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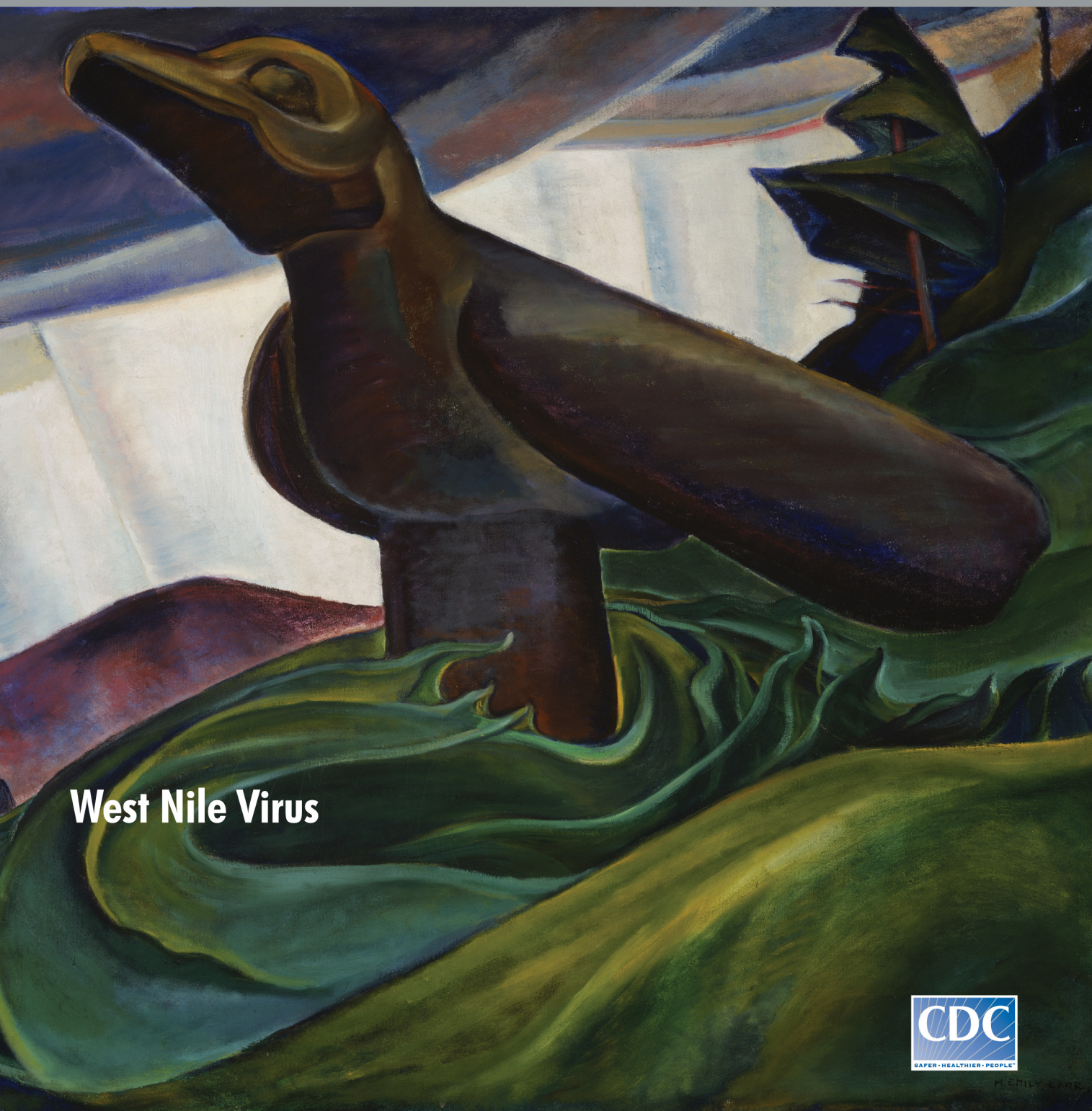

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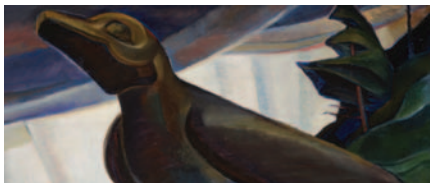

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Emily Carr (1871–1945). Big Raven (1931)
Oil on canvas, 87.3 cm x 114.4 cm, Vancouver Art Gallery, Emily Carr Trust

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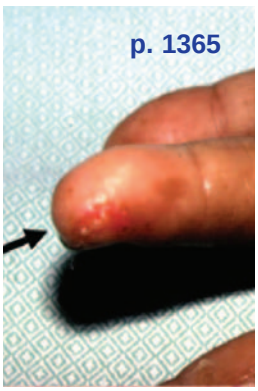

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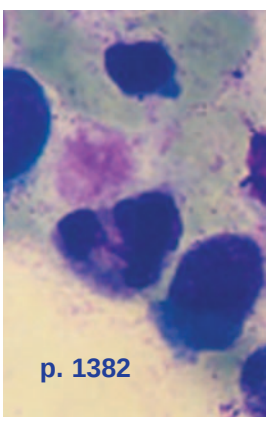

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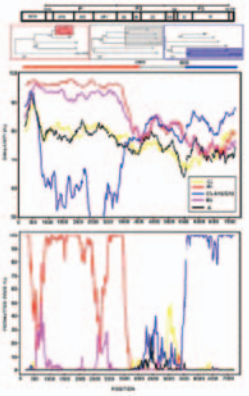

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Genomic-scale Analysis of Bacterial Gene and Protein Expression in the Host

John D. Boyce,* Paul A. Cullen,* and Ben Adler*

The developing complementary technologies of DNA microarrays and proteomics are allowing the response of bacterial pathogens to different environments to be probed at the whole genome level. Although using these technologies to analyze pathogens within a host is still in its infancy, initial studies indicate that these technologies will be valuable tools for understanding how the pathogen reacts to the in vivo microenvironment. Some bacterial pathogens have been shown to substantially modify their surface components in response to the host immune system and modify their energy metabolism and transport pathways to allow efficient growth within the host. Further detailed analyses of these responses will increase understanding of the molecular mechanisms of pathogenesis, identify new bacterial virulence factors, and aid in the design of new vaccines.

How do bacteria respond to the host environment during an infection? Bacterial pathogens must be able to gain access to, persist in, and replicate in normally privileged sites within a host. Moreover, they must produce certain factors that result in a level of host damage that perturbs homeostasis. Thus, pathogens must have specific mechanisms for mediating colonization, avoiding the host's immune system, and acquiring necessary nutrients. They must also produce factors that result (directly or indirectly) in host damage. Because the environment encountered within a living host will be quite different from the external environment, pathogens must be able to regulate the necessary genes in coordination as they move from the environment to the host and from one host niche to another.

The primary aim of investigating bacterial pathogenesis is to understand the way that pathogens interact with the host to cause disease. Central to this investigation is an understanding of what gene products are required and expressed during a natural infection and how this expression changes over time (from initial colonization to causation of disease and spread of the pathogen to new hosts) and space (in different cells or tissues within the host). We thus endeavor to understand how the pathogen adapts to the host microenvironment, what selective pressures are acting on the pathogen in each microenvironment, what bacterial factors are responsible for the host damage, and how the immune system is evaded. Although analyses that give information on the expression of a few genes provide insight and have been responsible for a large proportion of the bacterial pathogenesis literature currently available, our ultimate goal is to understand expression changes across the whole genome. The additional information generated by whole genome studies goes far beyond that derived by characterizing in isolation more genes and gene products, because analysis of the whole genome allows complete regulatory networks to be identified and characterized. These results cannot be achieved with a "one-gene-at-a-time" approach. Whole genome studies could be considered as an exponential and synergistic advance rather than a linear progression.

The host-pathogen interactions that define a disease are clearly complex, and, in many cases, the study of these interactions is limited by the lack of a suitable animal model. However, we now have a number of methods that allow identification of genes critical for survival in a host as well as methods that allow direct measurement of gene expression during interaction with a host. Two of these methods, signature-tagged mutagenesis and in vivo expression technology, do not directly measure gene expression and do not allow true genomic-scale analysis, but they have been devised to identify genes necessary for pathogens during real infections. Excellent reviews on these techniques are available (1,2), and they will not be discussed in this review. A second group of methods, which includes DNA microarrays and proteomics, have advantages that overcome the limitations implicit in signature-tagged mutagenesis and in vivo expression technology, namely, the ability to directly measure expression (gene or protein) levels on a true genome-wide scale, but

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their application to analysis of bacterial pathogens during real infections is still in its infancy. Another method, real-time reverse transcriptase polymerase chain reaction (RT-PCR) has qualities that bridge those of the other methods, allowing accurate gene expression measurements but on a subgenomic scale; thus, we will not discuss it in this review. However, real time RT-PCR is useful for coping with the low numbers of microorganisms that are often available during infections, and high-throughput whole-genomic scale real-time RT-PCR may become available in the near future. We summarize the current application of DNA microarray and proteomics techniques to the understanding of how bacteria modify their expression profiles within an infected host.

DNA Microarray Studies

DNA microarrays offer the promise of accurate gene expression measurements for every gene in a genome and allow this expression to be analyzed in response to any environmental variable. However, this huge potential for the understanding of bacterial pathogenesis has not yet been completely realized because of the substantial technical problems associated with accurately measuring bacterial gene expression during real infections. The main problems associated with their use in such situations include the following: the low numbers of bacteria in living tissues during infection, difficulty in purifying the bacteria (and therefore bacterial RNA) from the eukaryotic tissue, potential mRNA instability and possible differential degradation during purification, and the difficulty in finding an appropriate animal model for many diseases. The instability of bacterial mRNA can in part be overcome by using commercially available RNA stabilization reagents such as RNAlater (Qiagen, Hilden, Germany). Additionally, because of the specificity of DNA hybridizations, small amounts of co-purified eukaryotic RNA are unlikely to adversely affect the microarray results. However, this type of transcriptional analysis can currently only be applied to those infections that lead to high titers of infecting organisms in host tissues, since the experiments require at least microgram quantities of RNA. Thus far, no one has accurately measured gene expression throughout an infection, from the initial stage of invasion/colonization through multiplication and tissue spread to the final stages of disease with notable host damage. Such a complete analysis of gene expression remains the "holy grail" of gene expression measurements with regard to bacterial pathogenesis. However, we are beginning to see a number of experiments that provide insights into the way bacteria regulate gene expression at different phases of infection.

The first wave of DNA microarray experiments of relevance to bacterial pathogenesis focused on analyzing bacterial gene expression during growth in vitro under conditions chosen to mimic some aspect of infection. In many cases, the relationship to a specific condition that the bacteria will face during growth in the host is clear, and as the conditions are manipulated in vitro, the test conditions can be tightly controlled. Thus, these studies have allowed a detailed description of bacterial pathogen response to iron limitation (3,4), nutrient limitation (5,6), acidic environments (7,8), low oxygen (9,10), bacterial density (11,12), and biofilm formation (11,12). A number of studies have also analyzed the global effects of transcriptional regulators with the aim of defining complete regulatory networks (13).

Although the in vitro experiments have added substantially to our understanding of gene expression in bacterial pathogens, they can never completely model the conditions that a pathogen encounters in a host during infection. Thus, the second wave of microarray experiments has focused on directly measuring bacterial gene expression during interaction with eukaryotic cells or during growth within the host (Table). Three studies have directly analyzed whole-genome bacterial gene expression during growth in the tissues of a living eukaryotic host (18–20), while a fourth analyzed gene expression in bacteria recently exited from host tissue, the lumen of the bowel (21). All four experiments compared growth in vivo with growth in in vitro laboratory medium. Three of the studies carried out competitive hybridization of in vivo and in vitro RNA to directly compare gene expression in the two sites, whereas the third study compared gene expression levels of both in vivo and in vitro samples to a common reference sample (genomic DNA). Despite the analyses being carried out on different bacterial species (*Vibrio cholerae* and *Pasteurella multocida*), striking similarities between the gene expression changes were seen in all four experiments.

All experiments showed a substantial up-regulation of genes involved in amino acid metabolism, purine biosynthesis, and iron transport and metabolism. Genes in the *ilv* and *pur* operons were consistently up-regulated. Many of the changes also involved up-regulation of genes involved in transport of amino acids and carbohydrates. Indeed, a large number of ABC transport systems were measured as up-regulated. Therefore, in the in vivo environment, whether in rabbit ileal loops, blood, liver, or rice water stools, available nutrients are markedly reduced compared with those in the in vitro medium. Although the current studies have compared gene expression with growth in rich in vitro medium, the major reason for this approach has been the desire to identify potential virulence genes rather that those up-regulated in vivo simply in response to the in vivo nutritional environment. However, many of these genes have been identified by signature-tagged mutagenesis studies as necessary for in vivo survival (25–27).

Table. Large-scale measurement of bacterial gene or protein expression during real infections or interactions with host cells

Species	Experiment type	Description	Reference
Growth in the host			
Borrelia burgdorferi	DNA microarray	Growth in dialysis membranes implanted in rats	14
	DNA microarray	Adaptation to growth in dialysis membranes in rats	15
	DNA microarray	Alteration of lipoprotein expression during host adaptation in mice	16
	Antigenic profiling	Alteration of antigenic profile during host adaptation in mice	17
Pasteurella multocida	DNA microarray	Gene expression during growth in blood of infected chickens	18
	DNA microarray	Gene expression during growth in livers of infected chickens	19
Vibrio cholerae	DNA microarray	Gene expression during growth in rabbit ileal loops	20
	DNA microarray	Gene expression in rice water stools	21
Interaction with tissue culture cells			
Chlamydia pneumoniae	2-dimensional gel electrophoresis	Protein expression during growth in HEp-2 cells in response to interferon-γ	22
Neisseria meningitidis	DNA microarray	Interaction with epithelial and endothelial cells	23
	DNA microarray	Interaction with epithelial cells	24

These studies also found that a number of genes involved in energy metabolism were up-regulated during growth in vivo. Specifically, in each experiment some of the highest up-regulated genes included those encoding particular alternative electron acceptor complexes. In both *V. cholerae*, purified from rice water stools, and *P. multocida*, purified from the blood of chickens, the *nap* (periplasmic nitrate reductase) operon was highly up-regulated. In *V. cholerae*, grown in rabbit ileal loops, the *frd* (fumarate reductase) operon was up-regulated, and in *P. multocida*, purified from the livers of chickens, the *dms* (dimethyl sulfoxide reductase) operon was up-regulated. The appropriate terminal electron acceptor complex is likely determined by the pervading oxygen tension, and oxygen tension differs between different tissues in vivo. Indeed, the growth of *V. cholerae* in rabbit ileal loops and of *P. multocida* in liver indicated up-regulation of a number of genes expected to be regulated by anaerobiosis. Again, these measurements have been compared with growth in vitro in laboratory media so that anaerobiosis is only defined by comparison with the (likely) highly aerobic in vitro environment.

These in vivo experiments have so far shown variable expression of known virulence factors. In *V. cholerae*, in which virulence factors are fairly well defined, a small number of virulence factors were expressed in organisms purified from rice water stools (21), including genes involved in amino acid metabolism, purine metabolism, and the acid tolerance response. None of the genes in the ToxR/TcpP/ToxT virulence gene regulon was identified as differentially expressed in this host niche, which indicates that these genes are transiently expressed and are not necessary as the bacteria are exiting the host. However, a number of virulence genes were expressed in bacteria grown in rabbit ileal loops. Twelve of the top 300 expressed genes in vivo were part of the pathogenesis functional group and included the virulence regulators *tcpP*, *tcpH* and *toxR*, the hemolysin and hemolysin transporter genes *hlyA* and *hlyB*, and the hemagglutinin protease gene *hapR*. For *P. multocida*, one third of the genes identified as virulence genes by signature-tagged mutagenesis (26) were also identified as differentially regulated during growth in the blood of chickens.

Three studies of *Borrelia burgdorferi* have analyzed gene expression during infection (Table). Two of these analyzed whole-genome expression changes during growth in dialysis membranes implanted in rat peritoneal cavities (14,15), and one focused specifically on expression of lipoproteins during growth in mice (16). The gene expression profiles observed differed substantially from those observed for *P. multocida* and *V. cholerae* growing in tissue. Few changes were observed in genes involved in energy metabolism or in amino acid, carbohydrate, and iron transport and metabolism. This finding may be a result of the slow rate of *B. burgdorferi* growth in the mammalian environment. The most notable changes observed in *B. burgdorferi* gene expression involved expression of outer membrane components, particularly lipoproteins (although one study analyzed only lipoprotein genes). Thus, *B. burgdorferi* appears to respond primarily to the host innate or adaptive immune system, or both, resulting in the down-regulation of a large number of surface components, including about 100 lipoproteins.

For many human-specific pathogens, no well-defined animal model exists; conducting gene expression studies during real infections is thus very difficult or impossible. One experimental method that has been used to overcome this problem is analysis of gene expression in response to interaction with host cells. Two studies have analyzed the global transcriptional response of *Neisseria* species to interaction with eukaryotic cells (Table). These studies of *Neisseria meningitidis* and *N. lactamica* compared

expression profiles of bacteria in cell culture medium with bacteria in contact with epithelial or endothelial cells (23,24). The gene expression profiles observed in the two studies showed substantial similarity. Similar to the findings from the in vivo studies of *V. cholerae* and *P. multocida* (18–20), many up-regulated genes were identified that were involved in transport and energy metabolism. A range of transporters were up-regulated, especially those involved in amino acid and sulfate transport. Indeed, the sulfate transport system, which is strictly linked to sulfur-containing amino acid metabolism, was up-regulated in the pathogen (*N. meningitidis*) but not in the commensal species (*N. lactamica*), which indicates that this factor may play a role in virulence (24). The other major group of genes that were up-regulated in the bacteria in contact with host cells were those involved in adhesion. Many of these have been previously characterized as virulence genes.

Comparing the in vivo studies with defined in vitro studies may allow deconstruction of the stimuli acting in the in vivo microenvironment. This possibility is a promising aspect of gene expression studies that has not yet been fully explored. For example, in *P. multocida* grown in chickens, the gene expression profile of bacteria within two of three animals was similar to the genes observed to be up-regulated under in vitro iron starvation. Such comparative analyses can expand our understanding of the selective pressures acting on the pathogen during infection. In fact, this first analysis of *P. multocida* indicated that in at least one of the infections, the bacterial gene expression profile differed from that observed under iron-limiting conditions, which suggests that a bacterial response to low iron may occur only in some hosts or at certain stages of infection.

Proteomic Studies

Proteomics refers to any global analysis of proteins. Proteomics has the potential to show posttranslational modifications, translational regulation, the products of alternative splicing of mRNAs, and selective degradation of proteins, all of which cannot be accounted for when directly measuring mRNA transcript levels.

Although proteomic strategies abound, two main approaches exist for analyzing complex protein mixtures, each of which is quite distinct and possesses subtle advantages and disadvantages. One method relies on the separation of whole proteins by two-dimensional gel electrophoresis (2-DGE) and the subsequent identification of individual proteins through mass spectrometry. The other method, often referred to as multidimensional protein identification technology (MUDPIT), relies on the separation of proteolytic peptides by liquid chromatography and their identification by directly coupled electrospray ionization-tandem mass spectrometry.

A number of technical problems limit the coverage of proteomic analyses. Proteome analysis with 2-DGE often excludes proteins that are large, hydrophobic, or have extremely alkaline isoelectric points. Hydrophobic proteins are often missed because of their insolubility during isoelectric focusing, the problems in extracting hydrophobic peptides from gel matrices, and the difficulty in ionizing hydrophobic peptides for analysis by mass spectrometry (28). Large or basic proteins are often not resolved because they do not enter the isoelectric focusing gradient or do not remain soluble during focusing. The MUDPIT approach overcomes many of the limitations imposed by the solubility difficulties encountered during the isoelectric focusing step of 2-DGE. However, 2-D gels provide a visual reference of protein expression for comparison, while also permitting the experimenter to observe posttranslational modifications and protein cleavage events, which would not be evident by using MUDPIT. In addition, MUDPIT per se does not yield quantitative information (29). 2-DGE and MUDPIT are complementary technologies, and to achieve optimal coverage, both systems should be used (30). However, in contrast to mRNA-based approaches, the current technologies are not capable of elucidating the entire proteome.

Do data obtained by using microarray experiments correlate with proteomic data collected from the same biological system and should we expect them to? In a study performed on yeast in which protein expression with mRNA levels were compared with those obtained by using the serial analysis of gene expression technique, a correlation coefficient of approximately 0.4 was obtained, which indicated that protein expression levels correlated poorly with quantitative mRNA data (31). A more global study, in which mRNA levels obtained by microarray analysis were compared with those obtained with protein expression levels assessed by using a MUDPIT/isotope-coded affinity-tag approach, found that the expression of mRNA and protein sets involved in some biologic pathways were highly correlated while others were not. This finding suggests that posttranscriptional regulation mechanisms were operating in those instances when protein expression levels correlated poorly with quantitative mRNA data (32). Thus, the lack of correlation reported by many researchers (33–36) may result from technical hurdles associated with accurately measuring either mRNA or protein expression levels on a global scale. Nonetheless, if the tools used to measure gene and protein expression are accurate, the expression data should correlate for transcripts and proteins that comprise biologic pathways not subject to post-transcriptional or posttranslational regulation.

Using these proteomic methods to analyze bacterial pathogens has substantial promise, but no example yet exists of global protein expression analysis of a bacterial

pathogen growing inside its natural host or in an animal model. This situation has likely occurred because of the technical hurdles associated with separating bacteria from the host tissues and obtaining sufficient material to perform serial analyses required for statistical significance. The problem of contaminating host tissue has probably been overemphasized: small amounts of contaminating host proteins should not compromise the separation and can subsequently be withdrawn from the dataset when in silico searches show a match with the host organism. A far greater problem is obtaining sufficient sample because no techniques are available for signal amplification (as is the case for mRNA expression analysis). Even when enough material can be obtained, this will almost certainly be during the end stages of infection, when the bacterial expression may not differ markedly in quantity from that observed during growth in culture. To analyze global protein expression during the early stages of infection when virulence factors are likely to be expressed, we must await improvements in the technologies involved in separating bacteria from the host and protein expression analysis systems that have improved sensitivity for use with small amounts of sample.

Although no studies of bacterial protein expression inside the host have been published, several investigators have analyzed bacterial protein expression during growth in vitro under conditions that mimic some aspect of infection. These studies have included the response to temperature change, iron-limitation, and the presence of serum proteins (37), nutrient starvation (6), pH stress (38,39), magnesium limitation (40), and biofilm formation (41,42). Other studies have used cell culture systems to more closely mimic the host environment. An analysis of whole cell protein expression of *Chlamydia pneumoniae* (Table) during growth in HEp-2 cells and in response to treatment with interferon-γ was possible after radioactive labeling of the bacteria (22). This analysis indicated up-regulation of a small number of proteins involved in replication, energy metabolism, and peptidoglycan synthesis. An antigen profile analysis of *B. burgdorferi* allowed changes in the antigenic proteins expressed during growth in mice to be compared with changes during growth in in vitro laboratory medium (Table). This analysis allowed for the semiquantitative measurement of *B. burgdorferi* antigen expression in different mouse tissues and showed the differential expression of some known surface proteins (17) However, this analysis falls short of a whole-genome study because it can only measure antigenic proteins soluble in Triton X-114. As technical hurdles are overcome, whole-cell protein expression analysis of bacterial pathogens growing inside the host is poised to provide substantial insight into the mechanisms of bacterial pathogenesis.

