping on the zip code level is of value to the general public. It can focus efforts to reduce tick exposure and increase motivation to use appropriate preventive measures when tick exposure is unavoidable. Such mapping can also aid health-care providers in assessing the likelihood of a particular patient's having LD (19).

Meltzer et al. estimated how much the cost-effectiveness of LD vaccination depends on individual risk (20). Mapping

LD incidence in detail complements the Centers for Disease Control and Prevention's (CDC's) recommendation that the LD vaccine be administered based on residential, occupational, and recreational risk assessment (21). The CDC report recognizes the need to "develop maps of geographic distribution of LD with improved accuracy and predictive power" beyond the county-based national LD risk map. This level of detail would aid the "Healthy People 2010" goal of LD prevention through targeted vaccination (22).

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Christina Frank has a degree in geography from Cologne University, Germany, and is currently finishing a doctorate in epidemiology at the University of Maryland, Baltimore. She has conducted 18 months of field studies on hepatitis virus infections and liver cancer in Egypt. Her interests include geographic medicine and infectious disease mapping.

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Address for correspondence: G. T. Strickland, Department of Epidemiology and Preventive Medicine, University of Maryland - Baltimore, 660 W. Redwood Street, Baltimore, MD 21201, USA; fax: 410-706-8013; e-mail: tstrick@epi.umaryland.edu

Salmonella enterica Serotype Typhimurium DT 104 Antibiotic Resistance Genomic Island I in Serotype Paratyphi B

Danièle Meunier,* David Boyd,† Michael R. Mulvey,† Sylvie Baucheron,* Caterina Mammina,‡ Antonino Nastasi,§ Elisabeth Chaslus-Dancla,* and Axel Cloeckaert*

We have identified *Salmonella* genomic island I (SGI1) in an isolate of *Salmonella enterica* serotype Paratyphi B. This antibiotic-resistance gene cluster, which confers multidrug resistance, has been previously identified in *S. enterica* serotype Typhimurium phage types DT 104 and DT 120 and in *S. enterica* serotype Agona.

Multidrug-resistant *Salmonella enterica* serotype Typhimurium definitive phage type 104 (DT 104) has emerged during the last decade as a global health problem because of its association with animal and human disease (1). Multidrug-resistant strains of this phage type were first identified from exotic birds in the United Kingdom in the early 1980s and in cattle and humans in the late 1980s but have since become common in other animal species such as poultry, pigs, and sheep. The DT 104 epidemic has now spread worldwide, with several outbreaks since 1996 in the United States and Canada (2-5).

Multidrug-resistant *S.* Typhimurium DT 104 strains are commonly resistant to ampicillin, chloramphenicol/florfenicol, spectinomycin/streptomycin, sulfonamides, and tetracyclines. The antibiotic-resistance genes are clustered in part of a 43-kb genomic island called *Salmonella* genomic island I (SGI1), between the *thdf* and *int2* genes of the chromosome (6-10). The *int2* gene is part of a retron that has been detected only in serotype Typhimurium (7,8). Downstream of the retron sequence is the *yidY* gene, which is also found in the chromosome of other *S. enterica* serotypes (7,8). The antibiotic-resistance gene cluster represents approximately one third of SGI1 and is located at the 3' end of the structure (7,8). All resistance genes are clustered and are bracketed by two integron structures (Figure 1). The first integron carries the *aadA2* gene, which confers resistance to streptomycin and spectinomycin, and a truncated *sulI* resistance gene. The second contains the beta-lactamase gene bla_{PSE-1} and a complete *sulI* gene. Flanked by these two integron structures are the *floR* gene (6), also called *floSt* (11) or *cmlA*-like (9), which confers cross-resistance to chloramphenicol and florfenicol, and the tetracycline-resistance genes *tetR* and *tet* (G). Florfenicol resistance and detection of the *floR* gene by polymerase chain reaction (PCR)-based methods have been proposed as a means for rapidly identifying multidrug-resistant *S.* Typhimurium DT 104 strains (11), since phage typing is available only in specialized laboratories. However, recently SGI1 has been reported in another phage type of serotype Typhimurium and in serotype Agona, suggesting horizontal transfer of SGI1 (7,10,12). In serotype Agona, SGI1 has the same chromosomal location as in DT 104 strains, except that it lacks the retron sequence found downstream of SGI1; thus it is located between the *thdf* gene and the *yidY* gene of the chromosome (7).

Recently, Nastasi and Mammina reported the presence of the *floR* and *tet*(G) genes detected by PCR in an *S. enterica* serotype Paratyphi B strain of biovar Java isolated from a tropical fish in Singapore (13). We examined this isolate to determine the presence of SGI1.

The Study

The antibiotic-resistance phenotype of the serotype Paratyphi B strain was assessed by the disk-diffusion assay. All antibiotic disks except for florfenicol were purchased from Bio-Rad (Marnes-La-Coquette, France). Florfenicol disks and the drug itself were obtained from Schering-Plough Animal Health (Kenilworth, NJ). The serotype Paratyphi B strain showed a multidrug-resistance profile typical of serotype Typhimurium DT 104 or serotype Agona strains carrying SGI1, i.e., resistance to ampicillin, chloramphenicol and florfenicol, streptomycin and spectinomycin, sulfonamides, and tetracyclines. Moreover, this strain showed the same resistance level to florfenicol as serotypes Typhimurium DT 104 and Agona, i.e., a florfenicol MIC 32 µg/mL (12). The strain was susceptible to trimethoprim and the quinolones nalidixic acid, enrofloxacin, and ciprofloxacin.

No plasmids were detected in the serotype Paratyphi B strain, suggesting that all antibiotic-resistance genes were chromosomally located. PCR mapping of the typical antibiotic resistance genes and integrons associated with SGI1 was performed as described (Figure 1) (12). PCR amplifications yielded products from genomic DNA extracted from the serotype Paratyphi B strain of the size expected from DNA of serotype Typhimurium DT 104 control strain BN9181 (data not shown) (6,12). Partial nucleotide sequencing of the *floR* gene showed 100% identity with that of serotype Typhimurium DT 104. Thus, these PCR mapping results indicated that the serotype Paratyphi B strain contains the entire antibiotic-resistance gene cluster of serotype Typhimurium DT 104.

The conservation of the antibiotic-resistance genes' organization was further assessed by Southern blot of *Hin*dIII- or *Xho*I-digested genomic DNA, with the 12-kb *Xba*I insert as

*Institut National de la Recherche Agronomique, Nouzilly, France; †Health Canada, Winnipeg, Manitoba, Canada; ‡University of Palermo, Palermo, Italy; and §University of Florence, Florence, Italy

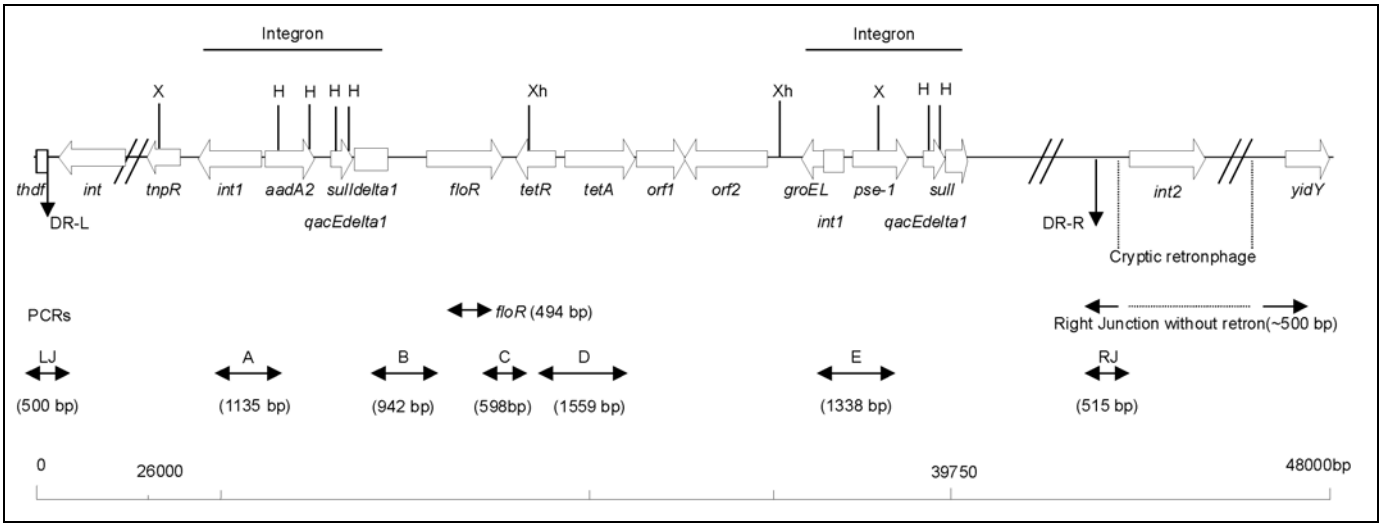


Figure 1. Genetic organization of the antibiotic-resistance gene cluster of SGI1 of *Salmonella enterica* serotype Typhimurium DT 104. DR-L and DR-R are the left and right direct repeats, respectively, bracketing SGI1. Polymerase chain reactions (PCRs) used to assess the genetic organization of the antibiotic-resistance genes (PCRs *floR*, A, B, C, D, and E) and the SGI1 junctions to the chromosome (PCRs LJ and RJ for left and right junctions, respectively) are indicated. Abbreviations for restriction sites: X, *Xba*I; H, *Hin*dIII; Xh, *Xho*I.

probe (see *Xba*I fragment, Figure 1) from recombinant plasmid pSTF3 containing the DT 104 antibiotic-resistance gene cluster as described (6,12). The Southern blot profiles of the serotype Paratyphi B strain were identical to those of the DT 104 control strain BN9181 and serotype Agona strain 959SA97 (Figure 2), confirming that the organization of the antibiotic-resistance genes in the serotype Paratyphi B strain is the same as in serotype Typhimurium DT 104 or serotype Agona containing SGI1 (7,12).