Conclusion

Techniques are now available to begin to meaningfully analyze bacterial expression during growth within eukaryotic hosts, and such studies will transform our understanding of the molecular mechanisms of pathogenesis. Although technical problems remain (such as how to cope with the limited amount of material present during infection and how to purify the pathogens from the eukaryotic host), methods are rapidly being developed to overcome or circumvent these problems. In fact, the necessary further advances will likely be gradual improvements in current technologies rather than new technologies. However, the new challenge may well become the analysis of the large datasets that are generated and the seamless integration with other genomic, proteomic, metabolic pathway, and phenotype data. Integrating these data types will delineate the pathogen's response to the host and help clarify the intricate cross-talk from host to pathogen and the environmental cues and regulatory networks that lead to the expression of bacterial virulence factors. Such a detailed understanding of bacterial pathogens will likely ultimately be available, and this knowledge will facilitate the design of improved vaccines and the rational design of antimicrobial compounds.

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Vibrio vulnificus in Taiwan

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Residents in Taiwan are often exposed to marine microorganisms through seafood and occupational exposure. The number of reported cases of infection attributable to this organism has increased since the first case was reported in 1985. The increasing number of cases may be caused by greater disease activity or improved recognition by clinicians or laboratory workers. We analyze a clinical-case series of 84 patients with *Vibrio vulnificus* infection from 1995 to 2000 and describe the molecular epidemiologic features of pathogens isolated from these patients. The spectrum of clinical manifestations and outcomes, options of antimicrobial therapy, and virulence mechanisms were investigated. Results of molecular typing of isolates from humans and marine environment in this country had a high genetic divergence among these isolates. Education and measures are needed to prevent this emerging disease.

Awareness of *Vibrio vulnificus* as a threat to human health has evolved during the past 30 years (1). In Taiwan, Yuan et al. first reported *V. vulnificus* infection in a patient with septicemia and leg gangrene in Kaohsiung County in 1985 (2). Chuang et al. described an additional 27 cases during a 5-year period from May 1985 to July 1990 and demonstrated three major discernible syndromes: primary septicemia, wound infection, and gastrointestinal diseases. The disease had a high mortality rate (41%) (3). Chuang et al.'s report was also the first to demonstrate the recurrent nature of this disease. Since then, many clinicians and researchers from Taiwan have reported risk factors and the clinical spectrum of this disease on the basis of an increasing number of reported cases (4–12). Many factors have been associated with increased vulnerability of Taiwanese people to *V. vulnificus* infection. These include the high prevalence of hepatitis B or C virus infection-related hepatic diseases (liver cirrhosis and hepatoma), the environment, and the popularity of preparing and eating raw or undercooked seafood (3,13). These factors have drawn considerable interest to finding optimal therapeutic regimens for this infection, as well as to identifying the pathogenesis, ecology, and the reservoirs of this microorganism.

We describe the clinical features of 84 recently identified patients with *V. vulnificus* infection treated from 1995 to 2000 in Taiwan and report the results of molecular typing of 50 isolates of *V. vulnificus* from these patients. We also summarize the recent advances in understanding this newly recognized disease from the Taiwan perspective.

Disease Prevalence

Taiwan is a small island situated off the southeast coast of the Asian continent with a population of >22 million people. Figure 1 shows the annual number of reported cases and the estimated prevalence of *V. vulnificus* infection (per 10^6 persons) from 1985 to 2000 in Taiwan (2–12). Two peaks occurred: one in 1988 to 1990 (0.354–0.450/10^6 persons) and the other in 1996 to 2000 (0.606–1.237/10^6 persons). Most reported cases (>90%) occurred in residents of southern Taiwan. In Taiwan, the temperature of surface seawater is usually >18°C, except for February, when it is 17°C–23°C (6). Nearly all cases

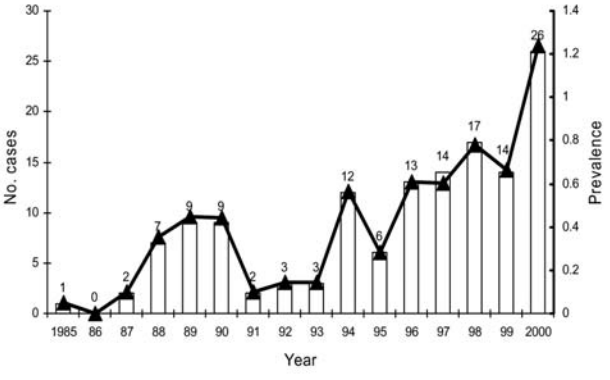

Figure 1. Estimated prevalence (per 10^6 population) and annual number of cases of *Vibrio vulnificus* infection reported from 1985 to 2000 in Taiwan. The line and triangles represent the prevalence and the bars the number of cases.

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occurred in the late spring to early fall (April–October), when the seawater temperature is 20°C–29°C. The peak months for infections were June–August (summer season) when the temperature of surface seawater in Taiwan was approximately 26°C–29°C (6).

The reasons for the increased rate of *V. vulnificus* during the past 2 decades are not fully understood. The extent to which the increasing number of cases may be caused by increasing disease activity or improved recognition by clinicians or laboratory workers is also unclear. Since the first report of *V. vulnificus* infection in 1987 and subsequent reports in both humans and environmental studies, clinicians in Taiwan have become increasingly aware of the clinical features of this disease, and laboratory workers more likely to understand how to isolate and identify this pathogen accurately.

Environmental Habitants and Reservoirs

The occurrence of *V. vulnificus* infections in cultured shrimp and eels has been reported in Taiwan (14). A monthly survey on the distribution of *Vibrionaceae* in seawater from five major harbors in Taiwan was conducted from July 1991 to February 1994 (15). Among the 1,167 *Vibrionaceae* isolates, *V. vulnificus* accounted for 67 (5.7%) (15). This finding indicates that the organism exists autochthonously around the coastal waters or aquatic habitats in Taiwan. Most isolates (91%) from marine water and oysters were indole-negative (biotype I) but some belonged to biotype II (ornithine decarboxylase- and mannitol-positive) (16). Strains of *V. vulnificus* serovar E (also belonging to biotype II) avirulent for eels, which were recovered from water and oysters, were reported (17). Ribotyping analysis of the environmental isolates indicated a great genetic divergence among these isolates (18). More than half of the environmental isolates exhibited virulence in mice, indicating that these isolates might be pathogenic to humans (16). In addition, saline and aqueous ethanol extract (lectins) from some marine algae collected from the northeastern coast of Taiwan had marked antibacterial activity against *V. vulnificus* isolates recovered from the northeastern coast of Taiwan (19). Further study is needed to explore the symbiosis between marine algae and their associated marine vibrios.

Clinical Features and Outcomes

Clinical information from 84 patients *V. vulnificus* infection treated from 1995 to 2000 was obtained from medical records from five hospitals in Taiwan (Table). These hospitals, with a capacities of 1,500 to 2,000 beds, included National Taiwan University Hospital, Taipei; Chi-Mei Medical Center and National Cheng-Kung University Hospital, Tainan; Chang Gung Memorial Hospital-Kaohsiung, Kaohsiung; and Kaohsiung Veterans General Hospital, Kaohsiung. Most of the patients (73%) were male. More than 80% of these patients had various underlying medical conditions with liver disease (particularly hepatitis B or C virus infection-related diseases), which accounted for more than half of the patients,

Table. Clinical characteristics of 84 patients with *Vibrio vulnificus* infections who were treated at five major hospitals, Taiwan, 1995–2000

Characteristic (no. of patients for whom information was available)	No. of patients (%)
Sex (n = 84)	
Male/female	61 (72.6)/23 (27.4)
Age, mean/range (yr)	60/9-87
Underlying disease (n = 84) [a]	
Chronic hepatitis B or C virus infection	10 (11.9)
Liver cirrhosis	35 (41.7)
Hepatitis B or C virus infection-related	21
Alcoholic	7
Hepatoma	7
Diabetes mellitus	13 (15.5)
Steroid use	10 (11.9)
Alcoholism	8 (9.5)
Renal insufficiency	6 (7.1)
Other malignancies	3 (3.6)
None	12 (14.3)
Type of infection (n = 84)	
Cutaneous infection	57 (67.9)
Cellulitis	15 (17.9)
With bacteremia	5
With septic shock	6
Necrotizing fasciitis	42 (50.0)
With bacteremia	2
With septic shock	32
Primary septicemia	20 (23.8)
With septic shock	3
Spontaneous bacterial peritonitis	6 (7.1)
Meningitis	1 (1.2)
Exposure history (n = 55)	
Injury from handling marine animals (fish, crab)	7 (12.7)
Preexisting skin wounds	11 (20.0)
Ingestion of raw seafood	2 (3.6)
None	35 (63.6)
Initial antibiotic treatment (n = 82)	
A third-generation cephalosporin[b] plus minocycline	38 (46.3)
A first-generation cephalosporin plus an aminoglycoside	15 (18.3)
Other combinations [c]	29 (35.4)
Surgical treatment (cutaneous lesions, n = 57)	
Incision and drainage, débridement and/or fasciotomy	43 (75.4)
Amputation	6 (10.5)
Outcome	
Survived	57 (67.9)
Died	25 (29.8)
Unknown	2 (2.4)

[a]Patients might have more than two underlying diseases.
[b]Include ceftazidime, cefotaxime, ceftriaxone, and moxalactam.
[c]Include a penicillin or a first -generation cephalosporin plus an aminoglycoside or minocycline.

followed by diabetes mellitus and steroid use. Nine patients (16.3%) had exposure to marine injuries (caused by fish or crab bones or eating raw fish) or marine environments (swimming in coastal seawater or raising fish). Although 11 (20%) patients had preexisting skin wounds, exposure of the skin wounds to salt water was not known. More than 60% of these patients had a cutaneous infection, and 50% had necrotizing fasciitis. Approximately three fourths of the patients with necrotizing fasciitis had septic shock. Characteristic cutaneous lesions in patients with necrotizing fasciitis and wounds associated with bacteremia attributable to *V. vulnificus* are shown in Figure 2. Twenty patients (23.8%) had primary septicemia, and 3 were complicated with septic shock.

Similar to the previous findings, we found no patients with gastroenteritis caused by to *V. vulnificus* (3). Most patients with gastroenteritis or diarrheal illness in Taiwan do not seek care at the large teaching hospitals; they also do not usually have a stool culture, which might explain the lack of patients with gastrointestinal illness attributable to *V. vulnificus*.

A third-generation cephalosporin plus minocycline was used as the definite treatment regimen in 46% of patients. Among 57 patients with cutaneous lesions, 49 (86.0%) had some form of surgical treatment (incision and drainage, débridement, fasciotomy, and amputation). The overall case-fatality rate was approximately 30% (Figure 3), which was similar to that reported previously among patients seen from 1995 to 1990 (3). Patients with spontaneous bacterial peritonitis had the highest case-fatality rate (50%), followed by necrotizing fasciitis (40.5%). Patients with cellulitis had the lowest case-fatality rate (6.7%).

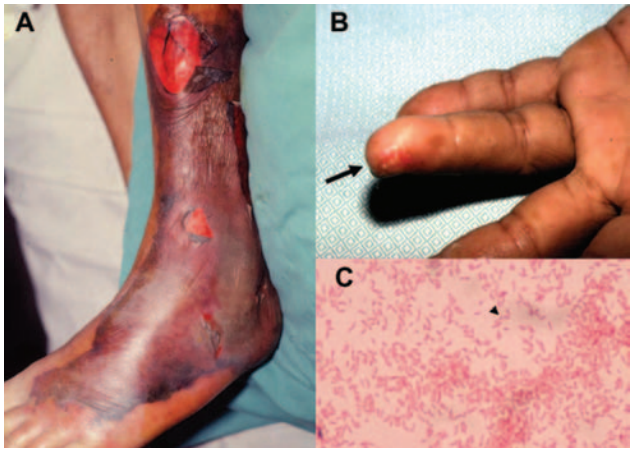

Figure 2. Characteristic skin lesions of *Vibrio vulnificus* infection and morphotype of the microorganism. A) Gangrenous change with hemorrhagic bullae over the leg in a 75-year-old patient with liver cirrhosis in whom septic shock and *V. vulnificus* bacteremia developed. B) *V. vulnificus* bacteremia developed 1 day after a fish bone injury on the fourth finger of the left hand (arrow) in a 45-year-old patient with uremia. C) Gram-negative curved bacilli (arrowhead) isolated from a blood sample of the 45-year-old patient with uremia.

Antimicrobial Drug Resistance and Treatment Options

MICs were determined and interpreted by using the MIC interpretive criteria for *Enterobacteriaceae* recommended by the National Committee for Clinical Laboratory Standards (20–23). All isolates of *V. vulnificus* in Taiwan, which were collected from the previous studies, were susceptible to the following agents (MIC_{90}): ampicillin (1 μg/mL), carbenicillin (4 μg/mL), cephalothin (4 μg/mL), cefamandole (2 μg/mL), cefotaxime (≤0.03–0.06 μg/mL), ceftriaxone (≤0.03 μg/mL), cefoperazone (0.12 μg/mL), aztreonam (8 μg/mL), imipenem (≤0.03–0.12 μg/mL), gentamicin (4 μg/mL), amikacin (8 μg/mL), tetracycline (0.25 μg/mL), minocycline (0.06–0.25 μg/mL), chloramphenicol (0.5 μg/mL), and fluoroquinolones: ofloxacin (≤0.03 μg/mL), lomefloxacin (0.12 μg/mL), ciprofloxacin (≤0.03–0.03 μg/mL), levofloxacin (0.03 μg/mL), moxifloxacin (0.06 μg/mL), gatifloxacin (0.06 μg/mL), and sparfloxacin (0.06 μg/mL) (20–23). Few isolates were not susceptible to ceftazidime (MIC 32 μg/mL) and moxalactam (MIC 32 μg/mL) (21). All isolates were resistant to clindamycin (MICs ≥256 μg/mL) (20). In vitro synergism between cefotaxime and minocycline against *V. vulnificus* isolates was documented by time-kill study (21). Time-kill study also demonstrated that fluoroquinolones at concentrations of two times the MIC had a persistent inhibitory effect on *V. vulnificus* for >48 hours (23).

In vivo study using a mouse model of *V. vulnificus* infection clearly indicated that combination therapy with cefotaxime and minocycline is distinctly superior to therapy with cefotaxime or minocycline alone (22). A similar effect of newer fluoroquinolones as single agents compared with the cefotaxime-minocycline combination was also demonstrated in the treatment of severe experimental *V. vulnificus* infection (23).

On the basis of the in vitro and in vivo animal studies, along with clinical outcome analysis, combination therapy with cefotaxime (2 g every 6 h intravenously) and minocycline (100 mg every 12 h intravenously) was recommended for treating adult patients with bacteremia and severe soft-tissue infection caused by *V. vulnificus* (21,22). For severe soft-tissue infection (necrotizing fasciitis, tissue necrosis with gangrene change, and myositis), early and aggressive surgical interventions (incision and drainage, débridement, fasciotomy, and amputation) are important in saving the life of the patient.

Pathogenesis

More than 90% of *V. vulnificus* isolates whose biotypes were determined belonged to biotype I, which is well

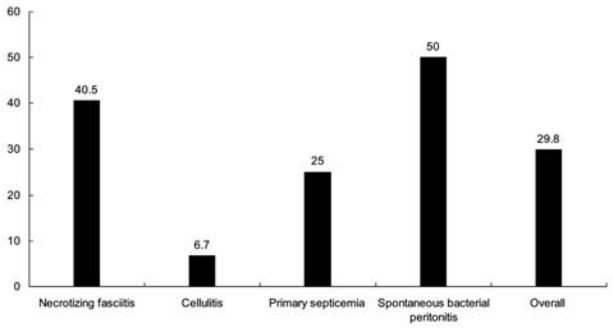

Figure 3. Rates of deaths according to different types of infection of 84 patients with *Vibrio vulnificus* infection.

known to be pathogenic for humans (15,16). In 1997, Chuang et al. first demonstrated that severe damage of the connective tissue of a mouse by *V. vulnificus* wound infection could be mediated by a recombinant extracellular metalloprotease (able to digest collagen and elastin) (24). Lee et al. also illustrated that extracellular products of *V. vulnificus* were lethal to fish (moribund black porgy, *Acanthopagrus schlegeli*) (25). Genes (*vvp* and *empV*) encoding the metalloprotease and gene (*vllY*) encoding a novel hemolysin of *V. vulnificus* were subsequently cloned and characterized (26–28).

Hor et al. showed that isogenic protease-deficient (PD) mutant of *V. vulnificus* was as virulent as its parent strains in mice infected intraperitoneally and was 10-fold more virulent in mice infected through the oral route (29). A metalloprotease- and cytolysin-deficient mutant of *V. vulnificus* also had similar virulence in mice, and its cytotoxicity for HEP-2 cells (cytotoxin) compared with those of the wild-type strains (30). These findings suggest that neither metalloprotease nor cytolysin is essential for the virulence or invasiveness of *V. vulnificus* in mice. A possible multifactor interaction in bacterial virulence might be present but to an extent that is not yet clear. However, two genes, *vvn* (encoding a periplasmic nuclease, *Vvn*) and *smcR* (encoding *SmcR*, which regulate metalloprotease gene expression), were not required for *V. vulnificus* virulence in mice (31,32).

Animal studies clearly demonstrated that iron could increase the growth rate of *V. vulnificus*, which quickly reached a lethal concentration with enhanced cytotoxicity in the iron-overloaded mice (33). A study of the survival of *V. vulnificus* in whole blood from patients with different degrees of liver disease showed that high serum ferritin levels and low phagocytosis activity of neutrophils were independent and important predictors of survival of the organism in blood (34). These findings indicated that patients with chronic hepatitis, liver cirrhosis, and hepatoma (high serum ferritin levels and lower phagocytosis) were at high risk for *V. vulnificus* infection (34). Although many putative virulence factors have been studied for this exceptionally virulent human pathogen in Taiwan, how these factors and other veiled factors (such as capsular polysaccharide and lipopolysaccharide) interact to produce dramatic infections and what host aspects (such as overproduction of proinflammatory cytokines) are essential to infection are yet to be elucidated (3).

Molecular Epidemiologic Features

Results of molecular typing by using restriction fragment length polymorphism analysis of rRNA (ribotyping) among 13 clinical and environmental (from seawater and eels in southern Taiwan) isolates of *V. vulnificus* and arbitrarily primed polymerase chain reaction analysis of 37 isolates (24 clinical isolates and 13 from seawater from coast areas around Taiwan) were previously reported (18,35). Both showed high genetic divergence among clinical and environmental isolates.

The concentration of *V. vulnificus* in recent clinical and environmental isolates in southern Taiwan indicates the possibility of clonal spread in this area. In this study, 50 isolates of *V. vulnificus* collected from 1995 to 2000 from southern (46 isolates) and northern (4 isolates) Taiwan were analyzed. These isolates included those from various clinical specimens (blood and wound pus) of 50 patients with *V. vulnificus* infection. All isolates of *V. vulnificus* were identified by using conventional methods and the O/129 susceptibility tests. Identification of the isolates was further confirmed by the API 32 GN system (bioMérieux Vitek, Inc., Hazelwood, MO). Pulsed-field gel electrophoresis (PFGE) analysis was performed by a method described previously by Tenover et al. (36,37). DNA was digested by the restriction enzymes *Sfi*I and *Not*I (Promega, Madison, WI). All isolates were not identical in PFGE profiles (50 pulsotypes were found), and only two isolates from southern Taiwan were closely related (within three bands of difference). These findings support the high degree of heterogeneity among isolates of *V. vulnificus* that cause human infections in Taiwan.

Preventive Measures

Residents of Taiwan, particularly those with preexisting liver and other chronic, underlying medical conditions (renal disease, diabetes mellitus, chronic steroid use), should be educated in measures to prevent acquiring *V. vulnificus* infections. This bacterium is present in warm coastal waters around Taiwan during the summer months, particularly in the southern region. Exposing open wounds or broken skin to warm salt or brackish water or to raw marine animals harvested from such waters should be avoided. Patients at high risk should wear protective clothing (e.g., gloves) when handling seafood (fish, oysters, clams, shrimp, eels, and other shellfish) and not eat raw or

improperly cooked seafood. Because this disease is rapidly progressive and deadly if not recognized promptly and treated aggressively, any illness (such as fever or skin lesions), which develops in patients at risk after contact with marine animals or waters or ingestion of seafood requires immediate medical care.

The government in Taiwan (Department of Health and Council of Agriculture) should encourage food companies to put warning labels on seafood containers, menus, and public health brochures. The wording of such labeling should be similar to the label required by the Florida Department of Natural Resources for all wholesale shell food and shucked products: "Consumer Information—There is a risk associated with consuming raw oysters or any raw animal protein. If you have chronic illness of the liver, stomach, or blood or have immune disorders, you are at a greater risk of serious illness from raw oysters and should eat oysters fully cooked. If unsure of your risk, consult a physician" (38).

Conclusion

Residents of Taiwan have a high prevalence of chronic liver disease and are often exposed to marine microorganisms present in the sea that surrounds the island or rivers, lakes, or ponds inside the island. The presence of high genetic divergence among *V. vulnificus* isolates from humans and the environment indicates that this virulent bacterium is ubiquitous in nature. When *V. vulnificus* is suspected as the cause of sepsis, empiric therapy that includes a third-generation cephalosporin and minocycline should be administered. It should be standard practice for physicians to advise patients with underlying medical illness to avoid eating raw or undercooked seafood and to avoid exposing wounds to seawater.

Dr. Hsueh is an associate professor in the departments of Laboratory Medicine and Internal Medicine of National Taiwan University Hospital and National Taiwan University College of Medicine. His research interests include mechanisms of antimicrobial resistance and molecular epidemiology of emerging pathogens.

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West Nile Virus in California

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West Nile virus (WNV) was first isolated in California during July 2003 from a pool of *Culex tarsalis* collected near El Centro, Imperial County. WNV transmission then increased and spread in Imperial and Coachella Valleys, where it was tracked by isolation from pools of *Cx. tarsalis*, seroconversions in sentinel chickens, and seroprevalence in free-ranging birds. WNV then dispersed to the city of Riverside, Riverside County, and to the Whittier Dam area of Los Angeles County, where it was detected in dead birds and pools of *Cx. pipiens quinquefasciatus*. By October, WNV was detected in dead birds collected from riparian corridors in Los Angeles, west to Long Beach, and through inland valleys south from Riverside to San Diego County. WNV was reported concurrently from Arizona in mid-August, and from Baja, Mexico, in mid-November. Possible mechanisms for virus introduction, amplification, and dispersal are discussed.

Since the arrival of West Nile virus (WNV, *Flavivirus*, *Flaviviridae*) into New York City in 1999, the public health community has chronicled the unimpaired spread of this virus across North America from the Atlantic to the Pacific Coasts (1) and from Canada (2) into tropical America (3) and the Caribbean (4,5). Regionally, the epidemic has been characterized by an initial introduction with a few human cases during the first season, followed by explosive amplification and an epidemic during the second season, and then subsidence to maintenance levels. Ongoing or recent transmission of closely related St. Louis encephalitis virus (SLEV) in Florida, Louisiana, and Texas seems to have had little dampening effect on WNV amplification, which contradicts the long-held premise that two closely related flaviviruses cannot co-exist (6). Minimal ecologic resistance or selection pressure has left the strains of WNV intact genetically (7,8), until relatively minor changes may have resulted in attenuation in Mexico (3).