To assess the presence of the entire SGI1 and its location in the chromosome of the serotype Paratyphi B strain, PCR was performed by using primers corresponding to the left and right junctions of SGI1 in the *Salmonella* chromosome. PCR took into account the presence or absence of the *int2*-retron sequence, which is located downstream of SGI1 in serotype Typhimurium DT 104 but not in serotype Agona (Figure 1) (7,8). PCR was positive for the left junction of SGI1, as for serotype Typhimurium DT 104 or serotype Agona. If a sequence of the *int2* gene of the retron was used as reverse primer, PCR was negative for the right junction of SGI1, but it was positive if the sequence of the *yidY* gene was used. The PCR products showed the expected sizes of approximately 500 bp for both the left junction and right junction PCR without the retron sequence in Figure 1. These data indicate that the serotype Paratyphi B strain contains SGI1 at the same chromosomal location as in serotype Typhimurium DT 104 or serotype Agona, that is, between the *thdf* and *yidY* genes, but lacks the retron sequence found in DT 104 strains and other serotype Typhimurium strains (7,8). The presence of entire SGI1 was also confirmed by Southern blot of *Xba*I-digested genomic DNA with the p1-9 probe containing a 2-kb *Eco*RI insert. This corresponds to a central region of SGI1, comprising parts of the S023 and S024 open reading frames, which code for putative helicase and exonuclease proteins, upstream of the antibiotic-resistance gene cluster (7). This probe revealed *Xba*I fragments of the 4- and 9-kb sizes expected in the serotype Paratyphi B strain and the control serotype Typhimurium DT 104 and serotype Agona strains (Figure 2B).

Macrorestriction analysis by pulsed-field gel electrophoresis of the serotype Paratyphi B strain DNA cut by *Xba*I showed that it is genetically distinct from both serotypes Typhimurium DT 104 and Agona (Figure 3), further indicating at the molecular level that the occurrence of SGI1 in the serotype Paratyphi B strain probably results from horizontal transfer and not seroconversion of known *S. enterica* serotypes containing SGI1.

Conclusions

We have identified SGI1 in a *S.* Paratyphi B strain. These data, in conjunction with the identification of SGI1 in *S.* Agona and *S.* Typhimurium strains, suggest horizontal transfer of this region (7,10,12). That SGI1 has the same chromosomal location in *S.* Typhimurium, *S.* Agona, and *S.* Paratyphi B suggests that its insertion occurred through a homologous recombination event, perhaps through phage transduction (14). This hypothesis is experimentally supported by the fact that resistance genes of serotype Typhimurium DT 104 can be efficiently transduced by P22-like phage ES18 and phage PDT17, which is released by all DT 104 isolates analyzed (14). However, the question remains why the retron sequence downstream of SGI1 in serotype Typhimurium DT 104 and DT 120 strains (7) is not present downstream of SGI1 in other serotypes. A possible explanation could be that in the horizontal transfers described here, the SGI1 donor strains are not serotype Typhimurium strains. Once SGI1 has been acquired, it may become stable in the chromosome, as in vitro excision experiments failed to demonstrate its loss in a DT 104 strain in the absence of antibiotic selective pressure (8). This factor may contribute to evolution of *S. enterica* pathogens, similar to the acquisition of pathogenicity islands (15,16).

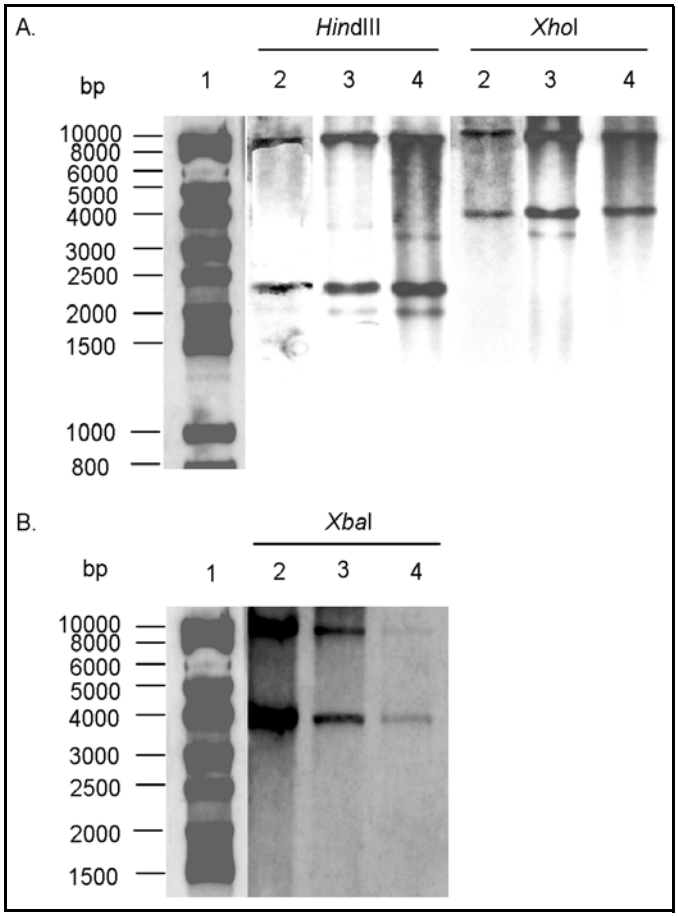
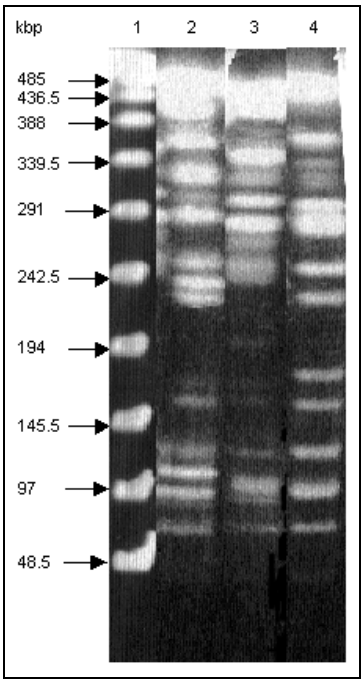


Figure 2. A. Southern blot hybridization with the *Xba*I probe (Figure 1) containing all antibiotic-resistance genes of *Hin*dIII- and *Xho*I-digested genomic DNAs of *Salmonella enterica* serotype Typhimurium DT 104 strain BN9181 (lanes 2), serotype Agona strain 959SA97 (lanes 3), and serotype Paratyphi B strain (lanes 4). Lane 1: DNA ladder. B. Southern blot hybridization with the p1-9 probe of *Xba*I-digested genomic DNAs of *S. enterica* serotype Typhimurium DT 104 strain BN9181 (lanes 2), serotype Agona strain 959SA97 (lanes 3), and serotype Paratyphi B strain (lanes 4). Lane 1: DNA ladder.

In addition, the use of antimicrobial agents in agriculture might be of importance for the selection and spread of SGI1 among *S. enterica* serovars (17). The export of contaminated food or animals contributes to the spread of such multidrug-resistant serovars and may be responsible for worldwide epidemics such as that caused by serovar Typhimurium phage type DT104. For example, florfenicol is a veterinary antimicrobial agent that has been used in aquaculture in Asia since the early 1980s and may have contributed to selection and horizontal transfer of SGI1 to phage type DT104 (7). Serovar Paratyphi B is the most frequently isolated serovar from imported seafood samples from Asian countries (18).

Acquisition of SGI1 may have been a key factor contributing to the DT 104 worldwide epidemic, perhaps not only through selection by agricultural use of antimicrobial agents (17) but also by possible virulence properties of SGI1 (10). Therefore, further surveillance is warranted for the emergence of horizontal transfer of SGI1 to *S. enterica* serotypes of public health importance.

Figure 3. Macrorestriction analysis by pulsed-field gel electrophoresis of genomic DNAs cut by *Xba*I of *S. enterica* serotype Typhimurium DT 104 strain BN9181 (lane 2), serotype Agona strain 959SA97 (lane 3), and the serotype Paratyphi B strain (lane 4). Lane 1: DNA ladder.



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Dr. Meunier is a postdoctoral fellow at the Institut National de la Recherche Agronomique in France. Her main interests are antibiotic-resistance mechanisms and spread of antibiotic-resistance genes among enterobacterial pathogens.

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Address for correspondence: Axel Cloeckaert, Unité de Pathologie Aviaire et Parasitologie, Institut National de la Recherche Agronomique, 37380 Nouzilly, France; fax: 33- 2 47 42 77 74; e-mail: cloeckae@tours.inra.fr

Multiply Resistant (MR) *Salmonella enterica* Serotype Typhimurium DT 12 and DT 120: A Case of MR DT 104 in Disguise?

Andrew J. Lawson, Miatta U. Dassama, Linda R. Ward, and E. John Threlfall

Multiresistant *Salmonella enterica* serotype Typhimurium definitive phage type (DT) 12 and DT 120 are more closely related to DT 104 than to non-multiresistant strains of their respective phage types. Multiresistant DT 12 and DT 120 appear to have arisen due to changes in phage susceptibility of DT 104 rather than horizontal transfer of resistance genes.