In 1999, when WNV was introduced into North America, few encephalitis virus surveillance programs remained intact, and most were structured to protect urban centers (9). Consequently, the initial detection of WNV in most areas occurred after introduction and amplification and frequently was heralded by the discovery of dead crows or horses and humans with neurologic illness. California is somewhat unique in that an extensive arbovirus surveillance program has remained intact statewide. Because of endemic SLEV and western equine encephalomyelitis virus (WEEV, *Alphavirus*, *Togaviridae*) transmission and nuisance mosquito problems, California residents have supported special local mosquito and vector control districts that currently protect ca. 33.9 million people (88% of the state's population) over a combined area of ca. 166,107 km^2. The associated California Encephalitis Virus Surveillance Program, which has been in place for more than 35 years (10), monitors mosquito abundance and infection rates as well as virus transmission to sentinel chickens. Local surveillance programs are coordinated at the state level by the California Department of Health Services, and supporting diagnostics currently are conducted by that agency and the Center for Vectorborne Diseases at the University of California, Davis. Recent ecologic studies on virus persistence and amplification by that center have been set against this extensive surveillance backdrop and have focused on wetlands along the Salton Sea (11,12). Our current study describes how this surveillance program, extended by associated field research projects, provided an early warning of the arrival of WNV in California and preliminary information on its ecology, surveillance, and dispersal during 2003. Highlighted information includes climatic conditions, the possible route(s) of introduction and subsequent dispersal, abundance of vector populations at the time of invasion, avian populations involved, and the comparative sensitivity of different surveillance indicators in different ecologic settings.

Materials and Methods

Climate data from Coachella Valley and the Los Angeles basin were downloaded from National Oceanographic and Atmospheric Administration weather stations from the California Integrated Pest Management

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website (http://www.ipm.ucdavis.edu/). These data were included to describe temperature conditions when virus was active and rainfall events associated with the intrusion of moist monsoon conditions from the Gulf of Mexico.

Mosquitoes were collected biweekly at permanent sites by using dry ice-baited CDC-style traps (CO_2 traps) operated without light (13) and gravid female traps (14). Sampling effort varied spatially. Six and 42 CO_2 traps were operated at wetlands and agricultural habitats in Imperial and Coachella Valleys, respectively, whereas 4–13 CO_2 and 6–20 gravid traps were operated per sampling occasion within an 8-km radius of the Whittier Dam area of Los Angeles. Mosquitoes were anesthetized with triethylamine, enumerated by species, grouped into pools of <50 females per species per site, frozen at –80°C, and then shipped on dry ice to the University of California at Davis for testing. There, mosquitoes were screened for infectious virus by cell culture by using an in situ enzyme immunoassay (EIA) (15) and for viral RNA using a robotic TaqMan system (16). Three separate TaqMan assays were conducted on each pool to detect WNV, SLEV, and WEEV by using primer sets evaluated previously against historical California lineages of SLEV and WEEV (E.N. Green and W.K. Reisen, unpub. data). Locations of mosquito pool collection sites statewide during 2003 are shown in Figure 1A.

Statewide, 212 sentinel flocks of 10 white leghorn hens each[1] were bled biweekly by lancet prick of the comb and samples mailed to the Viral and Rickettsial Diseases Laboratory, California Department of Health Services, where they were screened for antibody by WEEV or WNV/SLEV antigens with an EIA (17). *Flavivirus*-positive hens were re-bled, and whole serum specimens were tested by endpoint plaque reduction neutralization tests (PRNT) to separate those with antibody to WNV or SLEV. The locations of sentinel chicken flocks sampled during the summer of 2003 are summarized in Figure 1B. Three and six flocks, respectively, were located at research areas in Imperial and Coachella Valleys near the Salton Sea, whereas a single flock was located in the Whittier area of Los Angeles County. Seropositive birds were replaced at these study sites to track virus transmission activity through the season.

Free-ranging birds[2] were collected weekly at two wetland sites along the north shore of the Salton Sea by using 8 to 10 mist nets and 1 to 2 grain-baited ground traps, as described previously (18). Additional grain-baited traps were deployed at seven sites throughout Coachella Valley. Birds were identified to species, sex, and age; leg-banded with U.S. Geological Survey tags; bled by jugular puncture (0.1 mL whole blood into 0.9 mL of saline); and released. Samples were clarified by centrifugation and then screened for WEEV, SLEV, or WNV antibodies by using an EIA (19). Positive samples were retested by PRNT. Separation of SLEV and WNV infection was based on a fourfold or greater difference in endpoint PRNT titers.

Dead birds were reported to the California Department of Health Services by telephone. Carcasses appearing to be <24 hours old were submitted by local mosquito and vector control districts and public health agencies for necropsy to the California Animal Health and Food Safety laboratory at the University of California, Davis, where kidney, lung, and brain tissues were removed for testing. Kidney samples were screened for WNV RNA by using the robotic TaqMan system and primers described above. Virus isolation was attempted from pooled organs of RNA-positive birds by using a plaque assay on Vero cell culture.

Results

WNV was probably introduced into California during July 2003 and was detected initially in a pool of *Cx. tarsalis* mosquitoes collected near El Centro, Imperial County, on July 16, 2003 (Figure 2). During the following weeks, WNV was isolated from 16 pools of *Cx. tarsalis*, and transmission was detected by 51 seroconversions of sentinel chickens at six flocks positioned on wildlife refuges along the southern shore of the Salton Sea and in agricultural habitats near the Mexican border (Figure 2). WNV was detected concurrently along the Colorado River at Yuma and in eastern Arizona by the Arizona surveillance system (20). Multiple isolations of SLEV were made in Arizona before WNV was first detected in August. WNV was not reported from Baja, Mexico, until November 2003 (21). At the time of WNV amplification in Imperial County, mosquito catches in CO_2 traps along the southern shore of the Salton Sea had reached the typical midsummer minimum (Figure 3A) and were dominated by *Cx. tarsalis*, *Cx. erythrothorax*, and *Aedes vexans*. However, only pools of *Cx. tarsalis* contained WNV (Table 1). A comparable scenario developed in the Coachella Valley during mid-August (Figure 3B), with 10 isolations of WNV and 3 of SLEV made from *Cx. tarsalis* (even though 466 pools of other mosquito species were tested) and 20 seroconversions of sentinel chickens to both viruses detected at multiple flocks (Table 1). Despite intensive surveillance throughout the rest of Coachella Valley, WNV and SLEV

[1]Procedures for the bleeding and husbandry of sentinel chickens were described in Protocol 9608 approved by the University of California, Davis, Animal Use and Care Administrative Advisory Committee.

[2]The collection, banding, and bleeding of wild birds were conducted under Protocol 9605 approved by the Animal Use and Care Administrative Advisory Committee of the University of California, Davis, California Resident Scientific Collection Permit 801049-02 by the State of California Department of Fish and Game, and Master Station Federal Bird Marking and Salvage Permit 22763 from the U.S. Geological Survey Bird Banding Laboratory.

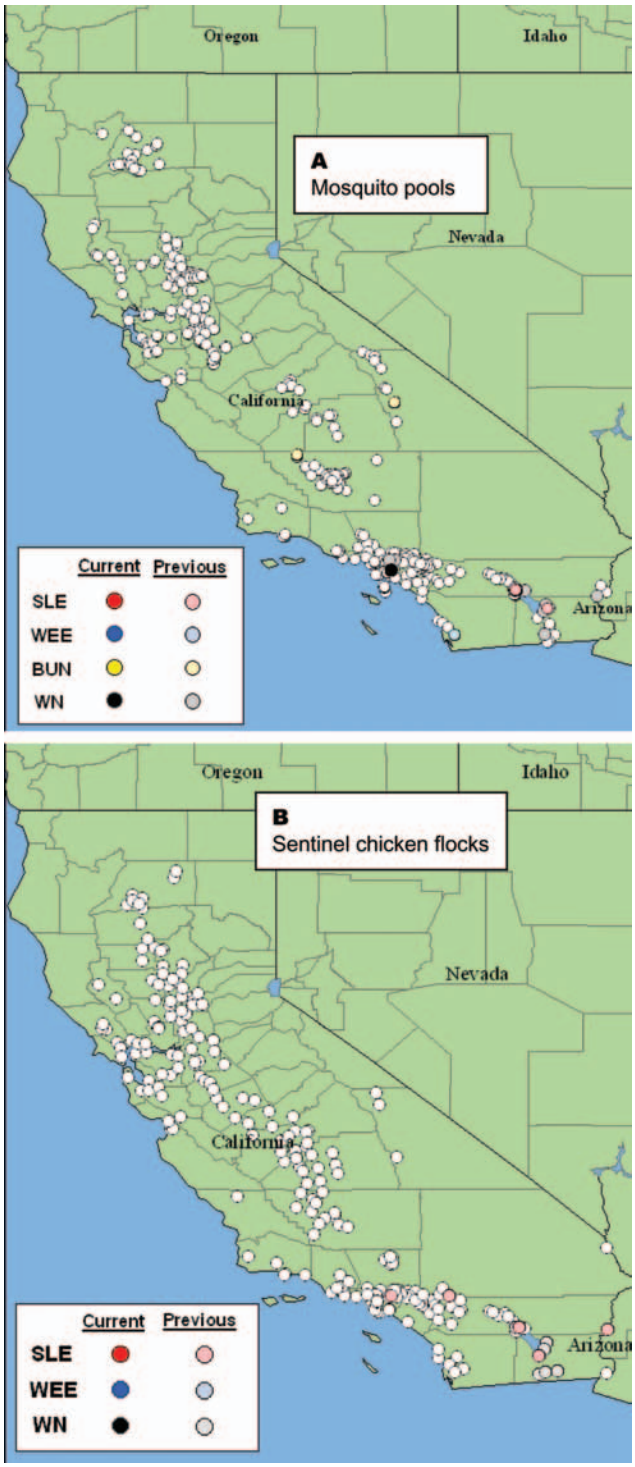

Figure 1. Map of California showing locations where A) 9,731mosquito pools were collected and B) 212 sentinel chicken flocks were located through November 1, 2003. Data are cumulative for 2003 and show negative, previously positive, and currently active sites as downloaded from http://www.vector.ucdavis.edu/. SLE, St. Louis encephalitis virus; WEE, western equine encephalitis virus; BUN, viruses in the California encephalitis virus complex, family *Bunyaviridae*; WN, West Nile virus.

activity was detected only along the north shore of the Salton Sea, even after the flooding of wetlands for migratory waterfowl in September resulted in a marked increase in *Cx. tarsalis* abundance (Figure 3A,B). No positive dead bird, human, or equine cases were associated with the initial invasion and amplification of WNV in rural southeast California, until a single human case was reported near El Centro in late October.

Serum samples from live free-ranging birds in Coachella Valley showed an increase in *Flavivirus* prevalence (Figure 4) in resident species (Table 2), with WNV, SLEV, and WEEV detected near sites where these viruses were isolated from mosquitoes or detected by sentinel chicken seroconversions (Figure 2). Confirmatory PRNTs showed that *Flavivirus*-positive birds were infected with both WNV and SLEV. Of 31 birds with demonstrable PRNT titers, 20 were infected with WNV, 8 were infected with SLEV, and 3 had equivocal titers against both viruses. Live bird sampling programs in Los Angeles, Bakersfield, and Sacramento did not collect antibody-positive birds despite comparable sampling and testing efforts (Table 1).

Climatic conditions at the time of WNV introduction included above average temperatures and several rainfall events associated with the extension of the southwestern monsoon into southeastern California (Figure 5). Normally, summer storms track north from the Gulf of Mexico into Arizona and New Mexico; however, during the summer of 2003 a persistent high pressure system over Nevada resulted in a frequent clockwise pattern flowing from Colorado south into Arizona and then into southeastern California (http://www.srh.noaa.gov/abq/climate/Monthlyreports/July/nams.htm).

WNV then dispersed from the Salton Sea area to the City of Riverside in Riverside County and to the City of Arcadia in the Los Angeles Basin during September and October (Figure 2). In urban Los Angeles, WNV was tracked by testing dead birds reported by the public and by virus isolations from *Cx. pipiens quinquefasciatus* collected by gravid female traps (Figure 6). Sentinel chickens situated near dead bird collection sites remained negative for WNV, although two chickens in Monterey Park, Los Angeles County, seroconverted to SLEV during the week of September 16, 2003. Virus movement into the City of Riverside was associated with the detection of the first locally acquired WNV human case in California, followed by single cases in Imperial County and then the City of Whittier in Los Angeles County.

WNV then seemed to disperse south and was tracked through dead birds submitted from inland suburban communities along Highways 215 and 15 from San Bernardino to San Diego (Figure 2). Included in the 57 dead birds that tested positive for WNV through October 30, 2003, were

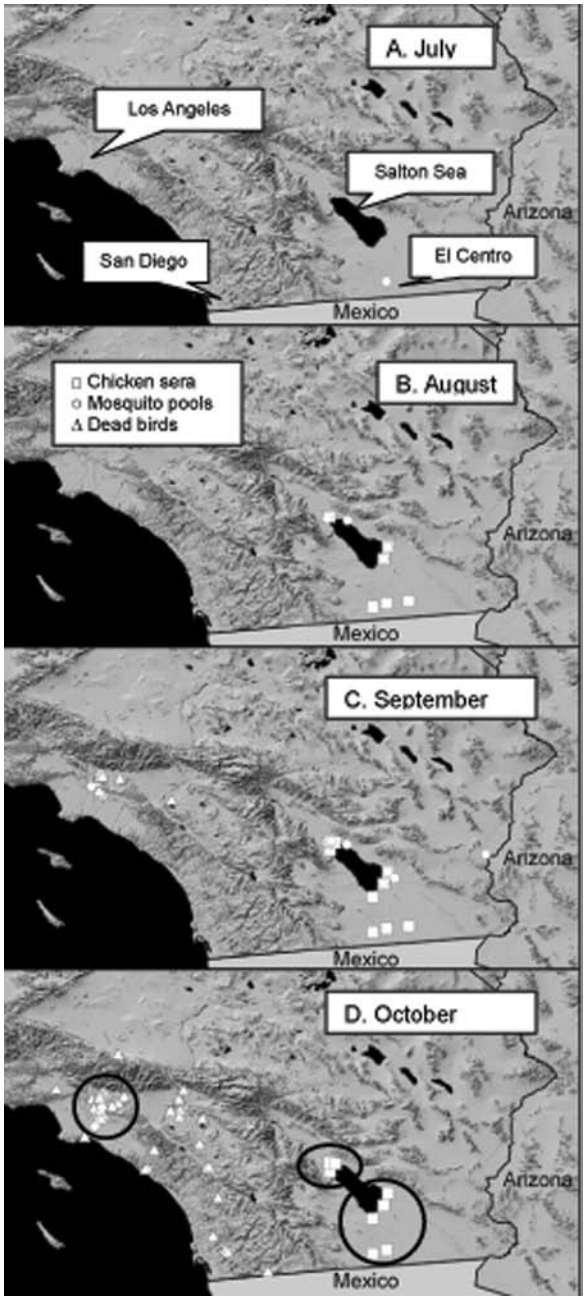

Figure 2. Introduction of West Nile virus into California. Panels show the locations of positive mosquito pools, sentinel chicken flocks with >1 seroconversion, and positive dead birds during each month. Encircled in panel D are the locations of the three foci studied in depth during 2003.

47 American Crows, 1 Brewer's Blackbird, 2 House Finches; 3 House Sparrows, 1 Northern Mockingbird, 1 Western Scrub-Jay, and 1 White-crowned Sparrow. WNV-positive dead raptors have yet to be reported, and sick or dead birds have not been reported from the Los Angeles or San Diego Zoos.

Discussion

Enzootic monitoring by the California Encephalitis Virus Surveillance Program and associated field research projects provided an effective early warning that detected the introduction of WNV into rural southeastern California before reported avian, equine, or human illness. Our observations provided information related to the potential modes of dispersal and amplification as well as the effectiveness of different surveillance indicators to track WNV.

Dispersal

The timing of initial WNV detection in California provided some insight into possible mechanisms for invasion and subsequent dispersal. WNV was first detected during mid-July in southeastern California concurrent with the detection and amplification of endemic SLEV. These events occurred approximately 7 months after the termination of reproductive diapause (22) and 2 months after the vernal peak in the *Cx. tarsalis* population (11), 2 months after the end of the nesting season for most resident avian species (18), 2 months after the passing of the northbound avian migrants, and 2 months before the arrival of the southbound avian migrants. This pattern of arbovirus appearance during midsummer, when temperatures are highest and vector populations lowest, has been documented repeatedly for SLEV in southeastern California and frequently occurs concurrent with the onset of the hot summer period associated with the southwest monsoon (12). Partial sequencing of SLEV isolates from southeastern California has indicated minimal genetic change during sequential years with SLEV activity but differences from isolates made after years with no virus detection (12,23) and from strains sequenced from Central and South America (24). Recently, minor genetic change has been detected in WNV isolated in the Yucatán (3).

Our attempts to detect WNV infection in both north- and southbound migrants along the Pacific flyway were unsuccessful, agreeing with our previous studies with SLEV and WEEV (18). Surveillance along the Pacific flyway from British Columbia Province, Canada, the northwestern United States, and western states in Mexico indicated that there was no WNV activity in these areas during the fall of 2002 or the spring of 2003 to provide a source of infection for migratory birds. In contrast, seropositive resident and migratory birds have been documented along the Atlantic and Mississippi flyways into the Caribbean (5) and tropical eastern Mexico (3,25), indicating WNV dispersal into these areas. During 2003, a total of 4,502 free-ranging birds from Sacramento, Kern, and Los Angeles Counties were tested for WNV antibody with negative results. An additional 3,178 birds collected in the Coachella Valley were tested through November 2003; 51 resident species had antibody to flaviviruses detected by

Table 1. Surveillance data for southern West Nile virus foci and the rest of California, January 1–November 1, 2003 [a]

Surveillance data	Research areas			Remaining agencies	Total
	Imperial	Coachella	LA		
Human cases	1	0	0	1	2
Horse cases	0	0	0	1	1
Mosquito pools	238	1,414	1,663	6,416	9,731
Culex tarsalis	150	948	121	3,176	4,395
WNV pos	16	10	0	0	26
SLEV pos	1	3	0	0	4
WEEV pos	0	0	0	1	1
Cx. pipiens complex	0	299	1,036	1,170	2,505
WNV pos	0	0	6	0	6
Others [b]	88	167	506	2,070	2,831
Sentinel chickens	6	10	5	191	212
WNV pos	51	18	0	0	69
SLEV pos	3	2	0	8	13
WEEV pos	0	0	0	0	0
Dead birds reported	23	15	1,218	6,294	7,550
Tested	6	5	256	1,118	1,385
WNV pos	0	0	38	21	59
Wild bird sera	0	3,178	1,452	4,502	9,132
WNV pos		51	0	0	51
WEEV pos		2	0	0	2

[a]LA, Los Angeles; WNV, West Nile virus; SLEV, St. Louis encephalitis virus; WEEV, western equine encephalitis virus; pos, positive.
[b]Other mosquitoes tested: *Anopheles franciscanus* , *An. hermsi* , *Ae. vexans* , *Culiseta inornata* , *Cs. incidens* , *Cx. erythrothorax* , *Cx. erraticus* , *Cx. stigmatosoma* , *Oc. sierrensis* , *Oc. dorsalis* , *Oc. melanimon* , *Oc. taeniorhynchus* , *Psorophora columbiae* .

EIA. Mourning Doves repeatedly were positive, and, although adults were present in Coachella Valley year-round, evidence from U.S. Geological Survey band recovery reports indicated considerable dispersal (23). Adult doves survive WNV infection and produce a moderate 3–5 $\log_{10}$ PFU/mL viremia of 5 days' duration (26) (W.K. Reisen, unpub. data).

The late summer increase in WNV transmission and dispersal coincided with postnesting movements by summer and year-round resident birds. Several passerine species, such as House Finches, form flocks at this time that forage widely and roost in various locations. Vagrants from these populations could be responsible for the movement of virus in rural agricultural sites. During the hot summer months, a short extrinsic incubation period in local vector populations feeding on sick and less mobile individual birds from these flocks could infect other local birds, resulting in the relatively rapid movement of virus by resident avian species.

Climate patterns can influence mosquito dispersal. Storm fronts previously have been proposed as dispersal mechanisms for mosquitoes and the arboviruses they transmit in Asia (27) and North America (28,29). Each summer, the southwest monsoon brings moisture from the Gulf of Mexico into the arid Southwest, and this movement often is characterized by intense local thunderstorm activity. High barometric pressure established over Nevada during 2003 created a persistent clockwise airflow pattern from Colorado into southeastern California through Arizona and northern Mexico. Surveillance in Arizona during 2003 detected WNV concurrent with that in southeastern California, perhaps indicating that a similar climate-driven mechanism brought virus southwest from the Colorado epicenter.

A final and perhaps more remote consideration in the East-West dispersal of WNV is the transport of infected mosquitoes by commerce. The main East-West highways in the United States, such as I-15, I-40, I-10, and I-8, enter southern California (Figure 7). Possibly produce or other trucks loading at night or early morning in areas of intense transmission could entrap infected mosquitoes that would disembark when truck contents are inspected or offloaded. If conditions for mosquito survival were suitable, these infected mosquitoes could be the source of virus introduction into new areas. Such a mechanism was considered among several possibilities as the source of several new mosquito species introductions into southeastern California (30,31). In this context, it is possible to conceptualize the introduction of WNV into southern California via I-8, followed by movement northward along Highway 86 into refuges near the Salton Sea in Imperial and Coachella Valleys, and then along I-10 and Highway 60 into Los Angeles and Riverside, respectively, and by movement down I-15 into San Diego. However, the WNV epicenter during 2003 was situated in the Colorado-Nebraska area, and most ground transport from this area would be expected to enter California by I-80 into the Sacramento area, where WNV has yet to be detected.

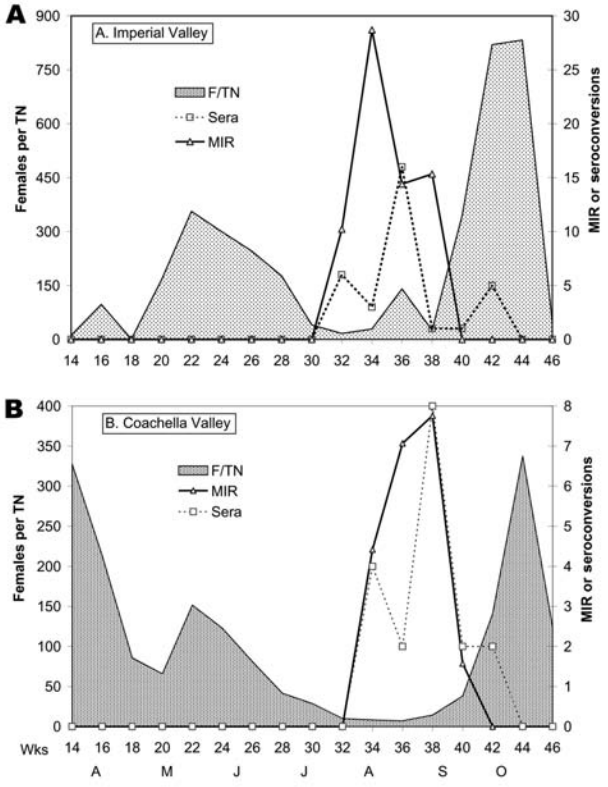

Figure 3. Virus temporal dynamics in relation to *Culex tarsalis* in A) Imperial and B) Coachella Valleys. Shown are female (F) *Cx. tarsalis* collected per CO_2 trap night (TN). West Nile virus minimum infection rates (MIR) per 1,000 tested adjusted for differential sample sizes, and the number of sentinel chicken seroconversions per 2-week period.