Multiresistant (MR) *Salmonella enterica* serovar Typhimurium definitive phage type (DT) 104 is now acknowledged as an internationally distributed zoonotic pathogen. Since 1991, this phage type has been second only to *S.* Enteritidis phage type 4 as the principal agent of human salmonellosis in England and Wales (1). MR DT 104 is characterized by resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (R-type ACSSuT). In MR DT 104 these antibiotic resistance genes have been accumulated in chromosomally encoded gene cassettes, a process mediated by the presence of class 1 integrons (2). Some isolates possess additional plasmid-mediated resistance to trimethoprim and low-level resistance to ciprofloxacin because of point mutations in the *gyr*A gene (1).

The potential exists for the horizontal transfer of genetic elements such as antibiotic resistance gene cassettes between *Salmonella* serotypes and phage types. Evidence of this transfer was reported in 2000, when the presence of an MR DT 104-like antibiotic resistance gene cluster was reported in *S.* Agona (3). Throughout the 1990s, the ACSSuT R-type was also identified in isolates of *S.* Typhimurium DT 12 and DT 120, although as yet numbers remain relatively small (27 of 84 and 22 of 109 of DT 12 and DT 120 isolates, respectively, were of R-type ACSSuT of a total of 2,651 *S.* Typhimurium isolates received at the Central Public Health Laboratory, London, in 2000). ACSSuT-resistant MR DT 12 incidence has remained fairly constant since the mid-1990s, but as isolations of sensitive DT 12 have diminished the relative proportion of MR DT 12 has increased (Figure 1). ACSSuT-resistant DT 120 has attained levels comparable with those of MR DT 12 only in the last few years but might well have been the predominant representative of DT 120, were it not for a large outbreak of antimicrobial-sensitive DT 120 in northern England during 1999-2000 (Figure 1) (4).

We investigated multiple antibiotic-resistant strains of MR DT 12 and MR DT 120 to determine if horizontal transfer of the antibiotic resistance genes had occurred between apparently unrelated phage types of *S.* Typhimurium.

The Study

Isolates of *S.* Typhimurium DT 12 and DT 120 from human, animal, food, and environmental sources received by the Laboratory of Enteric Pathogens from 1991 to 2000 were examined. For each phage type, 40 strains were selected, consisting of 15 antibiotic-sensitive strains, 12 strains of R-type ACSSuT, and 13 strains with non-ACSSuT R-types. Control strains of *S.* Typhimurium DT 104, both sensitive and MR, were also included.

All strains were characterized by phage type, in accordance with the scheme of Anderson et al. (5); antimicrobial susceptibility patterns were determined by using the breakpoint method (6), so that final concentrations (mg/mL^{-1}) were ampicillin, 8; chloramphenicol, 8; furazolidone (Fu), 8; gentamicin (G), 4; kanamycin (K), 8; neomycin (Ne), 8; streptomycin, 16; sulfonamides, 64; tetracyclines, 8; trimethoprim, 2; and ciprofloxacin, 0.125.

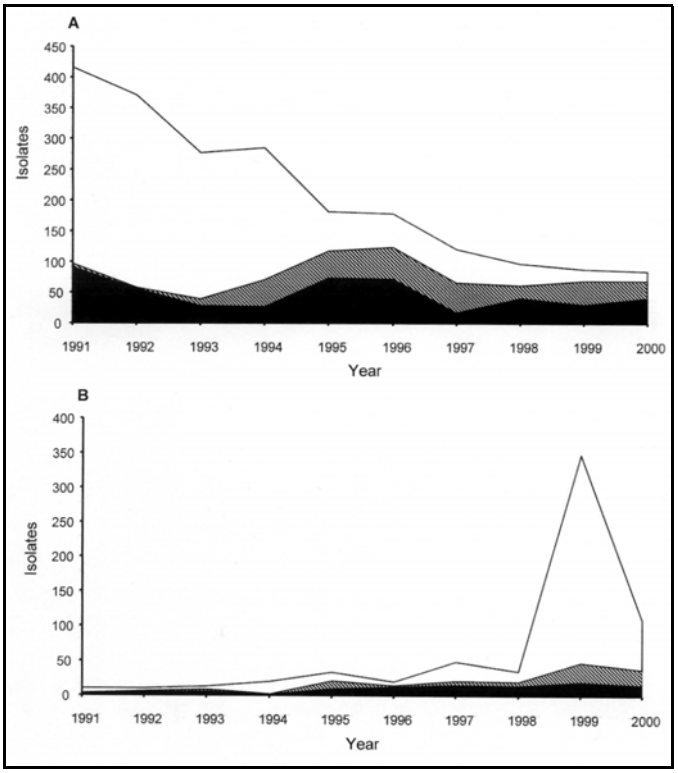

Figure 1. Antimicrobial susceptibility of *Salmonella enterica* serotype Typhimurium definitive phage type (DT) 12 and DT 120 isolates, England and Wales, 1991–2000. A, *S.* Typhimurium DT 12; B, *S.* Typhimurium DT 120. Clear bar, sensitive; diagonal screened bar, resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (ACSSuT; includes resistant-type ACSSuT and ACSSuT plus additional resistances to Tm, $Cp_{L,}$ or both); black bar, other resistance patterns.

Central Public Health Laboratory, London, United Kingdom

The presence of the 13 kbp ACSSuT resistance gene cluster was determined by a long-polymerase chain reaction (PCR) method developed by Walker et al. (7) that amplified the 10-kbp region between the *aad*A2 (streptomycin resistance) and *bla* $_{CARB-2}$ (ampicillin resistance) genes on the sequence of the antibiotic resistance gene cluster from *S.* Typhimurium DT 104 isolate H3380 (8). All strains of MR DT 104, DT 12, and DT 120 of R-type ACSSuT produced 10-kbp PCR amplicons; strains with other resistance patterns were negative.

The strains were examined for the presence of class 1 integrons by using a specific PCR assay (9). All strains of MR DT 104, DT 12, and DT 120 of R-type ACSSuT produced bands of 1.0 kbp and 1.2 kbp corresponding to the two integrons reported within the resistance gene cassettes (8,10). This corroborated the long-PCR result and demonstrated that there was no apparent variation in the arrangement of the genes within the ACSSuT resistance cassettes. Sensitive and non-ACSSuT-resistant strains were either negative by the class 1 integron PCR or produced bands other than 1.0 kbp and 1.2 kbp.

Pulsed-field gel electrophoresis (PFGE) analysis was performed by using the restriction enzyme *Xba*I according to the method of Powell et al. (11). Analysis showed that all sensitive and non-ACSSuT-resistant strains of DT 12 and DT 120 examined formed distinct groups of phage type-related PFGE profiles that clustered when analyzed by the nearest neighbor algorithm (12) (Figure 2). Nine distinct profiles (z1 to z9) were obtained from non-ACSSuT-resistant DT 12 and five different profiles (A to E) from the non-ACSSuT-resistant DT 120. Importantly, the ACSSuT-resistant strains of both DT 12 and DT 120 produced similar PFGE patterns that were distinct from those obtained by non-ACSSuT-resistant strains of the same phage type. However, these patterns were identical to those commonly observed in both sensitive and MR strains of DT 104 (profiles *xtm*1, *xtm*9, and *xtm*12). In contrast, the PFGE profiles of sensitive and ACSSuT-resistant strains of DT 104 typically differ by no more than a few bands. The PFGE profile of the ACSSuT-resistant DT 12 strains were all identical to that produced by most (80%-90%) DT 104 isolates, termed profile *xtm*1 (10). The ACSSuT-resistant strains of DT 120 were mainly (8 of 12 strains) profile *xtm*1; the remainder were profiles *xtm*12 (3 of 12) and *xtm*9 (1 of 12). PFGE profiles *xtm*9 and *xtm*12 are less common variants of the basic *xtm* 1 pattern in DT 104. These variants occur in <5% of isolates and differ from the predominant pattern by two bands and one band, respectively (A. J. Lawson, unpub. data) (Figure 2).

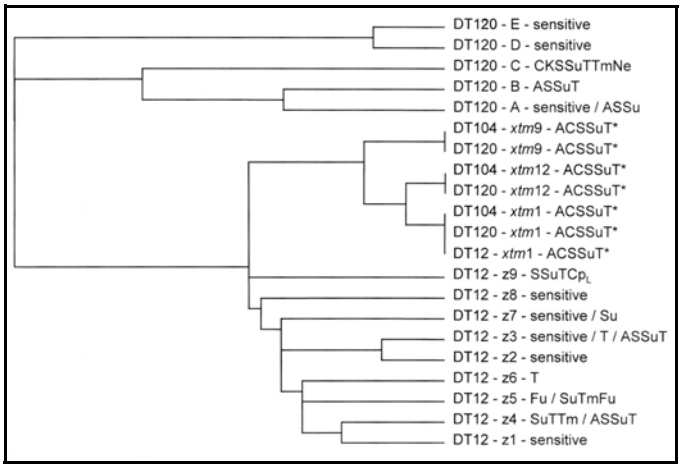

Figure 2. Dendrogram showing the relationships of pulsed-field gel electrophoresis profiles by the nearest neighbor technique.
*Includes resistant (R)-type ACSSuT and ACSSuT plus additional resistances to Tm, Cp_L or both. A = ampicillin, C = chloramphenicol, Fu = furazolidone, K = kanamycin, Ne = neomycin, S = streptomycin, Su = sulfonamides, T = tetracyclines, Tm = trimethoprim, Cp_L = ciprofloxacin.

Strains were analyzed for presence of plasmids by standard methods (13). All DT 104 and DT 12 strains contained a 90-kbp "serotype-specific" plasmid commonly found in *S.* Typhimurium (14). However, in DT 120 only those strains of R-type ACSSuT possessed this plasmid. Purified 90-kbp plasmid extracts from R-type ACSSuT strains of DT12, DT120, and DT104 were negative for integron-associated resistance gene cassettes by PCR (as described above), confirming the chromosomal location of the antibiotic resistance genes.

Results

Strains of *S.* Typhimurium DT 12 and DT 120 of R-type ACSSuT possess antibiotic resistance gene cassettes indistinguishable from those typically present in multiresistant strains of the internationally distributed zoonotic pathogen *S.* Typhimurium DT 104. Strains of MR DT 12 and MR DT 120 of R-type ACSSuT have PFGE profiles distinct from those of other strains of the same phage type. Furthermore, the PFGE profiles of ACSSuT-resistant MR DT 12 and MR DT 120 are identical to those previously reported in MR DT 104. Additionally, ACSSuT-resistant strains of MR DT 120 possess a 90-kbp plasmid absent in DT 120 strains of other R-types, but present in DT 12 and DT 104 strains.

These data suggest that the occurrence of R-type ACSSuT in *S.* Typhimurium DT 12 and DT 120 strains is probably due to changes in phage susceptibility in a small proportion of MR DT 104 strains rather than to horizontal transfer of a resistance gene cassette. The table shows selected phage reactions used in the *S.* Typhimurium phage typing scheme (5) and illustrates the similarity between the phage susceptibility profile of DT 12 and DT 120 and that of DT 104, especially the weaker reacting 104L profile. The causes of changes in phage susceptibility are complex and difficult to trace but are thought to result from the acquisition of new phages or plasmids and to changes in bacterial cell surface phage-receptors.

With regard to the international distribution of these strains, the ACSSuT-resistant DT 12 strains were all PFGE profile *xtm*1, and all originated from patients in England and Wales with no history of foreign travel. In contrast, the ACSSuT-resistant DT 120 strains consisted of a mixture of *xtm*1, *xtm*9, and *xtm*12 PFGE profiles and included cases from South Africa (*xtm*1), Sweden (*xtm*12), Northern Ireland (*xtm*9), and an isolate from a patient with a history of travel to the West Indies (*xtm*1). Continued surveillance coupled with molecular typing should be maintained to follow the spread of these new MR DT 104 variants in animals and humans.

Table. *Salmonella enterica* serotype Typhimurium phage typing scheme, selected phage reactions

Phage type	Selected routine phages																Additional phages				
	8	10	11	12	13	14	15	16	17	18	20	27	28	29	32	35	1	2	3	10	18
104	-/±	-	-	CL	CL	-	-	-	-	CL	+/±	+/±	-/±	-	-/±	+/±	-	-	-	OL	-
104L	-/±	-	-	++	++	-	-	-	-	+/ SCL	-	-	-	-	-	-	-	-	-	OL	-
12	-	-	-	CL	CL	-	-	-	-	-	-	-	-	-	-	-	SCL	SCL	SCL	OL	-
12L	-/±	-	-	CL	CL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	OL	-
120	-	-	-	-	-	-	-	-	-	CL	-	-	-	-	-	-	red	red	red	OL	-

L = light or reduced reaction with typing phages; CL = clear lysis; OL = opaque lysis; SCL = semi-confluent lysis; red = reduced zone; ±, +, ++ = increasing numbers of discrete plaques, - = no reaction.

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Dr. Lawson is a clinical scientist in the Laboratory of Enteric Pathogens at the Central Public Health Laboratory, London. His research interests include the molecular detection and epidemiology of *Salmonella*, *Campylobacter,* and *Helicobacter* species, with particular reference to the emergence of resistance to antimicrobials in these bacteria.

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Address for correspondence: A.J. Lawson, Laboratory of Enteric Pathogens, Central Public Health Laboratory, London, 61 Colindale Avenue, London NW9 5HT, United Kingdom; fax: +44 (0)20 89059929; e-mail: alawson@phls.org.uk

Andes virus and First Case Report of *Bermejo virus* Causing Fatal Pulmonary Syndrome

Paula Padula,* Marcelo González Della Valle,* María Garcia Alai,* Pedro Cortada,† Mario Villagra,‡ and Alberto Gianella§

Two suspected hantavirus pulmonary syndrome (HPS) cases from Bolivia occurred in May and July 2000 and were confirmed by enzyme-linked immunosorbent assay (ELISA)-ANDES using N-Andes recombinant antigen serology. Clot RNAs from the two patients were subjected to reverse transcription–polymerase chain reaction (PCR) amplification and sequencing. We describe two characterized cases of HPS. One was caused by infection with Bermejo virus and the other with Andes Nort viral lineage, both previously obtained from *Oligoryzomys* species. This is the first report of molecular identification of a human hantavirus associated with Bermejo virus.

Hantaviruses cause hemorragic fever with renal syndrome (HFRS) in Eurasia and hantavirus pulmonary syndrome (HPS) in the Americas. Infection occurs primarily by the respiratory route via aerosolized virus in rodent excreta. Field investigations have identified sigmodontine rodents as the host of the respiratory hantaviruses. HPS cases have been reported in several American countries during the last 8 years including Argentina, Bolivia, Brazil, Canada, Chile, Panama, Paraguay, United States, and Uruguay (1-5). *Andes virus* (ANDV) has been responsible for most of the >400 cases recorded in Argentina, Chile, and Uruguay; *Oligoryzomys* is the predominant rodent carrier. A different genus, *Calomys laucha,* was responsible for most cases in Paraguay associated with *Laguna Negra virus* (LNV) (6). Several additional hantaviruses have been detected in rodents in the Americas but have yet to be associated with human disease. Cases of HPS shown to be caused by ANDV (Andes Nort lineage) have been reported in northern Argentina in the Salta and Jujuy Provinces. No cases have been reported previously in Bolivia except in a Chilean HPS patient who was suspected of having acquired the infection during extensive travel in Bolivia prior to onset of illness in 1997. The viral characterization revealed an LNV variant that was 15% at the nucleotide level and identical at the deduced amino acid level relative to LNV (6). Molecular techniques have aided in the testing of Bolivian specimens from a museum collection and allowed the identification of a hantavirus named *Rio Mamore virus* (RIOM) from *Oligoryzomys microtis*. RIOMV has not been associated with human cases yet (7).

In 2000, a total of six male patients in Bolivia (15 to 49 years) were serologically confirmed to have HPS; five died. Five out of the six cases occurred between April and July; the sixth case occurred in November. All the patients lived and worked in rural areas in a 70-km ratio around Bermejo, although it was not clear whether one of them (Patient 2) was infected in Argentina or in Bolivia. In addition, two HPS cases were previously reported in Bolivia, one in June 1998 and the other in October 1999.

Case Reports

Patient 1

On May 18, 2000, a 49-year-old man from Bermejo, Bolivia, who worked as a woodcutter in San Telmo, had a onset of HPS symptoms. Seven days later he was hospitalized at the San Vicente de Paul Hospital and his case was confirmed by enzyme-linked immunosorbent assay (ELISA)-ANDES specific immunoglobulin(Ig) M and immunoglobulin(Ig) G test (8). The patient survived. He had respiratory distress with bilateral interstitial infiltrates. Initial laboratory results were as follows: serum creatinine 1.4 mg/dL, platelets 50,000/µL, hematocrit 46%, arterial O_2 pressure (PaO_2) 50mm Hg, arterial CO_2 pressure ($PaCO_2$) 32mm Hg, leukocytes 6,700 /mm^3, urea 0.36 g/L, bilirubin 0.52. mg/100 mL. Laboratory results 4 days after he was hospitalized were as follows: hematocrit 46%, 46.8%, 48%, 43%; platelets/mm^3 50,000, 32,000, 30,000, and 128,000; and white blood cells/mm^3 6,700, 15,400, 20,700, and 18,300.

Patient 2

In July 2000, a 20-year-old man from Bermejo was admitted to the San Vicente de Paul Hospital 4 days after symptoms began; a preliminary diagnosis of HPS was made. The patient's condition deteriorated rapidly, showing respiratory compromise with bilateral interstitial infiltrates and renal compromise with oliguria. Laboratory results were as follows: serum creatinine 2.3 mg/dL, lactic dehydrogenase (LDH) 572 IU/L, platelets 26,000 /µL, hematocrit 71%, PaO_2 73, $PaCO_2$ 35, and leukocytes 64,300 /µL. The patient died the same day he was hospitalized. HPS was confirmed by ELISA-ANDES-specific IgM and IgG test. Although the patient lived in Bolivia, he worked as a muleteer in Rio Blanco River in Salta Province, Argentina for 2 months and came back to Bolivia 3 weeks before his illness.