Amplification

Three foci of virus amplification were studied (Figure 2). Based on our surveillance data, WNV amplification in rural southeastern California initially occurred throughout Imperial Valley and around the northern shore of the Salton Sea in Coachella Valley. Based on virus isolations, *Cx. tarsalis* was the vector species and resident birds the presumed amplifying hosts in this rural irrigated desert biome. Recovery of WNV from *Cx. tarsalis* was expected because this species was infected frequently with SLEV and WEEV during previous ecologic studies (11,12,32) and ongoing surveillance in rural southeastern California. Although susceptible to infection (33), other species, including *Cx. p. quinquefasciatus*, *Cx. erythrothorax*, and *Ae. vexans* collected concurrently were not infected with WNV. Avian serosurveys showed highest antibody prevalence rates among resident columbiform and galliform species, which produce moderate-to-low viremias and do not die from infection (26). The lack of passerine positives may reflect elevated death rates among these species; however, few dead birds were reported from these areas, and none tested positive for WNV. The limited number of corvid species and the sparse human population in this desert environment may have combined to limit the utility of dead bird surveillance.

Once WNV dispersed into urban Los Angeles, virus was isolated from dead birds reported by the public and from *Cx. p. quinquefasciatus* collected by gravid traps. Positive bird species included mostly American Crows as well as small-sized species such as House Finches and House Sparrows. The Whittier Narrows and associated riparian corridors appeared to be the site of WNV introduction and subsequent amplification. This area supports a large American Crow communal roost during the postnesting season in late summer and fall that may have contributed to the receptivity of this area for WNV introduction and subsequent amplification.

Surveillance

WNV was monitored by using a wide variety of methods that varied in effectiveness. In rural southeastern California, WNV was tracked best by testing pools of *Cx. tarsalis* collected by CO_2 traps and by monitoring sentinel chicken sera. Free-ranging birds, such as quail and doves, which do not succumb to infection, also were useful sentinels; however, differentiating WNV from SLEV infections was problematic for birds collected before a definitive rise in immunoglobulin G antibody titer. None of these surveillance methods worked well in urban or periurban areas of Los Angeles. Few mosquitoes, including *Cx. tarsalis*, were collected there by CO_2 traps, and most positive pools to date have come from female *Cx. p. quinquefasciatus* collected by gravid traps. In urban neighborhoods, CO_2 traps and other methods collect relatively few mosquitoes in comparison to gravid traps (34,35).

The dense human population in Los Angeles County reported >1,200 dead birds by the end of October; 218 of these were tested, and 38 were positive for WNV. As expected because of their susceptibility and large size,

Table 2. Wild birds collected and bled in Coachella Valley, January 1–November 1, 2003

Species	Sera	% *Flavivirus*[a]	% WEEV[b]
Abert's Towhee	108	0.9	0.0
House Finch	251	0.4	0.0
Least Bittern	10	10.0	0.0
Gambel's Quail	643	3.3	0.2
Common Ground Dove	95	5.3	0.0
Mourning Dove	729	1.5	0.1
Domestic Pigeon	39	25.6	0.0
White-winged Dove	6	16.7	0.0
58 species	1,297	0.0	0.0
Total	3,178	1.6	0.1

[a]Positive by enzyme immunoassay (P/N ratio >2) . Some EIA-positive sera were by plaque reduction neutralization test, whereas some others were positive, but there was <4 fold difference between West Nile virus and St. Louis encephalitis virus titers.
[b]WEEV, western equine encephalitis virus.

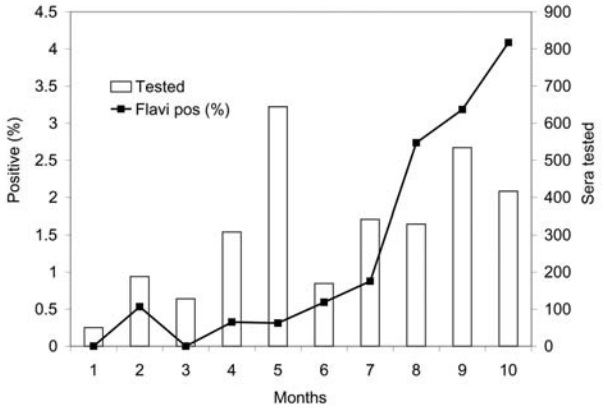

Figure 4. Wild bird *Flavivirus* seroprevalence rates (Flavi pos %) in Coachella Valley during 2003. Shown are percentages of total serum samples that tested positive each month by enzyme immunoassay. Positives include infections caused by West Nile virus and St. Louis encephalitis.

most positives were crows, but small-sized passerines also tested positive. In urban Los Angeles, sentinel chickens did not seroconvert to WNV during 2003, despite being situated near recoveries of WNV-positive dead crows and *Cx. p. quinquefasciatus* pools and being in the vicinity of the large Whittier crow roost. Differences in sentinel chicken sensitivity between rural and urban habitats may relate to vector mosquito dispersal and not to avidity for feeding on chickens. In agreement, of 78 serum specimens taken from backyard chickens of unknown age from this urban area along the Rio Hondo and San Gabriel riparian corridors, 7 had antibody confirmed by PRNT to be WNV. In California, *Cx. tarsalis* is very dispersive (36,37) and hunts along riparian corridors or vegetative transitions (38,39), whereas *Cx. p. quinquefasciatus* is less dispersive in urban environments and remains near the point of emergence (40). Therefore, infectious *Cx. p. quinquefasciatus* may be less likely to disperse in urban environments and encounter confined sentinel flocks than are *Cx. tarsalis* in rural environments, where farmhouse environs provide widely spaced "islands" of elevated vegetation used by birds for roosting and nesting and by *Cx. tarsalis* for host-seeking and resting. Southern California environments lack the contiguous canopy found in the eastern deciduous forest, and *Culex* mosquitoes feed readily at ground level (41,42). Therefore, positioning sentinels at ground level does not appear to have been a critical factor in effectiveness.

The number of dead bird reports in Los Angeles increased after WNV was introduced, presumably because of media coverage, public education concerning the dead bird surveillance program, and increased WNV-associated bird deaths. Our laboratory data indicated that approximately 80% of the dead birds tested after the invasion and media publicity were WNV-negative. These data indicated that at low-to-moderate levels of enzootic transmission, dead bird reports alone may not be a true indication of the level and location of WNV transmission. In addition, use of antibody testing of free-ranging birds collected in grain-baited crow traps (mostly House Sparrows and House Finches) did not seem to be a productive surveillance method in Los Angeles, and all birds to date have tested negative, including those trapped at Whittier Narrows.

Our data during 2003 clearly showed that WNV introduction, amplification, and widespread dispersal occurred with few human or horse cases, indicating that such cases are insensitive indicators of WNV presence and enzootic activity levels. Most humans in rural southern California reside in homes with some form of air-conditioning and thereby may be protected from mosquito contact during the evening (43). Unknown proportions of horses in California are vaccinated and thereby may be protected from disease. Epidemic transmission of WNV in southern California has been predicted for 2004, and it will be of interest to determine how well enzootic measures of virus activity forecast human infection.

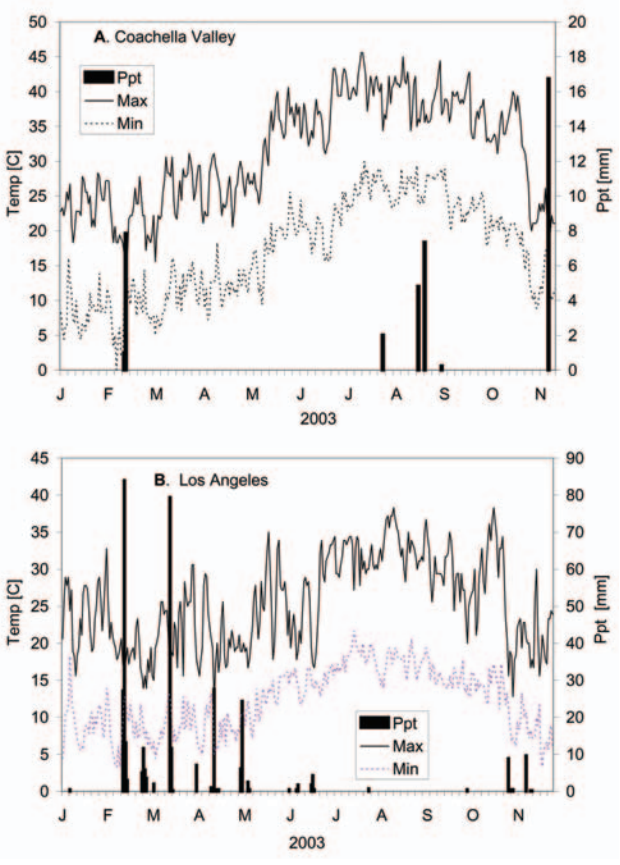

Figure 5. Climate conditions in A) Coachella Valley and B) Los Angeles at National Oceanic and Atmospheric Administration weather stations. Of interest was the dramatic drop in maximum temperature during early November coincident with the end of transmission. Ppt, precipitation.

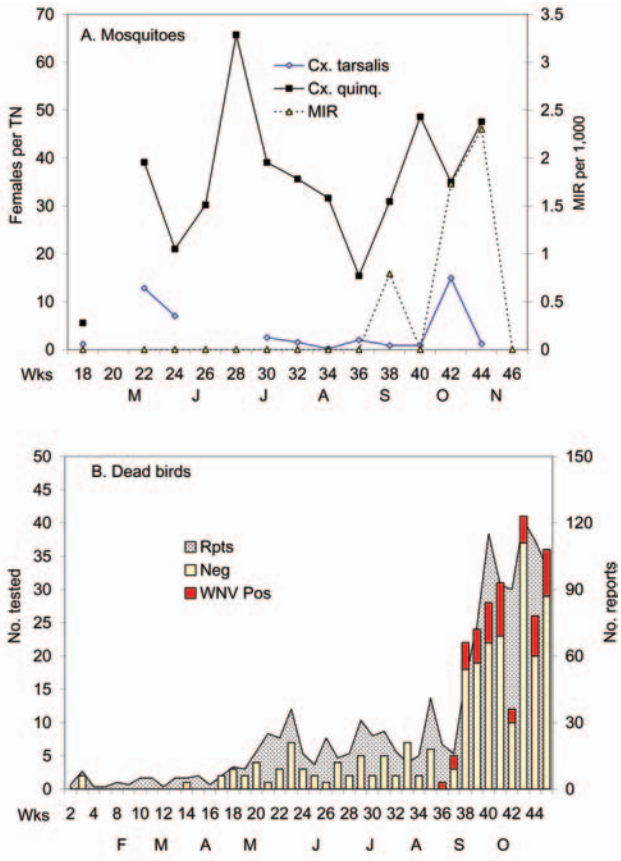

Figure 6. Virus temporal dynamics in relation to *Culex* abundance in the Whittier Narrows area of Los Angeles County. Shown are A) female *Cx. tarsalis* collected per CO_2 trap night (TN) and female *Cx. p. quinquefasciatus* collected per gravid TN, West Nile virus (WNV) minimum infection rates (MIR) per 1,000 *Cx. p. quinquefasciatus* tested, adjusted for differential sample sizes, and B) number of dead birds reported, tested, and positive for WNV in Los Angeles County.

Response

California health agencies and vector control districts have been preparing for the introduction of WNV since movement into the West seemed eminent, and state guidelines for escalated control responses to surveillance data have been prepared (http://westnile.ca.gov/Publications.htm). Initial responses included enhanced surveillance, expanded larval control operations, and preparation for emergency adult control. Extended surveillance in Imperial County by the Imperial County Health Department, Coachella Valley Mosquito and Vector Control District, and University of California, Davis, and the development of a dead bird surveillance program by the California Department of Health Services during 2002 are examples of new programs that proved useful in tracking WNV during 2003. Detection of WNV in southeastern California during 2003 triggered adult mosquito control operations to interrupt transmission at wetlands and to protect residents of the small towns of Niland in Imperial County and Mecca in Coachella Valley. Dead bird surveillance data in urban Los Angeles were used to direct focal larval control operations and to launch public education programs through various media events. Surveillance activities in southern California continued during the winter of 2003 to 2004 and have included mosquito pool submission, sentinel chicken testing, live bird sampling and testing, and dead bird reporting and testing. All findings have been negative through mid-February 2004, despite surveillance near wetlands along the Salton Sea and at the Whittier Narrows crow roost, perhaps indicating that transmission ceased, despite mild winter conditions. Positive after-hatching-year and second-year resident birds from Coachella Valley have been collected, but these birds presumably were infected during 2003; all winter resident birds, such as White-crowned Sparrows, have remained negative. Planned and ongoing operational responses during spring 2004 have been coordinated at the local, regional, and state levels but necessarily vary among agencies because of local ecology, politics, and funding. The introduction of WNV into California and its anticipated amplification during the next few years will provide a rigorous test of how well a widespread integrated vector management approach to mosquito control can protect the residents of California from mosquito-borne disease.

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Figure 7. Road map of California. Arrows indicate the points of entry of main U.S. highways into California from the East.

quito, chicken, and wild bird specimens from Imperial and Riverside Counties; J. Wilson for collecting mosquito, wild bird, and chicken specimens in the Whittier area of Los Angeles; C. Barker and B. Eldridge for directing data management; T. Scott for initially directing diagnostics; B. Lothrop and A. Gutierrez for assisting with field work in Coachella Valley; G. Estrada for assisting with surveillance in Imperial Valley; S. Kluh, J. Spoehel, P. O'Connor, and S. Tabatabaeepour for assisting with sampling in Los Angeles; C. Glaser, E. Tu, and E. Baylor for providing data on human cases and testing sentinel chicken sera; K. Linthicum, A. Hom, A. Houchin, L. Hui, and K. McCaughey for providing surveillance data and managing the dead bird reporting system; C. Barker for creating Figures 2 and 7; and M. Tyree and D. Cayan for providing insight into the dynamics of the Southwest monsoon.

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Crimean-Congo Hemorrhagic Fever in Turkey

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In 2002 and 2003, a total of 19 persons in Turkey had suspected cases of Crimean-Congo hemorrhagic fever (CCHF) or a similar viral infection. Six serum samples were tested; all six were found positive for immunoglobulin M antibodies against CCHF virus. Two of the samples yielded CCHF virus isolates. Genetic analysis of the virus isolates showed them to be closely related to isolates from former Yugoslavia and southwestern Russia. These cases are the first of CCHF reported from Turkey. Eighteen patients handled livestock, and one was a nurse with probable nosocomial infection. The case-fatality rate was 20% among confirmed CCHF case-patients (1 of 5 patients), and the overall case-patient fatality rate was 11% (2 of 19 patients). In addition to previously reported symptoms and signs, we report hemophagocytosis in 50% of our patients, which is the first report of this clinical phenomenon associated with CCHF.

Crimean-Congo hemorrhagic fever (CCHF) is an acute illness affecting multiple organ systems and characterized by extensive ecchymosis, visceral bleeding, and hepatic dysfunction; and it has a case-fatality of 8% to 80% (1). CCHF virus (CCHFV) (genus *Nairovirus*, family *Bunyaviridae*) is transmitted to humans by bites of infected ticks (several species of genus *Hyalomma*). CCHFV has also been transmitted to patients or viremic livestock through contact with blood or tissue (1). Epidemics of CCHFV have previously been reported from Eastern Europe, Africa, and central Asia (2–8). Many cases have been reported from the countries around Turkey, including Albania, Iran, Iraq, Russia, and the former Yugoslavia (7,9–12). Although serologic evidence indicated the existence of CCHFV in Turkey several decades ago (13), no clinical cases have been documented. We describe 19 patients from the eastern Black Sea region with hemorrhagic fever compatible with CCHF, who were admitted to Karadeniz Technical University Hospital during the spring and summer of 2002 and 2003.

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Patients and Methods

Patients

Several patients in May through July 2002 and 2003 were referred from surrounding county hospitals to our hematology unit with varying degrees of fever and hematologic manifestations. All of the patients had similar clinical and laboratory findings, including fever, petechiae, headache, abdominal pain, nausea, vomiting, liver enzyme elevations, and cytopenia. Bone marrow aspiration and routine serologic tests excluded hematologic malignancies and known viral or bacterial infections. Serum samples from several patients admitted in 2003 were stored at –80°C for further diagnostic testing for a possible hemorrhagic fever agent.

Laboratory Testing

Serum samples from seven patients were sent to Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, GA (CDC) for testing. Only six samples from five patients were available in sufficient volume. After we considered possible hemorrhagic fever viruses in the region, we performed immunoglobulin (Ig) M and IgG enzyme-linked immunosorbent assay (ELISA), using inactivated native CCHFV (Strain IbAr 10200) antigens grown in Vero E6 cells on serum samples (14). A test developed to detect CCHF viral antigens was also performed (15). Virus isolation attempts from the serum samples were conducted under biosafety level 4 conditions with Vero E6 cells.

For virus genetic detection and analysis, serum samples or infected Vero E6 cells were combined with Tripure Isolation Reagent (Roche Applied Science, Indianapolis, IN) in a ratio of 1:5 and incubated at room temperature for

a minimum of 10 min. Total RNA was isolated by using the RNaid Kit following manufacturer's recommendations (Qbioene Inc., Carlsbad, CA), and the extracted RNA was resuspended in 50 µL H_2O. Five microliters of the RNA was used in a 50-µL reverse transcription (RT) reaction with the Access RT-PCR System (Promega Biosciences, San Luis Obispo, CA). The primers that enabled the amplification of nucleocapsid-coding sequence (S segment) were previously described as was the polymerase chain reaction (PCR) method used, with slight modifications (16). Briefly, separate RT was performed by using CCHF-F2 primer at 42°C for 1 h. Ten microliters of the RT reaction was subsequently used in a 50-µL PCR reaction with FastStart Taq DNA Polymerase with GC-rich solution (Roche) and primers CCHF-F2 and CCHF-R3. The temperature profile for the PCR reaction was as follows: 2 min at 95°C (36 cycles of 1 min at 95°C and 1 min at 45°C), 2 min at 72°C, and a final elongation of 10 min at 72°C. Amplified DNA was analyzed by using a 1% low-melt agarose gel, and bands corresponding to 536-bp products were purified by using the Qiagen Gel Extraction Kit (Qiagen, Valencia, CA). Sequencing of both DNA strands was performed by using primers CCHF-F2 and CCHF-R3 in a BigDye Terminator v3.1 reaction on the 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The obtained sequences were analyzed with Sequencer (Gene Codes Corporation, Ann Arbor, MI).

Results

Serologic test results for hepatitis A, B, and C viruses (HAV, HBV, and HCV); herpes viruses; and HIV and PCR for HBV DNA and HCV RNA were negative. Although malaria does not exist in these provinces, peripheral blood smear examinations confirmed these specimens to be negative for *Plasmodium*. Bacterial blood cultures were negative in all patients. Serologic tests for *Brucella* and *Leptospira* were also negative in all patients. Samples were negative for anti-Alkhurma virus IgM, and IgG. Specific testing for CCHFV antigen detection, IgG and RT-PCR tests were negative for the six specimens from the five patients. However, all six specimens were positive for IgM antibodies reactive with CCHFV antigen. CCHFV (CDC, Special Pathogens numbers: 200310845 and 200310849) were isolated from two of the patients.

RT-PCR products of the correct predicted size (536 bp) were obtained for each of the viruses and sequenced. The resulting nucleotide sequences had high identity with previously characterized CCHFV strains, and 11 nucleotide differences were detected between the virus sequences obtained from the two patients. Comparison of the deduced amino acid sequences indicated that no amino acid differences existed between the two virus strains. Detailed genetic comparison was performed by using the CCHFV S segment sequences available from GenBank. The analysis indicated the close relatedness of the Turkish CCHFV isolates to CCHFV strains from Russia and Kosovo, with 97%–98% and 100% identity at the nucleotide and protein levels respectively (data not shown). A comprehensive phylogenetic analysis (Figure 1) by using PILEUP (Wisconsin Package Version 10.2,

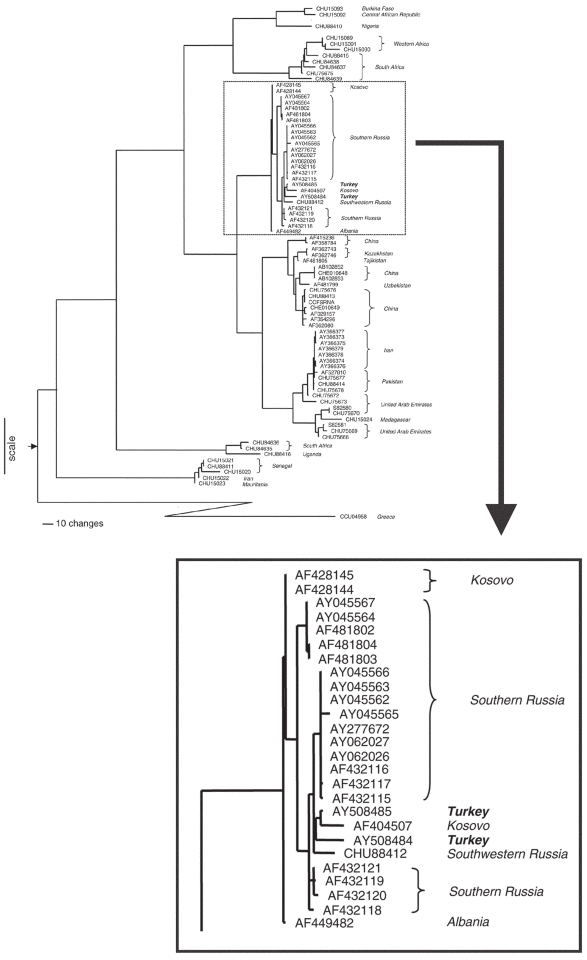

Figure 1. Phylogenetic analysis of Crimean-Congo hemorrhagic fever virus (CCHFV) genetic difference. Maximum parsimony analysis of the aligned sequences of a 488-nt region of CCHFV S segments and the equivalent genome region of Dugbe and Nairobi sheep disease viruses. Analysis was performed with the heuristic search method with stepwise addition, tree bisection-reconnection branch swapping, and transversions; transitions were weighted 4:1. The graphic representation of the results was outgroup rooted by using the Dugbe (GenBank accession no. AF434161, AF434162, AF434163, AF014014, AF434164, AF014015, AF434165) and Nairobi sheep disease virus (AF504293) S segment nucleotide sequences. The node attaching the outgroup to the CCHFV tree topology is shown by the arrow at the base of the tree. Horizontal distances within the CCHFV part of the tree are proportional to nucleotide steps (see scale bar), separating virus taxa and nodes. Vertical and diagonal lines are for visual clarity. Each virus sequence is indicated by the corresponding GenBank accession number. The two CCHFV sequences are in bold.