The Study

Clot samples from the two patients were used to prepare RNA with an RNA matrix (RNAid kit, BIO101, La Jolla, CA). The RNA was subjected to reverse transcription-polymerase

*Instituto Nacional de Enfermedades Infecciosas, Buenos Aires, Argentina; †Hospital San Vicente de Paul. Orán, Salta, Argentina; ‡Centro Nacional de Enfermedades Tropicales, Santa Cruz, Bolivia; and §Ministerio de Salud, La Paz, Bolivia.

chain reaction (PCR) amplification by nested or heminested reactions. Synthesized DNA products were separated on agarose gels, gel-purified, and directly sequenced with an ABI 377 sequencer. Three cDNA products were produced, allowing generation of nucleotide (nt) sequences of 289 nt in length for an S segment region encoding the most conserved region of the nucleoprotein (N), and 243 nt and 226 nt in length for two M segment regions of G1 and G2, respectively. The nucleotide sequences were determined and compared with known hantaviruses (Table). Sequence identity differences of 3.3% and 1.2% were seen at the nucleotide and amino acid levels respectively, when the G1 fragment sequence of Patient 1 was compared with those of other viral sequences associated with cases in North Argentina. Genetic analysis showed that Patient 1's viral sequence belonged to the Andes Nort lineage characterized previously (2,4). The hantavirus in Patient 2 was clearly identified as Bermejo virus, previously detected in one *O. chacoensis* captured in Orán City, Salta (2). We ruled out laboratory DNA contamination since this was the first available sample of RNA virus in our hands. Viral G1 fragment sequence from this patient showed 100% nucleotide identity compared with the Bermejo virus. Nucleotide and amino acid differences of 13.2% to 24.3% and 3.7% to 8.6%, respectively, were seen when the G1 viral fragment from Patient 2 was related with the more distantly ANDV and closely related lineages including Andes Nort, Andes Sout, and the three different Andes Cent lineages (4). To provide more information on the genetics of Bermejo virus, a highly variable fragment of the M segment (nt 88 to 442) was amplified and sequenced (GenBank accession number AF442564). Nucleotide and amino acid comparison of this fragment between Patient 2 and Andes Nort lineage showed a divergence of 17.2% and 9.3%, respectively. Additionally, a divergence of 6.2% and 0% in the S conserved fragment of Patient 2 and Andes Nort lineage at the nucleotide and amino acid level, respectively, was observed. Unfortunately, homologous fragment sequences from Bermejo virus were not available. Maximum parsimony phylogenetic analysis of the G1 fragment sequences of the two patients and other published hantavirus sequences showed the expected clusters between the viral sequence detected in Patient 1 and Andes Nort lineage virus and the viral sequence in Patient 2 and Bermejo virus (data not shown).

In previous studies, *O. chacoensis* was tentatively implicated as the predominant rodent reservoir for Bermejo virus; however, the sample size was too small since Bermejo virus was identified in only 1 rodent (2). Specific cases of HPS were not linked to the occurrence of the Bermejo virus-infected rodent at the presumed site of infection. Moreover, Andes Nort lineage was previously characterized in one rodent (*O. flavescens)*, two rodents (*O. chacoensis)* (9), and two rodents (*O. longicaudatus)* (2). Whether the viruses responsible for the infection in our report were harbored by any particular *Oligoryzomys* species is now being investigated.

Molecular and epidemiologic data showed the presence of Andes Nort lineage circulating in Bolivia. Considering that the incubation period was estimated to be 19.5 days (4) and since Patient 2 returned from Argentina 21 days before the onset of disease, in which country the infection occurred was unclear. This is the first report of molecular identification of a human hantavirus associated with Bermejo virus.

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Table. Nucleotide and amino acid sequence divergence comparisons between the 2 Bolivian patients and other hantavirus pulmonary syndrome (HPS)-related viruses of 2 fragments of the M segment[a], and 1 fragment of the S segment[b, c]

	Patient 1			Patient 2			ANDV Nort (Argentina)			Bermejo virus (Argentina)			RIOM (Bolivia)			HTN007 (Peru)			LN (Paraguay)		
	N	G1	G2	N	G1	G2	N	G1	G2	N	G1	G2	N	G1	G2	N	G1	G2	N	G1	G2
Patient 1	—	—	—	5,9	19,8	NA[d]	1,4	3,3	3,1	NA	19,8	15,5	12,8	NA	NA	14,2	NA	NA	15,6	24,3	17,7
Patient 2	0	8,6	NA	—	—	—	6,2	21	NA	NA	0	NA	14,2	NA	NA	14,9	NA	NA	17,3	29,2	NA
ANDV Nort (Argentina)	0	1,2	1,3	0	7,4	NA	—	—	—	NA	21	14,2	13,2	NA	NA	14,2	NA	NA	16,6	23,1	17,7
Bermejo virus (Argentina)	NA	8,6	2,7	NA	0	NA	NA	7,4	1,3	—	—		NA	NA	NA	NA	NA	NA	NA	29,2	19
RIOM (Bolivia)	11,5	NA	NA	11,5	NA	NA	11,5	NA	NA	NA	NA	NA	—	—	—	5,5	NA	NA	9	NA	NA
HTN007 (Peru)	11,5	NA	NA	11,5	NA	NA	11,5	NA	NA	NA	NA	NA	2,1	NA	NA	—	—	—	11,1	NA	NA
LN (Paraguay)	12,5	14,8	8	12,5	17,3	NA	12,5	13,6	6,7	NA	17,3	5,3	2,1	NA	NA	4,2	NA	NA	—	—	—

[a]GenBank accession numbers for the nucleotide sequences are: Patient 1 N:AF442561 G1:AF442559 G2:AF442560; Patient 2 N:AF442563 G1:AF442562; values above dashes are nucleotide sequence divergence and those below dashes are amino acid sequence divergence. The comparison analysis was performed using NALIGN and PALIGN programs from PCGENE 6.8 software from Intelligenetics Inc. (Mountain View, CA).
[b]M segment G1 (nt 1735 to 1977); M segment G2 (nt 2718 to 2943).
[c]S segment N (nt 48 to 336).
[d]NA= not available.

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Address for correspondence: Paula Julieta Padula, Felipe Vallese 3714, (1407) Buenos Aires, Argentina; telephone: (54 11) 4301-3146; e-mail: ppadula@cvtci.com.ar

Salmonella enterica Serotype Enteritidis Phage Type 4b Outbreak Associated with Bean Sprouts

Yvonne T.H.P. van Duynhoven,* Marc-Alain Widdowson,* Carolien M. de Jager,* Teresa Fernandes,* Sabine Neppelenbroek,* Winette van den Brandhof,* Wim J.B. Wannet,* Jan A. van Kooij,† Henk J.M. Rietveld,‡ and Wilfrid van Pelt*

In November 2000 in the Netherlands, an outbreak of *Salmonella enterica* serotype Enteritidis phage type 4b was investigated. Eating bean sprouts was the only exposure associated with *S.* Enteritidis pt 4b infection (matched odds ratio 13.0, 95% confidence interval 2.0-552.5). Contaminated seeds were the most likely cause of contamination of the sprouts. The sprout grower applied a concentration of hypochlorite solution that was too low for seed disinfection.

Salmonella is one of the most common causes of bacterial gastroenteritis in the Netherlands and is often implicated in foodborne outbreaks (1,2). Raw or undercooked meat, eggs, raw milk, and especially poultry are well-known vehicles for transmission of *Salmonella* spp. However, fresh produce, such as lettuce and unpasteurized apple or orange juice, has also caused outbreaks (3-5). In the last decade, multiple outbreaks of *Salmonella* spp. (and Shiga-toxin-producing *Escherichia coli* O157:H7) linked to seed sprouts have occurred throughout the world (6-8). In the Netherlands, a recent study of sprouted seed products showed that 0.9% of 666 samples of bean sprouts contained *Salmonella* spp. (9). We report the first confirmed outbreak of *Salmonella enterica* serotype Enteritidis phage type (pt) 4b associated with bean sprouts in our country.

The Study

On November 27, 2000, an outbreak detection algorithm identified a cluster of 12 cases with *S.* Enteritidis pt 4b in data from the National Reference Center for Salmonella at the National Institute of Public Health and the Environment (RIVM). This reference system covers 64% of all laboratory-confirmed salmonellosis in the country. Since the implementation in 1997 of the Colindale phage-typing system, no cases of this phage type had been reported. In the week before these cases were identified, the same phage type was found by the reference laboratory for an isolate detected during a quality control inspection of one batch of bean sprouts harvested on October 26, 2000 (typed as 4b on November 23).

In the first week of the outbreak, eight of the initial patients were interviewed by using a hypothesis-generating questionnaire adapted from the "*Salmonella* Trawl" (an existing questionnaire of the Communicable Disease Surveillance Centre in England). Three foods—chicken (reported by all), eggs (seven reports), and bean sprouts (six reports)—emerged as possible risk factors for infection. A case-control study was started on November 30.

We defined a case as diarrhea (≥3 loose stools in a 24-hour period) after November 1, 2000, with a stool specimen positive for *S.* Enteritidis pt 4b. Secondary cases were included to measure the magnitude of the outbreak but excluded from the case-control study. For each case, one control was found by searching for persons by street name in a web-based phone book, matching for neighborhood, age group (≤17, 18-40, >40 years), and sex.

Patients and controls were interviewed by telephone. The questionnaire addressed clinical manifestations (patients only), contacts with other symptomatic persons, and food consumption. Exposure factors were collected for the 7-day period before onset of illness for both patients and controls. A matched univariate analysis in Epi-Info 6.04 (Centers for Disease Control and Prevention, Atlanta, GA) was performed with maximum likelihood techniques to estimate odds ratios and exact confidence intervals (CI). To control for confounding, conditional logistic regression (exact method) was done in LogExact (Cytel Corp, USA.).

The sprout producer contacted the Inspectorate for Health Protection and Veterinary Public Health (hereafter referred to as Food Inspection Service) on November 30. The same day the company was inspected. Operational hygiene and cultivation procedures were reviewed. Three 25-g samples were taken from bean sprouts harvested that day; environmental samples were also obtained in case of air contamination during growth. All samples were tested for *Salmonella.*

Once it was determined by the epidemiologic study that bean sprouts were the suspected food source, patient data about the sales outlets for the sprouts were supplied to the Food Inspection Service. That agency then checked invoices from these businesses to identify the producer. No traceback was performed to the seed distributors of the implicated sprout producer.

The Netherlands participates in Enter-net, an international surveillance network for *Salmonella* and Verocytotoxin-producing *Escherichia coli* O157 infections, funded by the European Commission (10). All participants were informed about the cases and requested to forward information on recent cases of *S.* Enteritidis pt 4b or foodstuffs contaminated with this type. By this cross-the-border case-finding, international

*National Institute of Public Health and the Environment, Bilthoven, the Netherlands; † Inspectorate for Health Protection and Veterinary Public Health, the Hague, the Netherlands; and ‡Inspectorate for Health Protection and Veterinary Public Health, Region East, Zutphen, the Netherlands

problems could be identified. In addition, the international database was reviewed for this specific phage type for the period 1998-2000.

A total of 27 cases were identified in the Netherlands. In addition, a second *Salmonella* isolate from bean sprouts was received from the same sprout producer. It had been harvested on November 15 and typed as 4b on November 30. Patients were found in 7 of the 12 provinces. The 26 primary cases had dates of onset from November 3 through November 24 (Figure). The last patient's case was classified as secondary.

Of the patients, 67% were females. Ages ranged from 1 to 74 years, with 12 (44%) children <10 years of age. The median duration of illness was 8 days (range 5->25). The most frequently reported symptoms were diarrhea (100%; 56% bloody), abdominal cramps (92%), fever (76%), vomiting (40%), and headache (40%). Four female patients (16%) were admitted to the hospital for 3 to 7 days.

Data were collected for 24 matched pairs. The median age difference was 4 years in a pair (control older than the patient). In the univariate analysis, bean sprouts, chicken, tomatoes, celery, peppers, and onions were associated with illness at the 0.2 significance level (Table). Bean sprouts were the only variable associated with illness that was significant at the 0.05 level (matched odds ratio =13.0; CI 95% 2.0-552.5).Fifty-eight percent of patients recalled eating bean sprouts (Table). For seven patients who recalled the exact dates they ate the sprouts, the incubation period was 1 to 6 days (median 2 days). Of controls, 8% reported eating bean sprouts.

On the day the cluster was detected, the company that submitted the contaminated sprouts to the central laboratory was contacted and voluntarily took several measures 2 days later, including intensified testing and removal of the raw materials from which the contaminated sprouts were grown. In addition, as a routine practice, the room from which the contaminated sprouts were grown (separate rooms were used for each batch) was emptied, cleaned with a high-pressure equipment**,** and disinfected. No new cases of *S.* Enteritidis pt 4b have been reported since. The producer distributed sprouts almost nationwide and probably also exported to Germany. The Food Inspection Service's inspection the next day, November 30, demonstrated that Hazard Analysis and Critical Control Points systems were used for both disinfection of the facility and cultivation of the sprouts. For irrigation, the producer had a permit for a private water system, which was checked daily for microbiologic quality. The Food Inspection Service found no irregularities in the recorded microbiologic data in the days before harvest of the contaminated sprouts. None of the employees in the production facility had been ill before or at the time the contaminated sprouts were harvested. All product and environmental samples taken by the Food Inspection Service were negative for *Salmonella*. At the implicated production facility, every new batch of seeds and samples from each batch of sprouts harvested each day was routinely tested for *Salmonella* spp. Test results are available after 5 days (the end of the shelf life for sprouts). So far, no seeds have been positive for *Salmonella*; however, of the annually tested batches of sprouts, 0.5% were positive. The producer applied seed disinfection with approximately 5 ppm hypochlorite solution during presoaking.

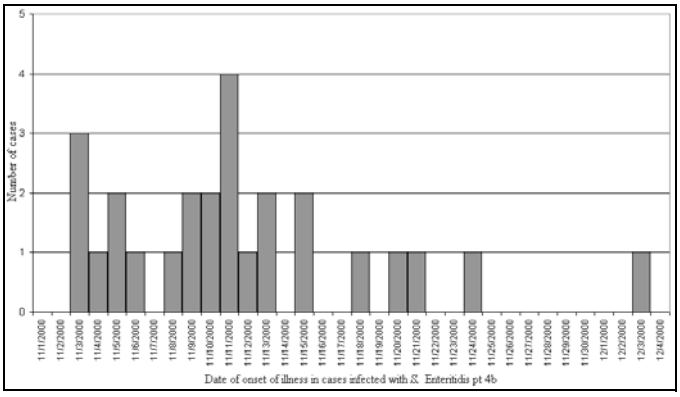

Figure. Epidemic curve of 27 identified cases (including 1 confirmed secondary case) in outbreak of *Salmonella enterica* serogroup Enteritidis phage type 4b, the Netherlands, November–December 2000

Table. Matched univariate analysis (24 pairs) of the association between *Salmonella enterica* serotype Enteritidis phage type 4b infection and various food items, the Netherlands, November–December, 2000

Food item	Exposed case, unexposed control No. of pairs	Unexposed case, exposed control No. of pairs	Matched OR[a] (CI)[b]	% Cases exposed
Chicken	8	2	3.0 (0.8-38.7)	71
Beef	2	5	0.4 (0.04-2.4)	71
Fish	6	3	2.0 (0.4-12.4)	54
Eggs	4	2	2.0 (0.3-22.1)	79
Tomato	9	3	3.0 (0.8-17.2)	71
Lettuce	5	8	0.6 (0.2-2.2)	50
Alfalfa sprouts	1	0	Undefined	4
Cabbage	5	5	1.0 (0.2-4.4)	38
Bean sprouts	13	1	13.0 (2.0-552.5)	58
Cucumber	6	4	1.5 (0.4-7.2)	63
Paprika	6	4	1.5 (0.4-7.2)	54
Peppers	4	0	Undefined	17
Onion	9	3	3.0 (0.8-17.2)	67
Carrots	6	8	0.8 (0.2-2.5)	46
Parsley	6	2	3.0 (0.5-30.4)	38
Celery	7	2	3.5 (0.7-34.5)	29

[a]Maximum Likelihood Estimate of odds ratio (OR).
[b]Exact 95% confident limits for Maximum Likelihood Estimate of OR.

Fourteen patients reported the places where they bought the bean sprouts. For seven, the invoices could be checked (starting December 12) and were traced back to the same sprout producer that was inspected.

Information from Enter-net participants arrived within a few days after the cluster was detected. No country reported a

recent increase in this phage type. In the Enter-net database, 15 (0.04%) of 33,773 and 24 (0.09%) of 26,336 *S.* Enteritidis isolates from 10 countries that were phage typed according to the Colindale system were type 4b in 1998 and 1999, respectively. In 2000, this was 139 (0.56%) of 24,961, mainly from Germany (57 since August), from our Dutch outbreak (27 isolates) and to a lesser extent from England and Wales (20 since June).

Conclusions

This outbreak of *S.* Enteritidis pt 4b was identified following routine surveillance and prompt investigation. This relatively small outbreak was noticed because of the unusual phage type 4b; outbreaks caused by more common types would likely be easily missed. Since only a minority of patients with gastroenteritis are known to seek medical care and fewer still have their cases laboratory confirmed, the real number of affected persons in this outbreak is likely to be several hundred.

Both the detection of the same, rare type of *Salmonella* during routine control procedures for the product and the epidemiologic study incriminated bean sprouts as the cause of the outbreak. The fact that not all patients reported eating bean sprouts may be due to recall bias, since interviews took place 16 to 32 days after the onset of illness and sprouts are often served inconspicuously in salads, sandwiches, and other meals (11,12). Other explanations might be secondary transmission and cross-contamination of other food items.

The predominance of women, the broad age category, and the wide geographic distribution correspond with previous sprout-associated outbreaks (7,8). In >60% of households, the confirmed case-patient was the only one who had symptoms, even though some entire families ate the sprouts. The low attack rate in these households might reflect a low-level contamination of the sprouts or, more likely, a high-level, but heterogeneous contamination, i.e., high numbers of *Salmonella* in sprouts grown from a contaminated seed and little or no *Salmonella* in the remainder.

Based on the 20-day interval between the *Salmonella*-positive harvests of bean sprouts and lack of evidence for other sources of contamination, seeds were the most likely cause of contamination, as in most sprout-associated outbreaks (9,13). Although, the seeds tested negative for *Salmonella* in the routine control operations of the producer, testing is probably ineffective, as contamination may be intermittent and low level (7,11).

To date, no single treatment has been demonstrated to completely eliminate pathogens without affecting germination of the sprouts (7). Therefore, research on methods to reduce or eliminate pathogens is still ongoing (7,13). The sprout grower implicated in our outbreak applied seed disinfection with 5 ppm hypochlorite solution; recommended concentrations range from 2,000 to 20,000 ppm (7,13).

To monitor trends and detect producers with above-average contamination rates, incorporating the investigation of sprouted seed products in the routine program of food law enforcement in the Netherlands might be considered. As a result of the outbreak, all sprout-producing companies are being investigated by the Food Inspection Services; sprouts are tested for *Salmonella* spp*, Listeria monocytogens,* and *Bacillus cereus;* and hygiene procedures and disinfection treatment practices are reviewed.

This outbreak also alerted the Netherlands to the fact that the country has no legal basis to stop distribution of known *Salmonella*-contaminated sprouts. Although the law states that food products should not contain pathogenic microorganisms in 25-g samples of the product, an explicit exception is made for raw, unprocessed foodstuffs. This exception is made because it is assumed that raw foodstuffs will be processed before consumption, eliminating microorganisms. However, sprouting products are generally eaten raw or undergo only a mild or rapid heat treatment, such as stir-fry. This concept was brought to the attention of the Minister of Health.