Genetics Computer Group, Inc.), followed by PAUP4.0b10 (Sinauer Associates Inc., Sunderland, MA, USA), showed that the Turkish CCHFV isolates clustered closely with the CCHFV strains from southwest Russia and Kosovo. Bootstrap analysis showed the clade containing the Russian, Balkan, and Turkish CCHFV to be well supported (99%), and these viruses are clearly distinct from those in other virus clades, including the clade containing the CCHFV detected in the CCHF outbreak in neighboring Iran in 2002 (GenBank accession no. AY366373–9).

Nineteen patients (including the five laboratory-confirmed patients) who fulfilled suspected-case criteria for CCHF of the European Network for Diagnostics of Imported Viral Diseases (ENIVD) were identified in 2002 and 2003 (17). Nine patients were admitted from May through July 2002, and 10 patients were admitted in June to July 2003. Most of the patients were female (15 female vs. 4 male), and the mean age was 42 ± 8 year. Twelve of 19 patients were from Gumushane, and the other 7 were from the neighboring cities of Giresun (4 patients), Artvin (2 patients), and Trabzon (1 patients) (Figure 2). All of them, except one, handled livestock; none of the patients described tick bites. However, six patients gave a history of removing ticks from livestock. The remaining patient was a nurse in a county hospital in Trabzon. Signs and symptoms observed in the patients are shown on the Table. The most commonly encountered signs and symptoms were malaise, fever, abdominal pain, myalgia, nausea, vomiting, petechiae, and bleeding from gingiva, nose, vagina, or gastrointestinal system. Complete blood counts showed thrombocytopenia in all patients (median 15 x 10^3/μL, range: 1–87 x 10^3/μL), leukopenia in 15/19 (median 1,700/μL, range 700–5,200/μL), and anemia in 5 of 19 patients (median 13.8g/dL, range 6.1–17.3 g/dL). Serum aspartate aminotransferase (AST) (median 693 U/L, range 178–5,220U/L), alanine aminotransferase (ALT) (median 248 U/L, range 66–1,438 U/L), and lactate dehydrogenase (LDH) (median 1,601 U/L, range 650–20,804 U/L) levels were elevated in all patients. Coagulation tests showed prolonged prothrombin time (PT) (median 13.4 s, range 12.1–18.5 s) and activated partial thromboplastin time (aPTT) (median 34.9 s, range 30.2–59.1 s) in 7 of 19 patients. Fibrinogen was decreased and D-dimer was elevated in one patient with suspected CCHF, which indicated disseminated intravascular coagulation. Fibrinogen and D-dimer levels were normal in other patients. Creatine phosphokinase (CPK) levels were elevated in 14 of 19 patients (median 568 U/L, range 81–2,500 U/L). Blood urea nitrogen and creatinine (median 0.8 mg/dL, range 0.5–6.2 mg/dL) were found to be elevated in 2 of 19 patients. Hematologic malignancies were excluded after bone marrow aspiration smear and trephine biopsy in 14 patients. In 7 of 14 patients (including 2 of 5 confirmed patients), hemophagocytosis with proliferation of histiocytes in bone marrow smears was present (Figure 3).

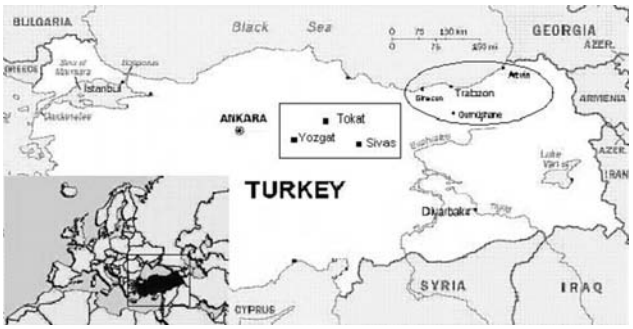

Figure 2. Geographic distribution of patients with Crimean-Congo hemorrhagic fever (CCHF), Turkey, 2002–2003. Residency of the patients with CCHF infection from our series is marked in the circle. Epicenter of a concurrent outbreak presented at the recent conference in Ankara is shown as a rectangle.

All patients received intensive clinical supportive measures, including platelets, fresh frozen plasma, and packed erythrocyte infusions, when indicated. Despite supportive treatment, one confirmed and one suspected CCHF patient died. The suspected CCHF patient was a nurse who had a history of taking care of similar clinical patients in a county hospital in Trabzon. She died of intraabdominal and pulmonary hemorrhage. The other patient died of massive gastrointestinal bleeding. The remaining 17 patients recovered within 5 to 10 days with clinical supportive measures.

Discussion

CCHF was first described in Crimea in 1944. In 1969, the pathogen that caused the disease was recognized to be the one responsible for febrile illnesses identified in the Congo. Since then, many human cases have been reported from different regions, namely Zaire, Uganda, Saudi Arabia, United Arab Emirates, Pakistan, European Russia, Iran, and South Africa (2–9). Additionally, sporadic cases, as well as large outbreaks, were reported from various regions, such as Kosovo and Kenya (10,12,18). Neither sporadic cases nor outbreaks have been previously reported from Turkey. All of the five patients' serum samples tested were found to be positive for IgM antibodies for CCHFV. Findings from the RT-PCR, antigen detection, and IgG tests were negative. These findings are in accordance with recent infection with CCHFV in these five patients. The negative RT-PCR findings are in accordance with the presence of IgM in all the samples; we usually find that we cannot detect infectious virus or virus RNA once detectable antibody has developed. Nevertheless, on this occasion, we were able to isolate CCHFV from two of the patients. IgM and IgG antibodies are usually not

Table. Signs and symptoms among clinically suspected and confirmed CCHF patients [a]

Signs and symptoms	Confirmed cases n = 5	Suspected cases n = 14	Total (%) n = 19
Malaise	5	14	19 (100)
Fever	4	12	16 (84)
Nausea and vomiting	3	13	16 (84)
Abdominal pain	3	13	16 (84)
Petechiae-ecchymosis	5	6	11 (58)
Myalgia	4	4	8 (42)
Bleeding from various sites	1	7	8 (42)
Diarrhea	3	4	7 (37)
Lymphadenopathy	1	3	4 (21)
Hepatomegaly	1	3	4 (21)

[a]CCHF, Crimean-Congo hemorrhagic fever.

detectable in early phase of illness, and they usually begin to rise during day 7–10 of infection. During the early phase, antigen detection and RT-PCR are usually the tests of choice for a sensitive laboratory diagnosis (19). All the patients were referred to our clinic, and blood samples were drawn ≥1 week after onset of illness.

These CCHF cases are among the first documented in Turkey. Similar cases have been reported in other provinces of eastern Turkey. Tokat, Yozgat, and Sivas seem to be the epicenter of the outbreak (Turkish Society of Clinical Microbiology and Infectious Diseases, unpub. data) (Figure 2). The cases in those areas are the subject of ongoing epidemiologic studies. No deaths were observed among the suspected CCHF patients during 2002; 2 of the 10 patients in the 2003 outbreak died of extensive visceral hemorrhages. One of the patients was a nurse in the emergency clinic of a local hospital with a possible exposure to a suspected CCHF patient. Nosocomial transmission of CCHFV through infected blood or body secretions from patients has been reported many times in the literature (12,20–22). The exact procedures performed by the nurse are not clear. She likely had an exposure to blood or infected body fluids of viremic patients affected by an unknown disease in the region. All the other patients handled livestock. In the eastern Black Sea region, women carry out most of the livestock handling, which may explain why most of the patients were female. Handling CCHF-infected animal materials, such as milk and meat, is a recognized means of infection (19) and the probable means of infection for most of our patients, since none had a reported history of tick bite. Some of our patients also gave a history of removing ticks from livestock, and this behavior has been incriminated in CCHF infections.

The most common clinical signs and symptoms reported in CCHF are fever, myalgia, dizziness, malaise, backache, headache, photophobia, nausea, vomiting, diarrhea, abdominal pain, petechiae, ecchymosis, and visceral bleeding. Most of these signs and symptoms were also observed in our patients. We observed elevated CK levels in 14 (75%) of 19 patients, including all of the confirmed CCHF patients. Elevated CK values can be explained with myositis, but the pathologic findings do not demonstrate myositis in the literature, and we did not have muscle biopsies from our patients. Rhabdomyolysis could be another explanation for elevated CK values, but urine samples

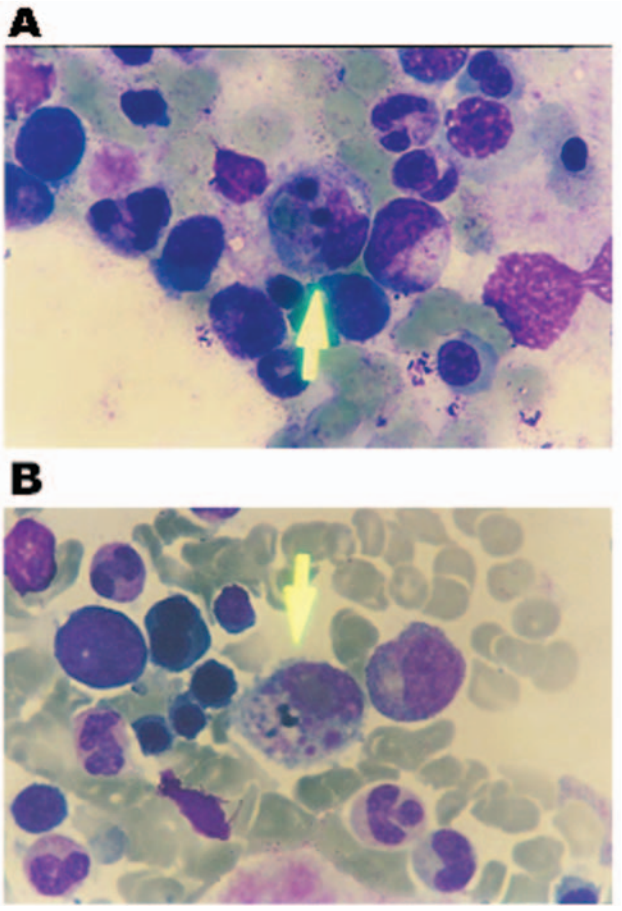

Figure 3. Bone marrow aspiration smear, stained with Wright, showing hemophagocytosis. A) phagocytosis of an erythrocyte and nuclear remnants by a macrophage. B) shows phagocytosis of platelets by a macrophage.

were also not tested for myoglobinuria. Among those patients with high CK levels, two had acute renal failure. Elevated CK values have also been reported in some other clinical series (23).

Hemophagocytosis, which has not been reported previously in CCHFV infections, was also found in our patients. This condition can develop secondary to many viral, bacterial, fungal, parasitic, and collagen vascular diseases (24). We detected reactive hemophagocytosis in 7 (50%) of 14 patients, which suggested that hemophagocytosis can play a role in the cytopenia observed during CCHF infection. Varying degrees of cytopenia are consistently found in CCHF infection (23), but to our knowledge, this is the first study demonstrating hemophagocytosis in CCHF patients. Only two case reports demonstrate hemophagocytosis with Hantaan and Puumala viruses (genus *Hantavirus*) among all the hemorrhagic fever viruses (25,26). Excessive activation of monocytes attributable to stimulation by high levels of Th1 cytokines, such as interferon-γ, tumor necrosis factor-α, interleukin (IL)-1 or IL-6, are proposed as possible immunopathologic mechanism of hemophagocytic lymphohistiocytosis (24). Cytokine studies are lacking in CCHFV infection and are needed for a better understanding of pathogenesis of the disease caused by CCHFV.

Prolongation of PT and PTT was thought to be caused by liver damage. However, in one of our patients, disseminated intravascular coagulation was clearly demonstrated. That patient was the nurse who died with pulmonary and intraabdominal bleeding. Contributing disseminated intravascular coagulation may be associated with a poor prognosis in CCHF infection. Although disseminated intravascular coagulation has been reported previously in some CCHF cases, the exact mechanism for hemorrhage remains unknown (23,27). Of the viral hemorrhagic fevers, CCHF infection has the most florid hemorrhage and highest frequency of large ecchymoses. Besides elevated PT, aPTT, and thrombocytopenia, damage to vascular endothelium directly by the virus can lead to bleeding tendencies (27,28).

Overall laboratory findings in our patients were consistent with the findings in other CCHF case series. Liver transaminase levels were high in our patients, and AST values were generally higher than ALT values, probably attributable to concomitant muscle damage. Beside the hepatic vascular involvement and resulting infarctions in liver parenchyma, direct hepatocellular involvement may also be responsible for elevated serum aminotransferases (23,27).

Any of the following clinical pathologic values during the first 5 days of illness were found to be ≥90% predictive of fatal outcome in a series of South African CCHF patients: leukocyte counts ≤10 x 10^9/L, platelet counts ≤20 x 10^9/L, AST ≥200 U/L, ALT ≥150 U/L, aPTT ≥60 s, and fibrinogen ≤110 mg/L (23). Although most of our patients have at least one or more of the risk factors described above, the overall death rate was low at 11%. Although very high death rates are reported in some series, low death rates in our patients can be explained with better supportive care of the patients. Regional strain differences in CCHFV may also play a role in the differential death rates.

Phylogenetic analysis of virus sequence differences indicates that at least two different genetic lineages of CCHFV are circulating within this current Turkish outbreak. These closely resemble virus lineages found in Kosovo and southwestern Russia and are clearly distinct from those associated with the recent CCHF outbreak in Iran in 2002 (9). The data are most consistent with CCHF's being enzootic in the affected areas in Turkey, rather than having been introduced from Iran by infected tick or livestock movement. The virus might also have come from Russia by birds migrating with their ticks across the Black Sea. Turkey is known to be on the flight path of some birds migrating from Russia to Africa during the winter. However, a number of recognized tick vectors and reservoirs have been known to occur in the region for many years (29), and serologic data from several decades in the past support the previous existence of the virus as well (13).

Our patients are among the first with documented cases of CCHFV infection in Turkey. Recognition of dozens of cases in many provinces of Eastern Turkey during the last 2 years led to the awareness of a previously unrecognized illness in the region. In addition, we documented, for the first time, the occurrence of reactive hemophagocytic syndrome in CCHFV infection, which may be responsible for some of the clinical manifestations. Tick bite, occupational exposure to the virus from infected animals, and nosocomial exposure to patients appear to have been the major transmission routes in this outbreak.

Dr. Karti is a hematologist with Karadeniz Technical University, School of Medicine. His research interests include nonmalignant hematology and chronic myeloid leukemia.

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Predicting Antigenic Variants of Influenza A/H3N2 Viruses

Min-Shi Lee* and Jack Si-En Chen*

Current inactivated influenza vaccines provide protection when vaccine antigens and circulating viruses share a high degree of similarity in hemagglutinin protein. Five antigenic sites in the hemagglutinin protein have been proposed, and 131 amino acid positions have been identified in the five antigenic sites. In addition, 20, 18, and 32 amino acid positions in the hemagglutinin protein have been identified as mouse monoclonal antibody–binding sites, positively selected codons, and substantially diverse codons, respectively. We investigated these amino acid positions for predicting antigenic variants of influenza A/H3N2 viruses in ferrets. Results indicate that the model based on the number of amino acid changes in the five antigenic sites is best for predicting antigenic variants (agreement = 83%). The methods described in this study could be applied to predict vaccine-induced cross-reactive antibody responses in humans, which may further improve the selection of vaccine strains.

Influenza viruses cause substantial medical and social problems throughout the world, and vaccination is the primary method for preventing influenza and its complications. Of the three types of influenza viruses (A, B, and C), only influenza A and B viruses cause epidemic human disease. Hemagglutinin (HA) and neuraminidase proteins are the two surface antigens that induce protective antibody responses and are the basis for subtyping influenza A viruses. Influenza B viruses are not categorized into subtypes (1). Since 1977, influenza A/H1N1, A/H3N2, and B viruses have been in global circulation, and these three viruses are currently included as vaccine components. Current inactivated vaccines provide essential protection when the vaccine antigens and the circulating viruses share high degree of similarity in the HA protein. Since new influenza virus antigenic variants emerge frequently from accumulation of point mutations in the HA protein (i.e., antigenic drift), influenza vaccine antigens need to be updated frequently, based on the results of global influenza surveillance (1), which includes clinical, virologic, and immunologic surveillance. In virologic surveillance, influenza viruses are characterized antigenically on the basis of ferret serum antibody cross-reactivity. Antigenic variants selected serologically are then tested for antibody cross-reactivity in human sera to evaluate the potential cross-protection against the antigenic variants provided by the current vaccines and to select vaccine strains for the next season (2,3).

The HA protein of influenza viruses is synthesized as a single polypeptide (HA0) that is subsequently cleaved into two polypeptides (HA1 and HA2) and forms into homotrimers. The HA1 polypeptide mutates more frequently than the HA2 polypeptide and plays a major role in natural selection (4,5). Three-dimensional (3-D) structure of the HA protein of A/Aichi/2/68 (H3N2) has been determined, and five antigenic sites on the HA1 polypeptide have been proposed conceptually (4–6). Of the 329 amino acid positions on HA1, 131 lie on or near the five antigenic sites (7,8). Twenty amino acid positions on HA1 have been mapped, based on laboratory variants selected in the presence of mouse monoclonal antibodies (9,10). In addition, 18 amino acid positions have been identified as being under positive selection by comparing 357 viruses isolated from 1984 to 1996 (7). In a recent study, 32 amino acid positions have been identified as diverse codons by comparing 525 viruses isolated from 1968 to 2000 (11). However, the importance of these amino acid positions in terms of predicting antibody cross-reactivity is unclear. Therefore, we conducted this study to explore the usefulness of these amino acid positions for predicting antigenic variants of influenza A/H3N2 viruses. The methods described in this study could be used to predict vaccine-induced cross-reactive antibody responses in humans, which may further improve the selection of vaccine strains.

Methods

Cross-Reactive Antibody Data

In the current global influenza surveillance system, influenza viruses are characterized antigenically based on ferret serum hemagglutinin-inhibition (HAI) antibody cross-reactivity. We first screened publications for

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influenza H3N2 virus cross-reactive antibody data. Then, we searched the H3N2 viruses with cross-reactive antibody data for their amino acid sequences of the HA1 polypeptide (www.flu.lanl.gov) (8). Table 1 shows the full name, abbreviation, identification (ID) by type, and accession code of the H3N2 viruses (12–16). Six sets of ferret serum HAI cross-reactivity data were available for analysis. The first set included 11 viruses (55 pairwise comparisons, virus ID: A to K) isolated from 1971 to 1979 (12). The second set included 8 viruses (28 pairwise comparisons, virus ID: J, L to R) isolated from 1979 to 1987 (17). The third set included 10 viruses (45 pairwise comparisons, virus ID: S to AB) isolated from 1989 to 1994 (13). The fourth set included 8 viruses (28 pairwise comparisons, virus ID: AC to AJ) isolated from 1994 to 1996 (18). The fifth set included 5 viruses (10 pairwise comparisons, virus ID: AE, AK to AN) isolated from 1995 to 1999 (15). The sixth set included 6 viruses (15 pairwise comparisons, virus ID: AN to AT) isolated from 1999 to 2002 (16). A mathematical method had been proposed to calculate "antigenic relatedness" between two viruses (presented as a percentage) as a geometric mean of two ratios between the heterologous and homologous antibody titers (19,20).

Since our study investigates the relationship between antigenic difference and amino acid changes in the HA1 polypeptide, the mathematical method was modified to calculate "antigenic distance" (i.e., reciprocal of antigenic relatedness). For example, if homologous titers of two viruses are 640 and 640 and two heterologous titers against each other are 320 and 320, the antigenic relatedness between these two viruses is $([320 \times 320]/[640 \times 640])^{1/2} = 50\%$, and the antigenic distance between these two viruses is $([640 \times 640]/[320 \times 320])^{1/2} = 2$. Table 2 shows the antigenic distances of the 55 pairwise comparisons among the 11 viruses in the first set. In total, 181 pairwise comparisons among 45 viruses were available for analysis. Among the 181 pairwise comparisons, 56 (31%) have an antigenic distance <4 (i.e., similar antigenicity), and 125 (69%) have an antigenic distance ≥4 (i.e., antigenic variant) (21).

Table 1. Full name, identification (ID), abbreviation, and accession code of influenza H3N2 viruses

Full name	ID	Abbreviation	Accession no.
A/Hong Kong/107/71	A	HK71	ISDNHK71
A/England/42/72	B	ENG72	ISDNENG72
A/Port Chalmers/1/73	C	PC73	ISDNPC73
A/Mayo Clinic/1/75	D	MC75	ISDNMC75
A/Victoria/3/75	E	VIC75	ISDNVIC75
A/Tokyo/1/75	F	TOK75	ISDNTOK75
A/England/864/75	G	ENG75	ISDNENG75
A/Allegheny County /29/76	H	AC76	Direct entry (12)
A/Victoria/112/76	I	VIC76	Direct entry (12)
A/Bangkok/1/79	J	BAN179	ISDNBK179
A/Bangkok/2/79	K	BAN279	ISDNBK279
A/Philippines/2/ 82	L	PHI82	ISDNPH282
A/Mississippi/1/85	M	MIS85	AF008893
A/Leningrad/360/86	N	LEN86	AF008903
A/Shanghai/11/87	O	SHA87	AF008886
A/Sichuan/2/87	P	SIC87	AF008884
A/Sydney/1/87	Q	SYD87	AF008882
A/Victoria/7/87	R	VIC87	AF008888
A/Beijing/353/89	S	BEI89	Z46391
A/Hong Kong/34/90	T	HK90	Z46409
A/Beijing/32/92	U	BEI92	Direct entry (13)
A/Hong Kong/23/92	V	HK92	Direct entry (13)
A/Guangdong/25/93	W	GUA93	Z46406
A/Madrid/252/93	X	MAD93	Z46411
A/Scotland/142/93	Y	SCO142	Z46413
A/Scotland/160/93	Z	SCO160	Z46414
A/Shangdong/9/93	AA	SHA93	Z46417
A/Hong Kong/1/94	AB	HK94	Z46407
A/Johannesburg/33/94	AC	JOH94	AF008774
A/Alaska/10/95	AD	ALA95	AF008748
A/Nanchang/933/95	AE	NCH95	AF008725
A/Wuhan/359/95	AF	WHN95	AF008722
A/Auckland/5/96	AG	AUC96	AF008714
A/Fujian/47/96	AH	FUJ96	AF008726
A/New York/37/96	AI	NY96	AF180650
A/South Africa/1147/96	AJ	SA96	Direct entry (14)
A/Sydney/5/97	AK	SYD97	ISDNASYD97
A/Ireland/10586/99	AL	IRE99	Direct entry (15)
A/Moscow/10/99	AM	MOS99	ISDN13277
A/Panama/2007/99	AN	PAN99	ISDNCDA001
A/Fujian/140/2000	AO	FUJ00	Direct entry (16)
A/Chile/6416/2001	AP	CHI01	Direct entry (16)
A/New York/55/2001	AQ	NY01	Direct entry (16)
A/Fujian/411/2002	AR	FUJ02	ISDN38157
A/Hong Kong/ 1550/2002	AT	HK02	Direct entry (16)

Sequence Alignment

Amino acid sequences of the HA1 polypeptide were downloaded from the Los Alamos Influenza Sequence Database (8) or entered from the original publications if they were not available from the Los Alamos Influenza Sequence Database. Amino acid sequences of the 45 viruses were harmonized to same length (329 residues) and were numbered according to A/Aichi/2/68 HA1 sequence because the 3-D structure of the A/Aichi/2/68 hemagglutinin protein has been determined (4–6). Pairwise alignments among the 45 sequences were conducted by using S-Plus 2000 (Insightful Corporation, Seattle, WA). Pairwise-aligned amino acid sequence data were transformed into 0 (without change) and 1 (with change) and were further linked with the pairwise antigenic distance data for predicting analyses.