During the outbreak investigation, Enter-net proved a rapid network for European communication on the occurrence of the specific phage type. Additionally, this database demonstrated an increase in the outbreak phage type in at least one other country in 2000, Germany; this increase is being investigated.

Seed sprouting products entering the retail market still might contain pathogenic microorganisms. Thus, persons who are at increased risk for complications, such as young children, the elderly, the immunocompromised, and the chronically ill, should avoid eating these products. Many Dutch consumers may be unaware that sprouts, usually considered a healthy food, can cause foodborne illness. To make them aware, publicity efforts are needed.

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Dr. van Duynhoven is a senior epidemiologist at the National Institute for Public Health and the Environment in the Netherlands. Her work focuses on epidemiologic research and disease-specific surveillance of infectious gastroenteritis and foodborne infections.

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Address for correspondence: Yvonne THP van Duynhoven, National Institute of Public Health and the Environment, Department for Infectious Diseases Epidemiology, P.O. Box 1, 3720 BA Bilthoven, the Netherlands; fax: 31 30 2744409; e-mail: y.van.duynhoven@rivm.nl

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms
- Known infections spreading to new geographic areas or populations
- Previously unrecognized infections appearing in areas undergoing ecologic transformation
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and *West Nile virus* infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Candida dubliniensis Infection, Singapore

To the Editor: We read with interest the letter by Marriott et al. (1) describing the first case of *Candida dubliniensis* fungemia in Australia.

We report the first two cases of *C. dubliniensis* infection in Singapore. We have been using the API 20C AUX on all yeast isolates from blood, sterile fluids, and tissue to screen for *C. dubliniensis* since May 2000. To our knowledge, this infection has not been previously reported from a Southeast Asian country.

The first patient was a 49-year-old woman with adult polycystic kidney and liver disease; she had mild chronic renal failure and a past history of nephrotic syndrome. She was admitted to the renal unit on May 9, 2001, for management of ascites. A septic workup showed leukocytosis (34.67 x 10^9/L) with predominance of neutrophils (95%). Blood culture received on May 31 grew yeast with two slightly different forms, one large and one small. The isolates, investigated separately, were germ-tube positive. The API 20C AUX profile for the larger isolate was 6072114 at 48 hours' incubation (96.2% certainty for *C. dubliniensis*) and 6072134 for the smaller isolate (99.8% certainty for *C. dubliniensis*). The yeasts grew well on potato dextrose agar at 35°C but poorly at 42°C and 45°C. In addition, electrophoretic karyotyping with pulsed-field gel electrophoresis showed that both isolates had identical patterns, with eight chromosome fragments, one of which was <1 megabase (Mb), indicating that the two morphologically different strains were the same karyotypically. The pattern obtained was in keeping with results obtained by Jabra-Rizk et al. (2), with *C. dubliniensis* showing a chromosome-sized band of <1 Mb. For *C. albicans*, by contrast, all bands were >1 Mb. Five control strains—*C. dubliniensis* (RCPA Microbiology QAP item 2001:2:7A), *C. albicans* ATCC 90028, and three clinical strains of *C. albicans*—were also run together with the two strains; the results obtained were consistent with those of Jabra-Rizk et al. (2). The MIC of the isolates to fluconazole by E-test was 0.75 μg/mL, indicating susceptibility. Disseminated intravascular coagulopathy due to sepsis from a possible ruptured liver cyst developed in the patient. Despite broad-spectrum antibiotics and amphotericin B, hemodialysis, and intensive-care support, she died 5 weeks after admission. Except for *C. dubliniensis* candidemia, *Candida* species isolated from the urine and endotracheal secretions (speciation not done), and *Acinetobacter baumannii* (cultured from the endotracheal secretions and femoral catheter tip), no other important microorganisms were isolated. Peritoneal fluid cultures did not yield any microorganisms.

Soon after the first case, *C. dubliniensis* was isolated from a sputum culture and bronchial alveolar lavage

cultures from a 50-year-old Chinese woman, who had myelodysplastic syndrome and was hospitalized for pneumonia. She had been previously treated with chemotherapy and had multiple admissions for infection. The same phenotypic and karyotypic methods as the first patient were used to identify the isolate. The API 20C AUX profile was 2152134 at 48 hours (98.5% certainty for *C. dubliniensis*). The MIC of fluconazole by E-test was also 0.75 µg/mL. Pancytopenia developed, and the patient's condition deteriorated. She died of pneumonia despite transfusions and treatment with broad-spectrum antibiotics and amphotericin B. Microbiologic investigations for bacteria, tuberculosis, pneumocystis, *Legionella,* and viruses did not yield positive results except for *Corynebacterium* species in the bronchial alveolar lavage fluid.

Although *C. dubliniensis* was first associated with oral candidiasis in HIV-infected persons (3), several reports now link the organism to non-HIV patients who were immunosuppressed due to chemotherapy, hematologic malignancy (4), and end-stage liver disease (1). Our two patients were not HIV positive but were immunosuppressed. In vitro susceptibility results showed that our patients should have responded to the usual antifungal treatment. However, they died despite appropriate therapy.

Acknowledgment

We acknowledge David Ellis for help in confirming the isolates.

Ai Ling Tan, Grace CY Wang, and Yoon Wan Chiu

Singapore General Hospital, Singapore

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O157:H7 Shiga Toxin-Producing *Escherichia coli* Strains Associated with Sporadic Cases of Diarrhea in São Paulo, Brazil

To the Editor: Shiga toxin-producing *Escherichia coli* (STEC) strains are associated with a spectrum of diseases ranging from mild to severe bloody diarrhea and complications such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (1). Since STEC was linked with hemorrhagic colitis in 1982 (2), strains—particularly serotype O157:H7—have been associated with numerous outbreaks and many sporadic cases of infections worldwide. STEC is now a major cause of foodborne disease, primarily in the United States, Canada, Japan, and Europe (1,3). Although most sporadic cases and outbreaks have been reported from developed countries, human infections associated with STEC strains have also been described in Latin American countries, including Argentina and Chile (3). In Brazil, STEC infections have been related to sporadic cases of nonbloody diarrhea caused by non-O157 strains (4,5); serotype O157:H7 has not been previously isolated from human infections in our country.

We report the characterization of three O157:H7 strains isolated in São Paulo State, Brazil. The first strain was identified among a laboratory collection of 2,573 *E. coli* strains that were retrospectively analyzed and isolated from patients with diarrhea in São Paulo State, from 1976 through 1999, at the Central Laboratory of Instituto Adolfo Lutz (IAL). This strain was isolated in 1990 from an 18-year-old patient with diarrheal disease who had AIDS. The two other O157 strains were recently isolated from a 4-year-old girl with bloody diarrhea and from an adult with severe diarrhea. Both patients were admitted to the same hospital at Campinas, São Paulo State, in June and July 2001, respectively. The strains, isolated by routine diagnostic procedures on MacConkey agar plates, were presumptively identified as *E. coli* O157 by standard methods with specific O157 antiserum. These last two strains were confirmed as sorbitol-negative *E. coli* O157 at the IAL Regional Laboratory at Campinas and were sent to the IAL Central Laboratory for further characterization.

Guidelines for Letters. Letters discussing a recent Emerging Infectious Diseases article (400-500 words, 5-10 references) should be received within 4 weeks of the article's publication. Letters reporting preliminary data (500-1,000 words, 10 references) should not duplicate other material published or submitted for publication, should not be divided into sections, and should avoid figures or tables. All letters have the same authorship, financial disclosure, and acknowledgment requirements as full articles and should include a word count. For more guidance on manuscript preparation, see Emerging Infectious Diseases Instructions to Authors. Send letters to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D 61, Atlanta, GA 30333, USA, or e-mail: eideditor@cdc.gov .

Letters Editor: Patricia M. Quinlisk, Des Moines, Iowa, USA

The three O157 *E. coli* strains underwent biochemical identification and serotyping by standard methods. Enterohemolysin production was determined according to Beutin et al. (5). Polymerase chain reaction assays were used to detect the *stx1* and *stx2* genes (6), and colony hybridization assays with specific DNA probes for *stx1* , *stx2* and *eae* genes were performed as described (7). Cytotoxicity to Vero cells was assayed as described (4). The strains were characterized as sorbitol-negative O157:H7, containing the *stx2* and *eae* sequences. The enterohemolytic phenotype and production of Stx were also observed in all isolates.

To our knowledge, these O157:H7 STEC strains are the first to be associated with human diseases in Brazil. Cultivation of stool specimens in sorbitol MacConkey agar is strongly recommended for screening O157 strains, and, indeed, all three strains were isolated from MacConkey agar plates. Laboratories should attempt to examine stool specimens from all patients (children and adults) with HUS, severe diarrhea (nonbloody and bloody stools) requiring hospitalization, or both, as well as from patients reporting a history of bloody diarrhea.

Despite the importance of O157:H7 serotype in causing life-threatening complications such as HUS and the isolation of this serotype from clinical specimens in São Paulo State, the relatively low prevalence of this serotype in healthy dairy and beef cattle in Brazil (8), as well as the occurrence of other non-O157 STEC strains associated with human infections (4,5,9), suggest that *E. coli* O157:H7 may be not as frequent as non-O157 STEC strains in our country.

Kinue Irino,* Tânia M. Ibelli Vaz,* Maria A.M.F. Kato,* Zita V.F.Naves,† Raquel Russo Lara,† Maria Elza Carvalho Marco,† Marilu M.M. Rocha,‡ Tânia P. Moreira,‡ Tânia A. T. Gomes,§ and Beatriz E.C. Guth§

*Instituto Adolfo Lutz, São Paulo, Brazil; †Laboratório de Análises Clínicas Dr João Antônio Vozza, Campinas, São Paulo, Brazil; ‡Instituto Adolfo Lutz, Campinas, São Paulo, Brazil; and §Universidade Federal de São Paulo, Escola Paulista de Medicina, São Paulo, Brazil

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Infection Highlights 2000-01

Mark H. Wilcox, Editor

Health Press Limited, Oxford, UK, 2001 (98 pages)

This 98-page paperback in the Fast Facts series contains concise updates on a diverse group of topics in infectious diseases. Chapters cover newly recognized and emerging infectious diseases problems including *Escherichia coli* O157:H7, *Helicobacter pylori*, and *Acinetobacter*. Other chapters provide therapeutic updates of a range of problems including exacerbations of chronic obstructive pulmonary disease, HIV infection, and onychomycosis. Most reviews are relevant to the clinician with the exception of discussions on alternative treatments for methicillin-resistant *Staphylococcus aureus* (MRSA) and antibiotic-resistance genes in plants. There are 12 chapters, and most of the authors are recognized authorities in their areas.

Page borders are color-coded by chapter with matching color schemes for tables. Each chapter contains a table of highlights with headings of "What's in," "What's out," and frequently "What's controversial," or "What's needed." This approach works with variable success. It does give a reader whose thumbing through the book a quick look at the major issues. But I can imagine authors struggling with what to include in this format leading to unhelpful entries such as "Over-prescribing of conventional antibiotics" under "What's out" in a chapter on alternative treatments for MRSA. In rapidly changing areas including HIV therapeutics, what was "in" at the time of writing is already "out" or "controversial" by the time of this review in the fall of 2001.

The chapters are, for the most part, well written and factual. The reviews on *Clostridium difficile* diarrhea (authored by the editor) and *E. coli* O157:H7 are especially well done. Unfortunately, the chapter on HIV chemotherapy contains a few inexplicable errors. Lopinavir, a protease inhibitor, is listed as a nucleoside reverse transcriptase inhibitor in both a table and the text. The authors also states that the nucleotide analogs, such as tenofovir are active in their native form. In fact, they are prodrugs that require phosphorylation by cellular enzymes. The HIV chapter is also the most dated, though I cannot fault the authors for this, given the dynamic nature of the field.

The editor does not tell us the intended audience for the book, but it appears to be geared for the infectious disease specialist rather than the generalist. The reviews average about six small pages of text, and the discussions are not sufficiently complete to serve as a background source for the uninitiated. The editor writes that review articles are often unwieldly or out-of-date at the time of publication. This text aims to summarize new information concisely. Concise it is – but perhaps too much so, as I came away from reading many of the reviews longing for more depth. Nonetheless, this volume generally succeeds with providing "fast facts" in a well-written and easy to read format.

James P. Steinberg

Emory University School of Medicine, Atlanta, Georgia, USA

Remote Sensing and Geographic Information Systems in Epidemiology

Simon I. Hay, Sarah E. Randolph, and David J. Rogers, Editors

Academic Press, London, 2000 (357 pages)

The applications of remote sensing and geographic information systems (GIS) to vector-borne and helminthic diseases have progressed far beyond the pretty pictures which dominated their early use. As Wood et al. indicate in the last chapter, the number of papers in the area has increased drastically over the last decade, in number and sophistication. The editors of this special volume of Advances in Parasitology have been in the forefront of applying statistical and biological

approaches to the mapping of vector-borne diseases and have brought together experts to review existing knowledge, identify gaps in understanding, and describe future applications of these powerful approaches.

This book is a timely overview of satellite imagery, GIS, and spatial statistics. The emphasis is on vector-borne diseases, with one chapter devoted to helminthic diseases. With the exception of the chapter on spatial statistic and GIS, there is little mention of other epidemiologic applications (e.g., GIS and cancer, AIDS, and environmental health). The book is divided into three parts: three introductory chapters describing the methodology; four chapters which review the applications and provide examples from the authors' experiences in studying African trypanosomiases, malaria, tick-borne diseases, and human helminthic diseases; and three concluding chapters which describe environmental variables, disease risk forecasting, and the education about and future of remote sensing in human health.

Although remote sensing, GIS, and spatial statistics have been reviewed separately elsewhere, the encompassing review, the inclusion of lists of URLs, and the extensive references make the introductory chapters timely and instructive for new users. The audiences that will benefit most from the book include researchers and public health administrators who want to integrate these tools into research, surveillance, and control efforts. This audience, as well as more experienced users, can gain much from the chapters that provide examples of specific applications derived from deep understanding of the biology of disease. The chapters by Rogers, Randolph, and Brooker and Michael, in particular, are based their own research and expertise in trypanosomiasis, tick-borne diseases, and helminthic diseases, respectively.

Remote sensing and GIS are particularly relevant to emerging infectious diseases. The chapter entitled Forecasting Disease Risk for Epidemic Preparedness provides a road map for developing early warning systems. While this chapter, like the rest of the book, is clearly written by advocates of the applications of remote sensing and GIS, the authors remain aware of critical issues, such as the distinction between statistical and biological models and the notion that insights gained by false negatives and positives predicted by models are as important as successful predictions. Other issues that have hampered more extensive applications of remote sensing and GIS to vector-borne diseases include lack of training, gaps in data (quality and quantity, particularly of epidemiologic and parasitologic data), inadequate tools for data gathering, and limits on management and understanding. This book goes a long way to address these issues and is likely to lead to more and improved applications of remote sensing and GIS.

Uriel Kitron

University of Illinois,
Urbana, Illinois, USA

About the Cover

México prehispánico, El antiguo mundo indígena (Indigenous World) (1929)

Diego Rivera
Fresco, Mexico City, National Palace, North Wall

Printed with permission from Banco de México Diego Rivera and Frida Kahlo Museums Trust; Avenue Cinco de Mayo No. 2, Col. Centro; Del Cuauhtémoc, 06059, México.

Along with colonial conquest, infectious diseases forever changed the Aztec world in the 16th century. These diseases included smallpox and an indigenous highly lethal hemorrhagic fever known as "cocoliztli." Tree-ring data suggest that the 16th-century cocoliztli epidemics occurred during extreme drought conditions, which may have contributed to the staggering demographic collapse of early colonial Mexico.

Until the colonial uprising of 1910, the indigenous people of Mexico had been oppressed, their individualism and Aztec origins discouraged. Diego Rivera's mural at the National Palace in Mexico City celebrates Aztec origins, promotes a Mexican identity, and promotes the reforms of the 1910 uprising.

The mural depicts Mexico's mythical and precolonial past. The bright orange sun against the blue sky and the erupting volcano are life symbols of Mexican ancestors. Quetzalcoatl, mythical creator of the world, appears in three forms: star, god, and human. At first, Quetzalcoatl, who was created by serpents, sails through space as a star that accompanies the sun at night. Then, he assumes human form and comes to earth to teach the Aztec people as their king and patriarch; he sheds his blood to give them life. Finally, having completed his earthly mission, he returns to the sky. Upon leaving earth, Quetzalcoatl assumes the form of the morning star Tlahuizcalpantecuhtli, which appears near the sun at sunrise. Quetzalcoatl's journey signifies the continuous cycle of life.

Abstracted from http://www.mexconnect.com/mex_/travel/jcummings/diegomural3.html .

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy Reviews, and Dispatches. 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 homepage at www.cdc.gov/eid. Articles by authors from non-English-speaking countries can be made simultaneously available in English and in the author's native language (electronic version of the journal only).

Instructions to Authors

Manuscript Preparation

Follow "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (Ann Intern Med 1997:126[1]36-47) (http://www.acponline.org/journals/annals/01jan97/unifreqr.htm).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

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). Also provide address for correspondence (include fax number and e-mail address).

Abstract and key words. Avoid citing references in the abstract. Include up to 10 key words; use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/mesh/meshhome.html).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Type only on one side of the paper and number all pages, beginning with the title page. Indent paragraphs 5 spaces; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use Courier font size 10 and ragged right margins. Italicize (rather than underline) scientific names when needed.

Electronic formats. For word processing, use MS Word. Send graphics in native format or convert to .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) formats. The preferred font for graphics files is Arial. Mac files should be sent in MS Word or RTF formats.

References. Follow the Uniform Requirements style. Place reference numbers in parentheses, not in 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 in full. List the first six authors followed by "et al."

Tables and figures. Create tables within MS Word's table tool. Do not format as columns or tabs; do not submit tables in Excel. For figures, use color as needed. Send digital files (see above) or camera-ready slides, photographs, or laser prints. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced. Place figure keys within the figure.

Access the journal's style guide at http://www.cdc.gov/ncidod/EID/style_guide.htm

Manuscript Submission

Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D61, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov

Types of Articles

Perspectives. Articles should be approximately 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.

Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be approximately 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 2,000 to 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.

These articles 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 (e.g., "Here is what we found, and here is what the findings mean").

Dispatches. Articles should be 1,000 to 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 10), 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.

Policy Reviews: Articles in this section report public health policies that are based on research and analysis of emerging disease issues.

Another Dimension: Thoughtful essays on philosophical issues related to science and human health.

Book Reviews: Short reviews (250 to 500 words) of recently published books on emerging disease issues are welcome.

Letters: This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

News and Notes: We welcome brief announcements (50 to 150 words) of timely events of interest to our readers. (Announcements can be posted on the journal web page only, depending on the event date.) In this section, we also include summaries (500 to 1,500 words) of conferences focusing on emerging infectious diseases. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.