Predicting Antigenic Variants

The first model was based on amino acid differences in the whole HA1 polypeptide (329 residues). The second

Table 2. Antigenic distance (upper right) and number of amino acid changes in the HA1 (lower left) in 55 pairwise comparisons among 11 influenza H3N2 viruses

	H3N2 virus										
Virus ID[a]	A	B	C	D	E	F	G	H	I	J	K
A. HK/71		27.7	19.6	39.2	55.4	39.2	48.0	39.2	110.9	67.9	110.9
B. ENG/72	15		4.0	26.1	16.0	64.0	156.8	4.0	64.0	78.4	181.0
C. PC/73	16	7		8.0	16.0	32.0	27.7	22.6	37.0	55.4	90.5
D. MC/75	21	12	12		9.2	32.0	45.3	32.0	90.5	55.4	90.5
E. VIC/75	30	19	19	15		11.3	27.7	1.9	5.7	78.4	128.0
F. TOK/75	20	17	18	16	20		78.4	45.3	26.1	39.2	90.5
G. ENG/75	27	18	17	8	17	22		32.0	4.6	6.9	19.6
H. AC/76	31	20	18	16	6	21	19		9.2	78.4	73.9
I. VIC/76	32	21	19	17	2	22	19	4		27.7	32.0
J. BAN/1/79	36	25	23	21	24	33	17	26	26		9.2
K. BAN/2/79	36	24	24	22	26	33	20	28	28	3	

[a]ID, identification.

model was based on amino acid differences in the five antigenic sites (131 residues) (online Appendix available at www.cdc.gov/ncidod/eid/vol10no8/04-0107.htm#app) (7,8). The third model was based on the 20 positions related to mouse monoclonal antibody binding (online Appendix) (9,10). The fourth model was based on the 18 positions under positive selection (online Appendix) (7). The fifth model was based on the 32 codons of substantial diversity (online Appendix) (11). For evaluating the qualitative performance of the five prediction models, an antigenic variant was defined as antigenic distance ≥4 (21). Positive predictive value (PPV), negative predictive value (NPV), and agreement of the five prediction models were calculated, and different cutoff levels of amino acid differences were compared by using the receiver-operating characteristic analysis (22).

Results

Model One

Figure A shows the scatterplot between antigenic distance and number of amino acid changes in the HA1 peptide (328 residues). Among the 181 pairwise comparisons, the antigenic distance ranged from 1 to 181, and the number of amino acid changes in the HA1 peptide ranged from 1 to 36. Overall, the antigenic distance correlated to the number of amino acid changes in the HA1 polypeptide ($R = 0.74$, $p < 0.001$). Different cutoffs of amino acid changes in the HA1 polypeptide were evaluated for predicting antigenic variants. The highest agreement was found with a cutoff of ≥7 amino acid changes, which shows that the NPV, PPV, and agreement were 66% (31/47), 81% (109/134), and 77% (140/181), respectively (Figure A).

Table 3 shows some unique pairwise comparisons with unusual patterns between antigenic distances and amino acid changes. A/Shanghai/11/87 and A/Victoria/7/87 were antigenically different (antigenic distance = 5.7), but they had only one amino acid difference (R247S). The position 247 is located at the antigenic site D. In addition to the amino acid change at position 247, A/Shanghai/11/87 had two more amino acid differences from A/Sichuan/2/87 (E156K, S186V) and A/Sydney/1/87 (A138S, N193K), but these three viruses were antigenically similar (antigenic distance <4). A/Victoria/7/87 had only two amino acid differences from A/Sichuan/2/87 (K156E, V186S) and A/Sydney/1/87 (S138A, K193N), but A/Victoria/7/87 was antigenically different from these two viruses (Table 3). The positions 156, 186, and 193 are located at the antigenic site B and the position 138 is located at the antigenic site A. Moreover, the positions 156 and 193 are also located at the mouse monoclonal antibody-binding sites (online Appendix).

The unusual patterns between antigenic distances and amino acid differences may be due to interaction between amino acid changes in the hemagglutinin or laboratory variability, which needs further experiments to clarify. In addition, A/Victoria/3/75 and A/Victoria/112/76 had only two amino acid differences (L3F, R229G), but they were antigenically different (antigenic distance = 5.7) (Table 3), which also requires further experiments to clarify. The position 3 is not located at any antigenic site, and the position 229 is located at the antigenic site D. We found that 3 of 80 pairwise comparisons with ≥12 amino acid changes had antigenic distance <4 (Figure A).

A/Sydney/5/97 and A/Panama/2007/99 had 12 amino acid differences, but these two viruses were antigenically similar (antigenic distance = 1.4) based on ferret serum HAI titers (Table 3). However, inactivated vaccines containing A/Sydney/5/97 induced low serum antibody titers against A/Panama/2007/99 in humans; therefore, A/Sydney/5/97 was replaced by A/Panama/2007/99 as the vaccine strain for the 2000–01 season (3). A/HK/1550/2002 had 12 amino acid differences from A/Chile/6416/01 and 14 amino acid differences from A/Fujian/140/2000, but A/HK/1550/2002 was antigenically similar to these two viruses (Table 3). These three com-

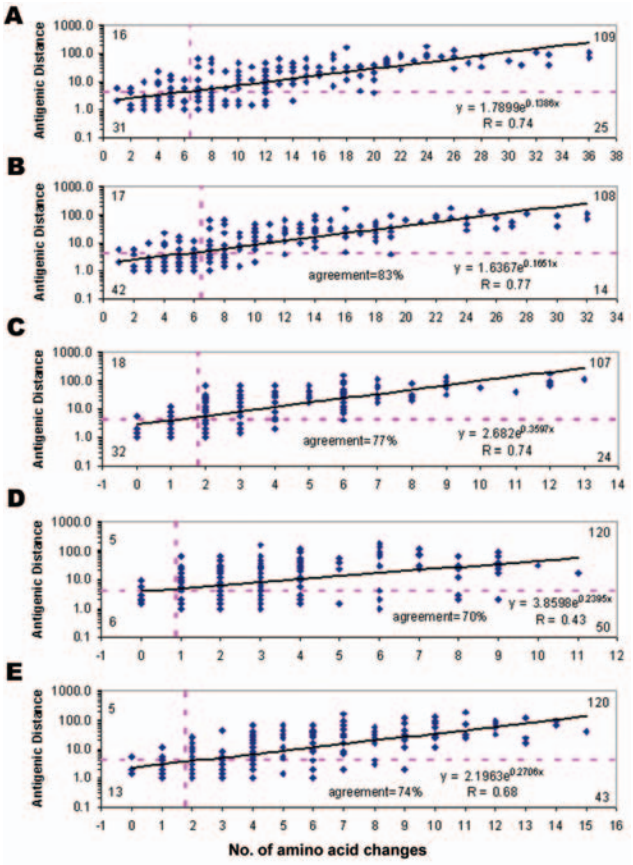

Figure. Performance of the five prediction models. Solid line at each plot, regression; horizontal dashed line, cutoff of antigenic distance >4; vertical dashed line, cutoff of number of amino acid changes. Numbers at the four corners indicate true negative (lower left), false negative (upper left), true positive (upper right), false positive (lower right) in each prediction model. A) The first model was based on amino acid differences in the whole HA1 polypeptide (329 residues). B) The second model was based on amino acid differences in the five antigenic sites (131 residues). C) The third model was based on the 20 positions related to mouse monoclonal antibody binding. D) The fourth model was based on the 18 positions under positive selection. E) The fifth model was based on the 32 codons with substantial diversity.

parisons may indicate that interaction of multiple amino acid changes could potentially preserve the 3-D structure of HA1. Alternatively, the ferret serum HAI assay system is not sensitive enough to detect the antigenic difference.

Model Two

Figure B shows the scatterplot between antigenic distance and number of amino acid changes in the five antigenic sites (131 amino acid positions). Among the 181 pairwise comparisons, amino acid changes in the five antigenic sites ranged from 1 to 32. Overall, the antigenic distance correlated to number of amino acid changes in the five antigenic sites ($R = 0.77$, $p < 0.001$). Different cutoffs of amino acid changes in the five antigenic sites were evaluated for predicting antigenic variants. The highest agreement was found by using a cutoff of ≥7 amino acid changes, which shows that the NPV was 71% (42/59), PPV was 89% (108/122), and agreement was 83% (150/181) (Figure B).

Model Three

Figure C shows the scatter plot between antigenic distance and number of amino acid changes in the 20 amino acid positions related to mouse monoclonal antibody binding. Overall, the antigenic distance correlated to number of amino acid changes in the 20 amino acid positions ($R = 0.74$, $p < 0.001$). Different cutoffs of amino acid changes in the previously defined 20 amino acid positions were evaluated for predicting antigenic variants. The highest agreement was found by using a cutoff of ≥2 amino acid changes, which shows that the NPV was 64% (32/50), PPV was 82% (107/131), and agreement was 77% (139/181) (Figure C).

Model Four

Figure D shows the scatterplot between antigenic distance and number of amino acid changes in the 18 amino acid positions under positive selection. Overall, the antigenic distance correlated moderately to number of amino acid changes in the 18 amino acid positions ($R = 0.43$, $p < 0.001$). Different cutoffs of amino acid changes in the 18 amino acid positions were evaluated for predicting antigenic variants. The highest agreement was found by using a cutoff of ≥1 amino acid changes, which shows that the NPV was 55% (6/11), PPV was 71% (120/170), and agreement was 70% (126/181) (Figure D).

Model Five

Figure E shows the scatter plot between antigenic distance and number of amino acid changes in the 32 codons with substantial diversity. Overall, the antigenic distance correlated moderately to number of amino acid changes in the 32 codons ($R = 0.68$, $p < 0.001$). Different cutoffs of amino acid changes in the 32 codons were evaluated for predicting antigenic variants. The highest agreement was found by using a cutoff of ≥2 amino acid changes, which shows that the NPV was 72% (13/18), PPV was 74% (120/163), and agreement was 74% (133/181) (Figure E). Overall, the model based on the number of amino acid changes in the five antigenic sites has the highest correlation to the antigenic distance ($R = 0.77$) and the best performance for predicting antigenic variants (agreement = 83%).

Discussion

Wilson and Cox proposed that a drift variant of epidemiologic importance usually contains ≥4 amino acid

Table 3. Some unique pairwise comparisons showing antigenic distance and amino acid changes

Viruses compared	Antigenic distance (ferret HAI titers) [a]	Amino acid changes (antigenic sites)
A/Shanghai/11/87 vs. A/Victoria/7/87	5.7 ([320 x 320]/[40 x 80]) ½	R247S(D)
A/Shanghai/11/87 vs. A/Sichuan/2/87	2.8 ([320 x 640]/[160 x 160]) ½	E156K(B), S186V(B), R247S(D)
A/Shanghai/11/87 vs. A/Sydney/1/87	2.0 ([320 x 320]/[160x 160]) ½	A138S(A), N193K(B), R247S(D)
A/Sichuan/2/87 vs. A/Victoria/7/87	5.7 ([320 x 640]/[4 0 x 160]) ½	K156E(B),V186S(B)
A/Sydney/1/87 vs. A/Victoria/7/87	4.0 ([320 x 320]/[80 x 80]) ½	S138A(A), K193N(B)
A/Victoria/3/75 vs. A/Victoria/112/76	5.7 ([640 x 2,560]/[640 x 80]) ½	L3F, R229G(D)
A/Sydney/5/97 vs. A/Panama/2007/99	1.4 ([5,120x 2,560]/ [2,560 x 2,560] ½	I3L, P21S, R57Q(E), Y137S(A), S142R(A), I144N(A), D172E(D), H183L, T192I(B), I194L(B), I226V(D), H233Y
A/Fujian/140/2000 vs. A/HK/1550/2002	2.0 ([640 x 640]/[320 x 320]) ½	G14C, A43V, R50G(C), E83K(E), N96S(D), S186V(B), V194I(B), P199S, V202I, W222R, G225D, I226V(D), C247S(D), S273P(C)
A/Chile/6416/01 vs. A/HK/1550/02	2.0 ([320 x 640]/[80 x 640]) ½	R50G(C), E83K(E), N96S(D), V106A, D144N(A), G186V(B), L194I(B), V202I, H221P, W222R, G225D, K246N(D)

[a]Hemagglutinin -inhibition (HAI) titers were shown as two homologous titers divided by two heterologous titers.

changes located on ≥2 of the five antigenic sites, but they did not specify the amino acid positions in the five antigenic sites (5). Our study further showed that the model based on the number of amino acid changes in the 131 amino acid positions in the five antigenic sites had the highest correlation to the antigenic distance and the best performance for predicting antigenic variants. Theoretically, not all 131 amino acid positions in the five antigenic sites play a critical role in determining antigenicity, and some immunodominant positions (i.e., major antibody-binding sites) could be identified by using bioinformatics models and reverse genetic techniques (23–25). A model based on the immunodominant positions can potentially have a better performance than the model based on the five antigenic sites.

The model based on the 20 amino acid positions related to mouse monoclonal antibody binding only have moderate performance for predicting antigenic variants (R = 0.74, agreement = 77%), which indicates that mouse and ferret antibodies may recognize different B-cell epitopes. In addition, that models four and five have a low performance for predicting antigenic variants is not surprising, since these two models identified the amino acid positions only on the basis of virus sequence data without incorporating antigenic properties.

Antigenic variants of influenza viruses are currently determined with the ferret serum HAI assay. The ferret serum HAI assay works well to distinguish major drift variants, but moderate differences are difficult to define reliably (26). As shown in Table 3, some unusual patterns between antigenic distance and amino acid changes in the HA1 may be caused by laboratory variability of the ferret serum HAI assay. The prediction models proposed in the present study may perform better if a more reliable assay system is used. Several studies have shown that neutralization assays are more sensitive for detecting influenza virus antibody responses than HAI assays (27,28). However, traditional neutralization assays based on cytopathic effect are labor-intensive and not suitable for a large-scale surveillance system. A simplified EIA-based neutralization assay may be the potential solution (29).

Several studies have documented that one to three amino acid changes in the HA1 of influenza H1N1 and H3N2 viruses could possibly reduce the antigenicity and efficacy of inactivated vaccines in animal models (30–33), which are consistent with our results (Table 3). In animal studies, single mutation at amino acid position 156 of the HA1 of two H3N2 viruses was linked to the reduced antigenicity (32,33). The position 156 is located at the antigenic site B and the mouse monoclonal antibody-binding site (see online Appendix). Overall, this evidence may indicate the existence of immunodominant positions in the HA1 and emphasize the importance of identifying the immunodominant positions to monitor the selection of vaccine strains and the process of vaccine manufacturing.

The current global surveillance system largely relies on ferret serum HAI data for selection of influenza vaccine strains (2,3). In some cases, human and ferret cross-reactive antibody data were not consistent (34,35). The methods described in this study could be applied to predict vaccine-induced cross-reactive antibody responses in humans, which may further improve the selection of vaccine strains (35).

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Mycobacterium ulcerans Disease (Buruli Ulcer) in Rural Hospital, Southern Benin, 1997–2001

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Data from 1,700 patients living in southern Benin were collected at the Centre Sanitaire et Nutritionnel Gbemoten, Zagnanado, Benin, from 1997 through 2001. In the Zou region in 1999, Buruli ulcer (BU) had a higher detection rate (21.5/100,000) than leprosy (13.4/100,000) and tuberculosis (20.0/100,000). More than 13% of the patients had osteomyelitis. Delay in seeking treatment declined from 4 months in 1989 to 1 month in 2001, and median hospitalization time decreased from 9 months in 1989 to 1 month in 2001. This reduction is attributed, in part, to implementing an international cooperation program, creating a national BU program, and making advances in patient care.

Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is the third most common mycobacterial disease in humans after tuberculosis and leprosy (1). Endemic foci exist in tropical Africa, the Americas, Australia, and Asia (1–3). In 1997, the World Health Organization recognized BU as an emerging public health problem. Prevalences have increased during the last few years, especially in West Africa (4–7).

M. ulcerans is an environmental mycobacterium associated with wetlands, especially slow-flowing or stagnant water (8–10). Infection is often related to specific trauma (11). Aquatic insects may play a role in transmitting BU to humans (12,13). Naturally acquired *M. ulcerans* infection in wild animals (14) suggests that the etiologic agent is an environmental organism. Most authorities divide BU lesions in the skin into three clinical categories: nonulcerative forms (papules, nodules, indurated plaques, or edema), ulcerative forms, and the healing or scarring form (1,6). Bone lesions also exist (15).

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Even though large numbers of patients have been reported, the epidemiology of BU remains obscure, even in disease-endemic countries. In 1997, a first report was published on 867 BU patients from the Republic of Benin (West Africa) for 1989–1996 (4). Our study covers the ensuing 5 years (1997 to 2001), during which a collaborative project was initiated to improve detection and control of BU. This study describes BU in Benin and presents demographic trends and epidemiologic data from the four southern regions of Benin (Zou, Oueme, Mono, and Atlantique), as seen in a rural hospital in the Zou Region.

Patients and Methods

Our observations are based on 1,700 consecutive patients diagnosed with BU and admitted from 1997 to 2001 to the Centre Sanitaire et Nutritionnel Gbemoten (CSNG), at Zagnanado in the Zou Region. Age, sex, origin, date of disease onset as reported by the patient, date of diagnosis, duration of hospitalization, clinical characteristics, and evolution of the disease were recorded. Clinical criteria for suspecting BU included: presence of a chronically developing lesion (several weeks or months), i.e., a "wound that will not heal"; no fever or regional lymphadenopathy; typical nodular, indurated plaque or edematous lesion; one or more painless chronic ulcers with undermined edges or a depressed scar; swelling over a painful joint, which suggested bone involvement; and patient age <15 years; patient living or traveling in a disease-endemic zone.

Seventy patients were excluded from the study: 13 were confirmed to have another disease (5 cases of cutaneous tuberculosis, 4 *M. chelonae* abscesses, 2 cases of mucormycosis, 1 case of cutaneous diphtheria, and 1 osteosarcoma), and 57 had recurrent BU (they constitute a particular group of patients with long hospitalization times or many recurrences), which left 1,630 patients for analysis. We define a recurrent case as occurring in a patient

with a previous history of BU who has another lesion at the same or different site of the body within 1 year of completing treatment (16). We define mixed forms as the simultaneous presence of different forms in the same patient at one or multiple body sites.

Specimens of tissue and exudates from 906 patients were analyzed by one or more of the following examinations to confirm the clinical diagnosis: direct smear examination for acid-fast bacilli (AFB), culture, IS*2404* polymerase chain reaction (PCR), and histopathologic examination (17). The remaining 724 cases were diagnosed clinically; all were typical of BU and did not present reasonable differential diagnostic problems.

Demographic data of the general population were taken from the most recent national census (1992) (18). Additional statistical information came from the "Benin Demographic and Health Survey" (19). For the 5-year period 1997–2001, demographic data were derived from 1992 statistics that assumed an annual 3.2% growth rate, corrected by projections of 1996 of the National Institute of Statistics and Economic Analysis of Benin.

Data were analyzed with EpiInfo (Centers for Disease Control and Prevention, Atlanta, GA) and SPSS v. 9.0 (SPSS, Chicago, IL) for Windows. Contingency tables were analyzed by the Pearson chi-square test, and nonparametric tests of Mann-Whitney and Kruskal-Wallis were applied to compare medians of asymmetric distributions. These medians are presented with the first quartile (q1) and the third quartile (q3). Cases were excluded from each analysis when information was missing for a specific variable.

Figure 1. Map of Benin with the four Buruli ulcer–endemic regions: the region of Zou (Z), the region of Atlantique (A), the region of Mono (M), and the region of Oueme (O).

Results

Geographic Origin of Patients and Changes in Buruli Ulcer Admissions

CSNG ordinarily receives patients from the regions of Zou, Oueme, Mono, and Atlantique (Figure 1). Most of the patients whose data were analyzed came from the region of Zou, where CSNG is located, followed by the regions of Oueme, Atlantique, and Mono. Twelve patients were from neighboring countries (Nigeria, Togo, Côte d'Ivoire, and Ghana), and the origin of 24 was not recorded (Table 1). BU has not been reported from the two northern regions of Benin (Atacora and Borgou).

Figure 2 includes additional data going back to 1992 (4) that show an increased number of patients in all regions of southern Benin from 1992 to 1997, with a decrease in certain regions from 1998 to 2001. A gradual increase is seen in the number of patients from the Oueme and Atlantique regions admitted to CSNG from 1992 through 2001. However, the number of patients from the Zou and Mono regions increased from 1992 to 1998 and then decreased in each region from 1999 to 2001.

Table 1. Origin of Buruli ulcer cases, 1997–2001[a]

Region	1997	1998	1999	2000	2001	Total (%)
Zou	224	229	221	196	195	1,065 (66.3)
Oueme	35	45	90	46	64	280 (17.4)
Atlantique	11	26	35	25	55	152 (9.5)
Mono	17	42	26	6	6	97 (6.0)
Other	3	2	2	3	2	12 (0.8)
Total	290	344	374	276	322	1,606

[a]Of cases in which region of origin was known. A total of 24 cases in the 5-year period were unknown.

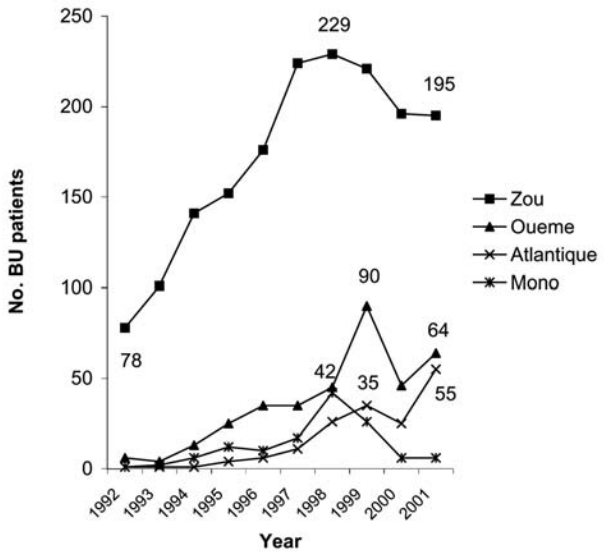

Figure 2. Number of Buruli ulcer (BU) patients by region, 1992–2001.

Table 2 shows the number of BU patients for some districts of Oueme, Atlantique and Zou regions. For the Zou region, detection rates are also presented. In this region, the number of BU patients coming from Abomey district remained relatively constant from 1997 to 2001. The number of BU patients from Zogbodomey and Agbangnizoun increased. During the same period, the number of patients from Zagnanado and Ouinhi decreased. In the Oueme region, patients from Bonou district increased, and patients from the Adja-Ouere district decreased. In the Atlantique region, the number of patients coming from the Ze district increased notably from 1997 to 2001.

Data from 1992 are represented in Figure 3 for five districts in the Zou region and in Figure 4 for two districts in the Oueme region and one district in the Atlantique region. In the five districts in Zou, the number of patients coming to CSNG was higher in 1997 than in 2000, except for Zogbodomey, where the number increased. From 1992 to 1997, the number of patients coming from Zagnanado and Ouinhi districts increased. After 1997, these numbers decreased to the 1992 level. For the three other districts, the number of BU patients progressively increased from 1992 to 2001. Numbers of BU patients were highest in 1999 in the two districts of Oueme. The number of patients coming from Ze in the Atlantique region doubled between 2000 and 2001. Data before 1992 are not reported because they concern only 71 patients (4).

Clinical Form of Buruli Ulcer

Different forms of the disease are presented in Table 3. Over the observation period, the percentage of ulcers decreased from 41.0% to 18.3% while mixed forms increased from 12.4% to 24.8%. The percentages of nodules decreased from 1997 to 2001. A total of 7.6% of the patients had osteomyelitis with no active cutaneous form. The percentage of patients with osteomyelitis reached 13.2% when all patients were included. The clinical form of the lesions was not reported for 19 patients.

If bone and mixed forms are divided into ulcerated and nonulcerated forms, the percentages of ulcerated and nonulcerated forms remained relatively constant for the entire study period. No statistical difference was found between the percentage of ulcerated and nonulcerated forms from 1997 to 2001 (data not shown).

Delay in seeking medical attention was related to clinical form of the disease (Figure 5) (all patients during entire study period). Median was 30 (q1 = 23, q3 = 58) to 46 days (q1 = 15, q3 = 101) for nonulcerated forms (nodule, edema, and plaque) and 61 days (q1 = 30, q3 = 122) for ulcerated forms. Median delay for bone lesions was 91 days (q1 = 30, q3 = 213).

Comparison of the duration of hospitalization with the clinical form is shown in Figure 6. Except for patients with a nodule, who spent 20.5 days (q1 = 11, q3 = 32) at the hospital, median times of hospitalization for all patients with each form of disease during the study period was from 23.0 (q1 = 21, q3 = 52) to 49.5 days (q1 = 18.5, q3 = 90).

Median patient delay in seeking medical care (for all clinical forms) over the study period was 46 days (Table

Table 2. Changes in number of Buruli ulcer patients in some districts of the Zou, Oueme, and Atlantique regions, 1997–2001[a]

Region	District	1997, N (DR)	1998, N (DR)	1999, N (DR)	2000, N (DR)	2001, N (DR)
Zou	Abomey	11 (13.9)	12 (14.7)	16 (19.0)	10 (11.5)	13 (14.6)
	Agbangnizoun	15 (26.2)	11 (18.6)	22 (36.2)	16 (25.5)	22 (34.1)
	Ouinhi	73 (204.3)	65 (176.6)	53 (139.8)	38 (97.3)	32 (79.6)
	Zagananado	76 (185.9)	68 (161.5)	37 (85.3)	41 (91.8)	52 (113.0)
	Zogbodomey	18 (25.8)	23 (32.0)	32 (43.2)	32 (42.0)	25 (31.8)
Oueme	Adja-Ouere	7	14	15	6	4
	Bonou	23	23	58	29	32
Atlantique	Ze	3	14	22	21	47

[a]DR, detection rate per 100,000 population.

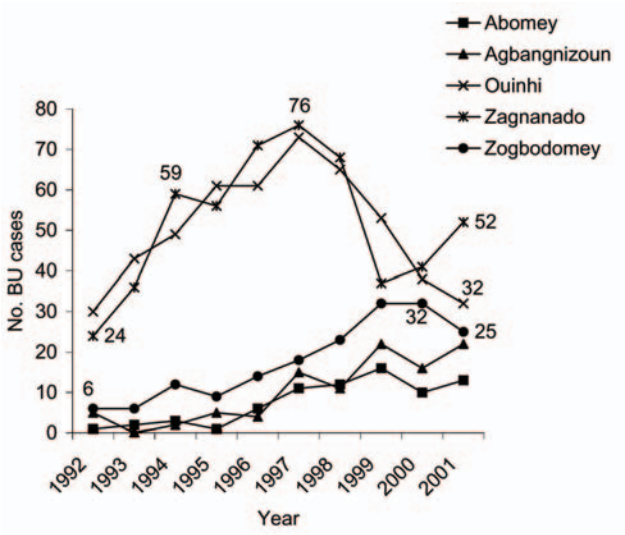

Figure 3. Number of Buruli ulcer patients from five districts of the Zou region who were admitted to the Centre Sanitaire et Nutritionnel Gbemoten (Benin), 1992–2001.

4). In 1997, median delay was 57 days, while in 2001 delay was reduced to 30 days. Overall median delay at CSNG from 1989 to 2001 declined from approximately 4 months to 1 month (Figure 7). Median hospital stay at CSNG from 1989 to 2001 declined from approximately 9 months to 1 month (Figure 8).

In 2000, the method of referral of BU cases to CSNG was recorded. Patients previously treated at CSNG recommended treatment at CSNG for 68.3% of the patients; 22.1% were referred by a family member acquainted with CSNG, and 5.9% were referred by village outreach activities of CSNG. Only 3.7% of the patients were referred by a government health center or a health professional.

Discussion

In 1997, Aguiar et al. (4) reported characteristics of 867 BU patients in southern Benin for 1992 to 1996. Our study supplements their data with an analysis of BU patients seen at the same medical center over the succeeding 5 years.

As was shown in 1992 to 1996, data collected from 1997 to 2001 indicate that CSNG receives patients mainly from the Zou region, where the center is located. Patients choose CSNG for a variety of reasons, including accessibility, financial concerns, and cultural compatibility. However, two new developments somewhat altered the data for the two periods. The first development was that in 1998, a new treatment center for BU was established at Lalo in the Mono region. This development moderately decreased the number of patients coming to CSNG from this region. Approximately 400 BU patients were treated at Lalo from 1998 through 2001. The second development was that from 1999 through 2001, more patients from the Atlantique and Oueme regions came to CSNG because of active public health programs that raised awareness of BU and the availability of treatment at the facility. During this period, these regions had no treatment centers.

Active case finding performed in the Zou region in 2000 did not result in an increase in the number of BU patients coming from this region. CSNG is well known and highly respected by the population, but some patients refuse to go to it, usually for cultural reasons. Aujoulat et al. (20) published a report on the psychosocial aspects of health-seeking behaviors of patients with BU in southern Benin. Their study indicates that some patients are reluctant to seek treatment at any health center. In addition, our own experiences confirm that some BU patients actively avoid detection and would never be included in official reports. These patients, therefore, would not be identified by active or passive detection methods. We conclude that rates for the Zou region are a valid estimate of the incidence of the disease, even if the rates are slightly underestimated. A comparison of detection rates of BU in the Zou region with those of leprosy and tuberculosis in 1999 shows a higher rate of BU (21.5/100,000) than of leprosy (13.4/100,000) and tuberculosis (20.0/100,000) (19). regional differences in the prevalence of BU exist, and the disease is believed to be severely underreported.

A few BU-endemic countries have reported national data on prevalence and incidence. For Uganda in 1972, Barker (8) reported incidence >500/100,000 in some regions. In Ghana, Amofah et al. (5) estimated a prevalence of 22% in some villages of the Amansie District and a national prevalence of 20.7/100,000 (21). Marston et al. (7) found a local prevalence of 16.3% in the Daloa region of Côte d'Ivoire. Seasonal variations in the frequency of BU have been reported in several countries (9,10). Environmental alterations may cause changes in BU frequency (9). Moreover, search for environmental *M. ulcerans* DNA detected variations in positivity rates of specimens over time, and these changes are reflected in

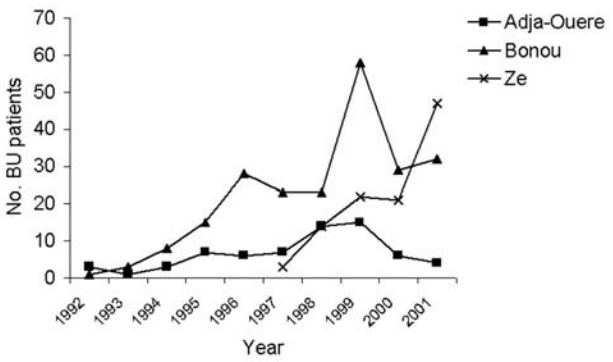

Figure 4. Buruli ulcer (BU) in two districts of the Oueme region and one district of Atlantique region, 1992–2001.

Table 3. Clinical signs and symptoms of Buruli ulcer by year

Clinical form	1997, n (%)	1998, n (%)	1999, n (%)	2000, n (%)	2001, n (%)	Total, n (%)
Nodule	35 (12.1)	35 (10.1)	13 (3.5)	20 (7.2)	19 (5.9)	122 (7.6)
Edema	1 (0.3)	6 (1.7)	1 (0.3)	3 (1.1)	1 (0.3)	12 (0.7)
Plaque	66 (22.8)	98 (28.2)	123 (32.9)	72 (25.9)	111 (34.4)	470 (29.1)
Ulcer	119 (41.0)	99 (28.4)	109 (29.1)	72 (25.9)	59 (18.3)	458 (28.4)
Bone	29 (10.0)	23 (6.6)	31 (8.3)	16 (5.8)	23 (7.1)	122 (7.6)
Mixed	36 (12.4)	69 (19.8)	78 (20.9)	79 (28.4)	80 (24.8)	342 (21.2)
Healed ulcer	4 (1.4)	14 (4.0)	14 (3.7)	15 (5.4)	29 (9.0)	76 (4.7)
Other	0	4 (1.1)	4 (1.1)	1 (0.4)	0	9 (0.6)
Total bone [a]	40 (13.8)	49 (14.1)	55 (14.7)	35 (12.6)	34 (10.5)	213 (13.2)
Total	290	348	373	278	322	1611

[a]Combined osteomyelitis with no associated active cutaneous form and mixed forms with bone lesions.

corresponding alterations of frequency of BU patients in the same foci (Portaels et al., unpub. data). The focal nature of BU prevalence is important in determining the overall disease rate.

Discrepancies between some published reports and our data are partly explained by factors that influenced frequencies in different BU-endemic regions in Benin. In the Zou region between 1992 and 1997, treatment facilities at CSNG developed markedly. This effort became even more efficient after 1997, as a result of aid from the Directorate-General for Development Cooperation (DGDC, Belgium), beginning in 1998. In the Mono region, the Médecins Sans Frontières–Luxembourg established a BU treatment facility in 1998 and conducted rural public health training and publicity programs. In the Oueme and Atlantique regions, Raoul Follereau France and Luxembourg Foundations conducted population surveys in 1999 for the future development of treatment centers in these regions.

The drop in frequencies after these peak years, perhaps related to reduced rural public health education activities, fell to pre-1997 levels, when detection was totally passive. This effect could be explained by cyclic environmental changes, such as excessively dry or wet periods that differ from region to region. Data for Ouinhi and Zagnanado in the Zou region have been collected since 1992 (Figure 3). From 1992 through 1996, frequencies in these two districts increased rapidly, probably because more BU patients had become aware of the effective therapy offered by CSNG. Detection of BU reached its highest level in 1997.

Reductions in new cases from Ouinhi and Zagnanado, beginning in 1998, may be attributable to any number of factors. After the intensive publicity on BU was discontinued, inhabitants may have begun to lose interest. After the intensive campaign, traditional practitioners' interest in the disease may have increased, and they may have promoted their treatment methods in their respective villages. Because of fear of surgery and lack of local access to practitioners, patients may initially prefer traditional therapists, who do not perform surgery. Transportation costs are minimized by frequenting local practitioners (22). The active program may have reduced the reservoir of untreated patients. Possibly, disease-endemic sites may have become less contaminated with *M. ulcerans.* Environmental studies show that some disease-endemic sites in the district of Ouinhi became less frequently positive for *M. ulcerans* DNA (Portaels et al., unpub. data).

As shown in Figure 3, BU frequencies remain nearly constant for three districts (Abomey, Agbangnizoun, and Zogbodomey). Frequency in the Ze district (Figure 4) increased in 2001, which may be attributed to active public health publicity campaigns. Abomey and Agbangnizoun districts are in the Kouffo River basin (Figure 1) rather than the Zou and Oueme basins, and Zogbodomey is more closely related to the Kouffo than to the Zou basin. This finding suggests that changes in the

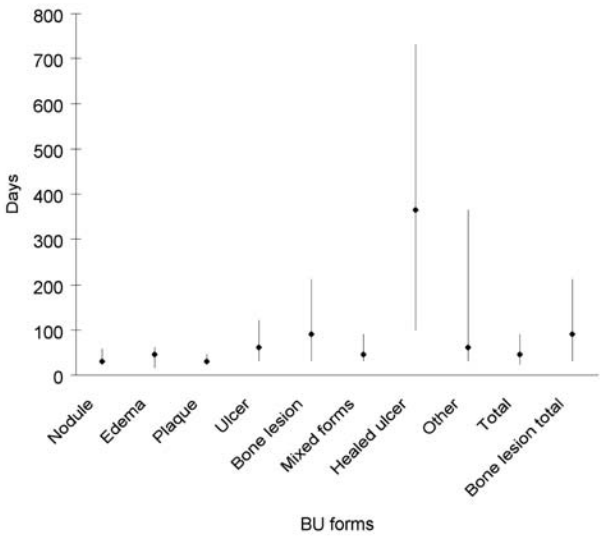

Figure 5. Median patient delay and interquartile range by Buruli ulcer (BU) clinical form.

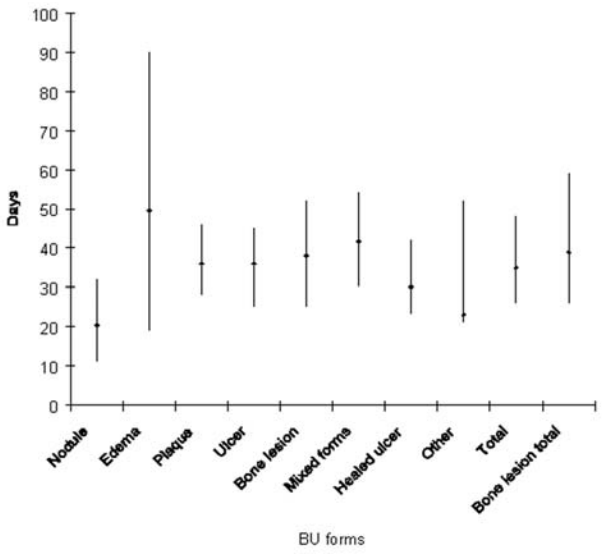

Figure 6. Median duration of hospitalization and interquartile range by Buruli ulcer (BU) clinical form.

BU rates in the Abomey, Agbangnizoun, and Zogbodomey districts remained stable because of common hydrologic relationships. Differences in number of cases coming from the districts of Ouinhi/Zagnanado and Agbangnizoun/Abomey/Zogbodomey could be related to uninvestigated environmental differences in the two different basins.

Other scientists (23,24) reported osteomyelitis in BU patients; in this study, bone involvement was frequent (13.2%). As shown in Figure 5, the form of disease is related to the period of delay in seeking medical attention. Nonulcerated forms have a median delay of 1 to 1.5 months, ulcers 2 months, and patients with osteomyelitis 3 months. This finding has several possible explanations. The nonulcerated form is the first stage of the disease in the nodular, edematous, or plaque form. After a variable period of time (a few weeks to several months), these forms ulcerate. Also, disseminated bone lesions take approximately 3 months to develop. Because open skin lesions may not be visible at the site of the bone lesion, the disease may go undetected or disregarded for long periods. Lesions may also arise by reactivation of subclinical latent foci (15).

In 1997, Aguiar et al. (4) described 867 BU cases, of which 94% were ulcerated. Improved knowledge on clinical classification of BU has led to recognizing a higher percentage of nonulcerated and mixed forms and fewer patients with ulcers. Nodules are less common in Benin. The present study shows that the percentage of ulcerated and nonulcerated forms of the disease was approximately 50% from 1997 to 2001 and that the fluctuation in the percentage of ulcerated and nonulcerated stages was insignificant. We attribute the difference in the percentage of ulcerated forms before 1997 to delayed admission to the hospital in 1989 to 1996. However, in spite of the reduced delay in admission after 1997, we have not observed an increased number of nodules. The reason for the reduced rate of nodular disease in Benin remains obscure.

Median patient delay in admission to hospital decreased from 1997 through 2001. From 1998 through 1999, the difference was not significant, but it became significant between 1999 and 2000 (Table 4). Introduction of the DGDC's "Ulcère de Buruli au Bénin" Program in 1998 was an important factor in the marked reductions in patient delay. Moreover, in 2000, promotional sessions on BU were organized by DGDC and the National BU Program PNLUB (Programme National de Lutte contre l'UB) in the Zou, Oueme, and Atlantique regions. After these efforts, patients reported earlier to the center than in 1999.

Median duration of hospitalization decreased from 1997 through 2001. These changes could be attributed to reduced patient delay (earlier care-seeking by patients with less severe lesions, especially those with ulcers and bone involvement) or improved patient care at the health center. Except for patients with nodules, patients with all other forms of the disease are usually hospitalized for 1 to 2 months (Figure 6). Under field conditions in BU-endemic countries, we believe this period of hospitalization is unlikely to be further reduced significantly for advanced BU disease with the current therapies.

Referral of patients to CSNG for treatment depended largely on word-of-mouth suggestions by former BU

Table 4. Changes in patient delay and duration of hospitalization in Buruli ulcer patients, 1997–2001

Year	Median patient delay (q1–q3)	p	Median duration of hospitalization (q1–q3)	p
1997	57 (30–91)	NS[a]	39 (31–53)	NS[a]
1998	61 (30–91)	NS[a]	39 (28–54)	< 0.001[a]
1999	46 (23–122)	0.001[a]	35 (26–43)	0.009[a]
2000	30 (23–61)	NS[a]	33 (23–42)	NS[a]
2001	30 (23–61)		32 (24–44)	
Total	46 (23–91)	< 0.001[b]	35 (26–48)	< 0.001[b]

[a]Mann-Whitney nonparametric test. Significance level = 0.0125 (Bonferroni correction for pair comparison = 0.05/4). NS = nonsignificant.
[b]Kruskal-Wallis nonparametric test. Significance level = 0.05.

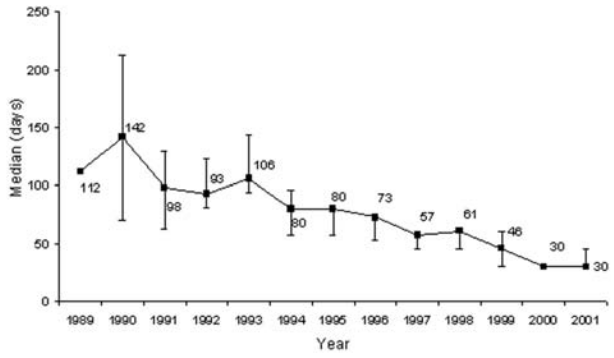

Figure 7. Median patient delay, Centre Sanitaire et Nutritionnel Gbemoten (Benin), 1989–2001.

patients. Patients now tend to bypass traditional therapists and go to CSNG. Accepting surgical treatment remains a deterrent to seeking institutional therapy (20). In these cases, former patients often provide the incentive to seek appropriate therapy. In 2000, a high percentage (68.3%) of patients was referred to CSNG by a previously treated patient.

While some health workers suggest that clinical features are sufficient to diagnose BU, in our experience, bacteriologic and histopathologic evaluations remain important for disease confirmation. This fact is especially true for all research projects on BU. Numerous conditions may present differential diagnostic problems, including parasitic infections, mycotic diseases, neoplastic conditions, tropical phagedenic and stasis ulcers, and cutaneous tuberculosis. In our study a disease other than BU was confirmed in 13 (1.4%) of 906 patients by bacteriologic or histopathologic analysis.

In conclusion, data from a rural hospital at Zagnanado show that BU is highly endemic in southern Benin. Our study highlights the importance of a team approach for optimal management of *M. ulcerans* disease, both at the village and treatment center levels. Such strategies should include efforts in early diagnosis and effective therapy that

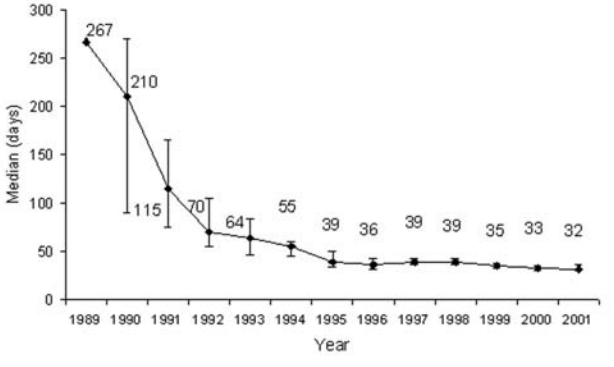

Figure 8. Median duration of hospitalization for Buruli ulcer, Centre Sanitaire et Nutritionnel Gbemoten (Benin), 1989–2001.

are compatible with the socioeconomic structure (25). These goals were largely achieved in Benin because of the implementation of an International Cooperation Program and the creation of a national BU Program.

We believe that a multidisciplinary approach that involves educating the population, training healthcare workers, adequately managing cases, and simplifying surgical procedures reduced hospitalization time and stimulated patient initiative. All these approaches improve patient outcome and lower the socioeconomic effect of the disease on rural populations.

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SARS in Three Categories of Hospital Workers, Hong Kong

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We analyzed attack rates for severe acute respiratory syndrome (SARS) in three categories of hospital workers (nurses, nonmedical support staff, and other technical or medical staff) in all public hospitals in Hong Kong that had admitted SARS patients. Of 16 such hospitals, 14 had cases. The overall attack rate was 1.20%. Nonmedical support staff had the highest attack rate (2.73%). The odds ratios of group nonmedical support staff versus those of nurses and of nonmedical support staff versus other technical or medical staff were 2.30 ($p < 0.001$) and 9.78 ($p < 0.001$), respectively. The number of affected staff and attack rates were significantly correlated with the number of SARS patients admitted ($r = 0.914$ and 0.686, respectively). Affected patients were concentrated in three hospitals and in the earlier phase of the epidemic. Cleaning and clerical staff on hospital wards were at a much higher risk.

The global epidemic of severe acute respiratory syndrome (SARS) occurred in Hong Kong, mainland China, and other countries from March to June in 2003. The cases in Hong Kong and mainland China accounted for 84.1% of all cases worldwide (1,755 and 5,327, respectively); the number of deaths accounted for 70.9% of all SARS-related deaths worldwide (298 and 348, respectively) (1). The first major outbreak in Hong Kong occurred in the Prince of Wales Hospital around March 10, 2003. It resulted in 138 SARS patients, 69% of whom were hospital workers (2). In Hong Kong, 360 hospital workers contracted SARS, a figure that represented 20.5% of all case-patients on the island (3). A study reported that 40 hospital workers in a community hospital in Hong Kong were affected during a 6-week period (March 25–May 5, 2003); the attack rates were 6.1, 10.2, 8.8, 2.0, 0.0, and 0.0 per 1,000, respectively, in these 6 weeks (4).

In Canada, the first large SARS outbreak also occurred in a community hospital, affecting 128 patients (36.7% of all hospital staff). The attack rates among nurses ranged from 10.3% to 60.0%, depending on which department they were serving (5). In mainland China, nosocomial infections played an important role in the SARS outbreak, especially in the first phase of the epidemic (6–8). Nosocomial infection was the most important cause of the SARS outbreak in the Haidian district, Beijing (7). Hospital workers were therefore at high risk of contracting SARS. Improved hospital infection control likely contributed substantially to the control of the SARS epidemic in Hong Kong (9).

A case-control study of 72 hospital workers with SARS and 144 matched controls found that inconsistent use of goggles, gowns, gloves, and caps (unadjusted odds ratio [OR] = 2.42–20.54, $p < 0.05$), as well as perceived inadequate training and perceived inadequate supply of protective equipment were significantly associated with higher risk for nosocomial infection (10). Another study in China showed that good ventilation, isolation of SARS patients, and use of personal protection equipment were key means of preventing healthcare workers from becoming infected (11).

Because a substantial number of hospital workers contracted SARS in Hong Kong and in other places, documenting the attack rate in different hospitals in Hong Kong was warranted. Such information would reflect the degree of exposure to relevant occupational hazards among different types of hospital workers in Hong Kong.

This study gives an account of the attack rates of workers in all public hospitals in Hong Kong that had admitted SARS patients. The attack rates of three categories of hospital workers, as well as relevant attack rates in the earlier and later phases of the epidemic, were compared. The attack rates were also correlated with the number of SARS patients admitted to the individual hospitals.

Methods

For all hospitals that had admitted SARS patients in Hong Kong, the numbers of probable SARS patients and of hospital staff in three job categories were obtained. These three categories included nurses (group N), nonmedical support staff (group S; healthcare assistants, ward assistants [cleaning staff], general service assistant

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[clerical staff]), and other medical or technical staff (group O; physicians, allied health workers, technicians, pharmacists, dieticians, radiologists, radiographers, and medical students, and the like). All were full-time staff. The Hospital Authority and individual hospitals kept lists of infected workers who were hospital staff members. These lists were provided to the authors, with data already grouped into the three categories and the two time periods; no further breakdown of the data was available. Most data were obtained from the Hospital Authority; supplementary data were obtained from a few hospitals. The number of these three types of workers who became probable SARS patients, according to the World Health Organization definition (1), was recorded. These figures were further stratified into two groups: patients whose onset of symptoms occurred 1) before April 17, or 2) on or after April 17, which was approximately the mid-point of the epidemic. (The first patient was admitted on March 4, 2003, and the onset of the last case was on May 31, 2003). Attack rates for the three categories of hospital workers were obtained by dividing the relevant number of hospital care workers contracting SARS by the total number of relevant staff members.

Chi-square test and Fisher exact test were used to test the significance of differences in proportions. Spearman correlation analysis was performed to examine the association between the number of SARS patients admitted into a hospital and the number of healthcare workers who contracted SARS in the same hospital. Analysis of variance (ANOVA) and Kruskal-Wallis test were used to compare differences in attack rates among the three types of workers. SPSS for Windows Release 11.0.1 (SPSS Inc., Chicago, IL) was used for the data analysis; $p < 0.05$ was considered to be significant. Differences in attack rates among the 16 hospitals were tested by using Fisher-Freeman-Halton test (StatXact-4 version 4.0.1, Cytel Software Corporation, Cambridge, MA).

Results

Infected Staff and Attack Rates

A total of 1,755 SARS patients were reported in Hong Kong; they were hospitalized in 16 of the 27 hospitals governed by the Hospital Authority. Fourteen of these 16 hospitals had at least one hospital staff member who contracted SARS. In other words, 2 of the 16 hospitals (hospital 2 and hospital 4, which admitted 7 and 17 SARS patients, respectively) had a zero attack rate (Table 1).

The total number of affected hospital workers in these 16 hospitals was 339 (i.e., 94.2% of all 360 affected hospital workers in Hong Kong). The other 21 (5.8%) affected hospital staff worked in six other hospitals that had not admitted SARS patients. The distribution of the 339 cases is analyzed in this article.

Table 1. Number of hospital workers, SARS affected hospital workers, and SARS patients admitted to hospitals and attack rates

Hospital no.[a]	No. of SARS patients admitted to hospitals	No. of hospital staff who contracted SARS				Attack rates (%)			
		Group N	Group S	Group O	Overall[a]	Group N	Group S	Group O	Overall[b]
1	3	0	0	2	2	0.00	0.00	0.73	0.18
2	7	0	0	0	0	0.00	0.00	0.00	0.00
3	17	6	2	0	8	1.19	0.96	0.00	0.79
4	17	0	0	0	0	0.00	0.00	0.00	0.00
5	24	1	3	0	4	0.33	1.29	0.00	0.64
6	29	7	3	1	11	0.95	0.83	0.23	0.71
7	36	2	0	0	2	0.12	0.00	0.00	0.07
8	53	1	2	1	4	0.26	1.80	0.41	0.54
9	82	5	5	1	11	0.42	1.11	0.18	0.50
10	83	8	6	0	14	0.51	1.15	0.00	0.48
11	85	6	6	1	13	0.47	1.41	0.14	0.54
12	114	18	14	4	36	3.58	6.93	1.37	3.61
13	128	15	7	0	22	0.87	1.42	0.00	0.68
14	188	13	14	2	29	1.01	3.36	0.26	1.17
15	326	64	54	2	120	4.66	13.30	0.21	4.38
16	563	35	18	10	63	2.76	3.92	1.53	2.64
Pooled	1,755	181	134	24	339	1.21	2.73	0.29	1.20
p value						< 0.001[c]	< 0.001[c]	< 0.001[d]	< 0.001[c]
Spearman correlation coefficients		0.883***	0.928***	0.525*	0.914*	0.737*	0.865***	0.390	0.686**

[a]All hospital workers, including all three groups (group N, nurses; group S, nonmedical support staff; and group O, other technical and medical staff).
[b]All hospitals that had at least admitted one SARS patient.
[c]p values derived from Pearson Chi-square test and comparing the attack rates among all the 16 hospitals.
[d]p values derived from Fisher-Freeman-Halton test and comparing the attack rates among all the 16 hospitals: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

The number of affected staff in 2 hospitals (hospitals 15 and 16) accounted for 54.0% of the 339 cases in the 16 Health Authority hospitals (Table 1). The number of affected staff in an individual hospital ranged from 0 to 120 (median = 11, interquartile range = 24.8) (Table 1). The overall attack rates for all three types of hospital staff was 1.20% for the 16 hospitals. These rates ranged from 0% to 4.38%, a significant variation ($p < 0.001$, Table 1). The overall mean and median of the 16 hospital attack rates for all workers were 1.06% and 0.59%, respectively (Table 2). The overall attack rates for all workers were >2% in three hospitals (hospitals 12, 15, and 16; Table 1). When these three hospitals were removed from the analysis, the overall attack rate was 0.54% and the mean and median of the 13 hospital attack rates were 0.48% and 0.54%, respectively (data not shown in table).

Attack Rates by Category of Hospital Worker

Attack rates in the three job category groups (group N, S, and O) of hospital workers are listed in Table 1. The ranges of attack rates for the three groups were 0%–4.66% (group N), 0.0%–13.3% (group S), and 0.0%–1.53% (group O). The pooled attack rates for these three groups were 1.21%, 2.73%, and 0.29%, respectively, in the 16 hospitals (Table 1). ORs for comparing the S and N, O and N, and S and O groups were 2.30 ($p < 0.001$), 0.24 ($p < 0.001$), and 9.78 ($p < 0.001$), respectively. The differences in both the mean and median attack rates for the three categories were also significant ($p = 0.035$, ANOVA test, and $p = 0.015$ and $p = 0.015$, Kruskal-Wallis test) (Table 2).

Associations between Numbers of SARS Patients Admitted and Hospital Attack Rates

The number of affected staff was strongly correlated with the number of admitted SARS patients for all the three groups: group N (Spearman $r = 0.883$, $p < 0.001$), group S (Spearman $r = 0.928$, $p < 0.001$), and group O (Spearman $r = 0.525$, $p < 0.05$) (Table 1). Similar significant associations between attack rates and number of admitted SARS patients were observed for groups N and S but not for group O (Spearman r = 0.737, 0.865, and 0.39, respectively) (Table 1).

Comparison of Attack Rates in First Two Phases of Epidemic

The overall attack rates for all hospital workers in the first phase of the epidemic (before April 17, 2003: 0.98%) were higher than those for the second phrase (on or after April 17, 2003: 0.22%) (Table 3). This finding was true for all three groups of workers (group N: 0.99% vs. 0.22%; group S: 2.24% vs. 0.50%; group O: 0.21% vs. 0.07%). When data from individual hospitals were examined, however, the trend was not always consistent.

Discussion

The overall attack rate for all workers in the 16 hospitals was 1.2%. Staff members working in 14 hospitals contracted SARS, although the attack rates varied significantly among hospitals. The attack rate was >2% in three hospitals, which had 219 (60.8%) of the total 360 cases in Hong Kong. Compared to the overall attack rate (1.2%), the attack rate was much lower (0.54%) for the other 13 hospitals; the pooled overall attack rate for the 16 hospitals was also much lower (0.47%) in the second phase of the epidemic. In other words, nosocomial infection of hospital workers in Hong Kong was concentrated in three hospitals and in the earliest phase of the epidemic (the overall attack rates in the earlier phase were 2.18%, 3.37%, and 3.81% for these three hospitals).

Attack rates were also associated with the number of SARS patients admitted into the individual hospitals. Five of the 16 hospitals admitted >100 patients. In terms of number of affected workers and attack rates, these five hospitals were also the top five of the 16 studied hospitals (except for hospital 13, which ranked seventh in terms of attack rates). Theoretically, viral load, inadequate manpower, inadequate equipment, and inadequate time for training were possible explanations for the observed associations. If the SARS epidemic resurges on a sizable scale, some consideration should be given to the number of patients to be admitted to a hospital. Yet, without further data, the exact reasons were not clear.

The attack rates also differed significantly among the three studied occupational groups. Support staff, such as healthcare assistants, cleaners, and clerical staff working on the wards (group S), had much higher attack rates,

Table 2. Mean and median attack rates of the 16 hospitals by job categories

	Mean of attack rates (%)	SD	Median of attack rates (%)	Interquartertile range	Range
Group N	1.07	1.38	0.49	0.99	0.00–4.66
Group S	2.34	3.43	1.22	2.76	0.00–13.30
Group O	0.32	0.49	0.16	0.37	0.00–1.53
Overall[a]	1.06	1.31	0.59	0.82	0.00–4.38
p value	0.035[b]		0.015[c]		

[a]Overall: all the hospital workers, including all three groups (group N, nurses; group S, nonmedical support staff; group O, other technical and medical staff)
[b]p values for testing differences among group N, S, and O (ANOVA).
[c]p values for testing differences among group N, S, and O (Kruskal-Wallis test).

Table 3. Attack rate among hospitals by job categories and time period

	Group N		Group S		Group O		Overall[a]	
Hospital no.	Onset before 7/4 (%)	Onset after 7/4 (%)	Onset before 7/4 (%)	Onset after 7/4 (%)	Onset before 7/4 (%)	Onset after 7/4 (%)	Onset before 7/4 (%)	Onset after 7/4 (%)
1	0.00	0.00	0.00	0.00	0.36	0.36	0.09	0.09
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.40	0.80	0.00	0.96	0.00	0.00	0.20	0.60
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.33	0.43	0.86	0.00	0.00	0.16	0.48
6	0.00	0.95	0.28	0.55	0.23	0.00	0.13	0.58
7	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.07
8	0.26	0.00	0.90	0.91	0.41	0.00	0.40	0.14
9	0.42	0.00	1.11	0.00	0.18	0.00	0.50	0.00
10	0.26	0.26	0.19	0.96	0.00	0.00	0.17	0.31
11	0.47	0.00	1.41	0.00	0.14	0.00	0.54	0.00
12	3.38	0.21	6.44	0.53	1.02	0.34	3.31	0.31
13	0.87	0.00	1.42	0.00	0.00	0.00	0.68	0.00
14	1.01	0.00	2.88	0.49	0.13	0.13	1.05	0.12
15	4.08	0.61	11.82	1.68	0.21	0.00	3.87	0.53
16	2.36	0.40	3.27	0.68	1.07	0.46	2.18	0.47
Pooled (16 hospitals)	0.99	0.22	2.24	0.50	0.21	0.07	0.98	0.22
Ratio of pooled rates (phase 1 vs. 2)	4.50:1		4.48:1		3.00:1		4.45:1	
p values (among 16 hospitals)	< 0.001[b]	< 0.001[c]	< 0.001[c]	0.036[c]	0.002[c]	0.048[c]	< 0.001[b]	< 0.001[c]

[a]Overall: all the hospital workers, including all three groups (N, S, and O).
[b]Pearson Chi-square test.
[c]Fisher-Freeman-Halton test.

when compared to nurses (group N) and other categories of hospital workers, including physicians (group O). The attack rate of nonmedical support staff (group S) was higher than those of the other two groups in 10 of the 16 studied hospitals. Nonmedical support staff accounted for 134 (39.5%) of the 339 SARS patients among hospital workers, despite the fact that only approximately 17.3% of all Hospital Authority staff belonged to this group. Furthermore, 12 of the 16 hospitals had cases in nonmedical support staff (attack rate 0.83%–13.3% [mean 2.34%]). Even when the later phase of the epidemic was considered, the attack rates of nonmedical support staff were still relatively high (Table 3). Three of the six Hospital Authority staff who died of SARS also belonged to this group. In short, nonmedically trained support staff were exposed to a disproportionately high risk for nosocomial transmission of SARS. Apparently, infection control training was also offered to this group. However, the exact amount of training or assessment of how well the training was understood by this group was not documented.

Extra protection is required to protect this staff group in the infection control campaign in Hong Kong. Insufficient emphasis may have been given to address the special needs of this group during the first SARS epidemic in Hong Kong, as well as during the post-SARS period. Infection control training and policies may need to be tailored for different occupational groups.

Nonmedical support staff, in general, were not contacting SARS patients as frequently as nurses did. This finding suggests that the fomite theory and the aerosol theory of transmission could not be dismissed. Another study investigating nosocomial infection in Hong Kong (10) reported that breakthrough transmission was likely responsible for nosocomial infection of hospital workers. Inconsistent use of personal protection equipment, perceived inadequate supply of such equipment, inadequate training, and perceived lack of knowledge about infection control were all statistically significant predictors of such breakthrough transmissions.

Nonmedical support staff may have been more likely to be lacking infection control knowledge, either as a result of receiving inadequate training or being unable to benefit fully from it. Whether they were adequately trained to use their personal protective equipment correctly is not clear. For instance, some preliminary studies mentioned that some workers were wearing or taking off such equipment in the wrong sequence (Lau et al., unpub. data). In Hong Kong, many support staff were likely to be middle-aged persons, who had a relatively low level of education (many of them did not finish secondary

schools). Tailored infection control training and surveillance programs are hence warranted to ensure that this group of workers is sufficiently protected from the occupational hazard of contracting SARS. Practice exercises may also be an effective preventive measure.

These findings do not mean that nurses were not under a high level of occupational hazard. More than 50% of the affected workers were nurses. In six hospitals, nurses' attack rates were close to or higher than 1%; the maximum was 4.66%. The correlations between number of SARS patients admitted and the number of affected workers were much stronger in the nonmedical support and nursing groups, when compared with that of other medical staff). This finding is understandable as most members of these two groups were working on the frontlines.

Most affected staff (94.2%) were working in hospitals that had been taking care of SARS patients. Further transmission through social contacts had not been a major factor of transmission among hospital workers (10). Nosocomial infection was therefore likely to be responsible for most transmission. Hospital workers in Hong Kong were well supported and appreciated by Hong Kong citizens and the mass media for their professionalism in treating SARS patients. Whether such strong media interest in their experiences and commitment influenced hospital workers in their decision to serve in high-risk environments, at times even when protection might not have been adequate, is of interest. Questions such as how conformity and peer pressure affected the decisions of individual workers who felt that they had to work under suboptimal infection control conditions are worth exploring.

The pooled attack rates (for 16 hospitals) were 0.22, 0.50, and 0.07, respectively, for nurses, nonmedical support, and other medical and technical staff when only cases of later onset (on or after April 17, 2003) were considered. When cases with earlier onset (before April 17, 2003) were considered, the rates were higher: 0.99, 2.24, and 0.21, respectively. The respective ratios of the two phases of the epidemic were 4.50:1, 4.48:1, and 3.00:1 for the three types of hospital workers and 4.45:1 for hospital workers overall (Table 3). This may be due to a reduction in the number of patients admitted after April 17 (approximately 18.3% of all cases; the number of patients admitted in the two phases was hence 3.92:1) or to improvement of infection control measures. The overall attack rate ratio (4.45:1, 0.98%/0.22%) was very similar to the overall admission rate for the two phases (4.46:1), although the two ratios were not conceptually equivalent. It, however, gives a clue that the decrease in exposure may have played a relatively important role in the decreased attack rate in the second phase. Improved infection control in the second phase may not be the primary reason for the decrease in the attack rate over time. If proper training, supply of personal protective equipment, infection control procedures, nonexcessive number of patients per hospital, and other measures are ensured, nosocomial infection of hospital workers should be avoidable. On the other hand, hospital workers, especially nonmedical support staff, should be aware that they are facing a certain level of occupational risk.

The study has some limitations. First, only macro-level data were used. Since no individual data were available, factors associated with nosocomial infection could not be studied. Similarly, no clinical data were reported. Some hospital workers may have been infected in the community. However, another case-control study showed that social contact with other infected colleagues was not a significant factor associated with likelihood of infection among hospital workers (10). The chance of nosocomial infection was therefore very high for these staff who contracted SARS. Another study suggested that asymptomatic transmission among hospital workers was not prevalent (5). Second, different types of workers were included in the three studied job categories, and some heterogeneity across these different types of workers with the categories may exist. The available data do not permit further breakdown. The classification of affected workers into the three categories was arbitrary and may also have affected results. Further, no additional data exist to compare the conditions on infection control and other measures taken within the two analyzed phases of the epidemic, making definite interpretation impossible. The study, however, documents one of the most important scenarios of nosocomial infection among hospital workers. The results should help us to learn from this very costly lesson.

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Long-Term Prognosis for Clinical West Nile Virus Infection

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Relatively little is known about the long-term prognosis for patients with clinical West Nile virus (WNV) infection. We conducted a study to describe the recovery of New York City residents infected during the 1999 WNV encephalitis outbreak. Patients were interviewed by telephone on self-perceived health outcomes 6, 12, and 18 months after WNV illness onset. At 12 months, the prevalence of physical, functional, and cognitive symptoms was significantly higher than that at baseline, including muscle weakness, loss of concentration, confusion, and lightheadedness. Only 37% achieved a full recovery by 1 year. Younger age at infection was the only significant predictor of recovery. Efforts aimed at preventing WNV infection should focus on elderly populations who are at increased risk for neurologic manifestations and more likely to experience long-term sequelae of WNV illness. More studies are needed to document the long-term sequelae of this increasingly common infection.

West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus*) has become endemic throughout much of the United States since its introduction in 1999 (1). In 2003, a total of 2,866 laboratory-confirmed human cases of neuroinvasive illness and 264 deaths were caused by WNV infection (2). Older persons are at substantially increased risk for severe WNV disease, a hallmark of which is profound muscle weakness (1), often with acute flaccid paralysis or other motor disorder (2–4).

Investigators of the first WNV disease outbreak in North America in 1999 documented that older persons and persons with diabetes are at increased risk for death after WNV infection (1,5–7) However, few epidemiologic studies have examined the sequelae or time course of recovery from WNV meningitis or encephalitis in survivors. A recent investigation of neurologic manifestations of WNV infections showed persistent symptoms at 8 months after infection, particularly in those patients who experienced flaccid paralysis (8).

We conducted an 18-month follow-up study on a cohort of New York City (NYC) case-patients identified as being ill with WNV infection in 1999 (1). The investigation had the following objectives: 1) to describe the physical, cognitive, and functional outcomes in patients recovering from WNV meningitis or encephalitis over the 18 months after acute illness and 2) to determine whether the severity of the initial clinical syndrome, the patient's age, and the patient's underlying illness affected the likelihood of recovery.

Methods

The medical records of all patients hospitalized with WNV infections were reviewed by using a standard form to abstract chart information. Follow-up interviews were conducted and blood was collected at approximately 6-month intervals from laboratory confirmed case-patients whose WNV infections were diagnosed in 1999. Three distinct health outcome areas—physical, cognitive, and functional health status—were each assessed at 6, 12, and 18 months after illness onset. Baseline health status was assessed by recall at the 12-month interview. Physical and cognitive health status outcomes were assessed at each interview by asking about the frequency of selected symptoms (Table 1). Functional ability was evaluated by administering the Instrumental Activities of Daily Living Scale (IADLS) (9) to assess daily functioning before and after WNV illness. The prevalence of physical, cognitive, and functional symptoms at baseline (by recall at 12 months) and at the 6-, 12-, and 18-month interviews was calculated. Underlying illness and initial clinical syndrome were ascertained from the medical chart.

The initial clinical syndrome was classified as WNV encephalitis, indicated by fever and altered mental status or other cortical signs (e.g., seizures) and cerebrospinal fluid (CSF) suggestive of viral infection; WNV meningitis, indicated by fever, meningeal signs (documentation of

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Table 1. Health outcomes assessed during follow-up telephone interviews of New York residents with clinical West Nile virus infection in 1999[a,b]

Physical health	Cognitive health	Functional health
Difficulty walking [c]	Confusion	Heavy chores
Fatigue	Depression	Laundry
Headache	Irritability	Light housekeeping
Insomnia	Lightheadedness	Managing medications
Joint pain	Loss of concentration	Managing money
Muscle pain	Loss of memory	Meal preparation
Muscle weakness		Shopping
Seizures		Telephoning
Stiff neck		Transportation

[a]At 12 months post -onset, baseline status for each outcome was assessed; for each outcome, patients were asked to report the degree to which they experienced the signs and symptoms at baseline (by recall) and at 12 months postonset.
[b]Each outcome w as scored 0–2 according to the following scale: always = 2, sometimes = 1, never = 0. Functional health was scored according to how frequently the patient had difficulty performing the task. Recovery was calculated as the sum of the baseline score in each category, divided by the sum of the 12-month score.
[c]Difficulty walking was given twice the weight as other outcomes in the recovery score calculation.

Kernig sign, Brudzinski sign, or nuchal rigidity), and CSF suggestive of viral infection; or WNV fever with headache. CSF suggestive of viral infection was defined as a negative bacterial stain and culture, with elevated leukocyte count ($\geq$5 cells/mm^3) or elevated protein (>4.5 g/L). Proxy interviews were conducted when case-patients could not be interviewed because of poor health, hearing difficulties, or a language barrier.

Laboratory Methods

Laboratory evidence for recent WNV infection (10) was confirmed in all patients and defined by any of the following test results: 1) isolation of WNV by culture or amplification of WNV RNA by reverse transcriptase–polymerase chain reaction testing from human tissue specimens; 2) demonstration of immunoglobulin (Ig) M antibody to WNV in CSF by IgM-capture enzyme-linked immunosorbent assay (ELISA); 3) greater than fourfold serial change in WNV-specific neutralizing antibody as measured by the plaque-reduction neutralization test (PRNT) in paired, appropriately timed serum samples; or 4) demonstration of both WNV-specific IgM (by ELISA) and IgG (screened by ELISA and confirmed by PRNT) in a single serum specimen. Patients with WNV-specific IgM in a single serum sample were classified as having a probable recent infection. Patients with anti-WNV IgG only in a single serum specimen were also classified as having a probable WNV infection if the antibodies were found to be WNV-specific by PRNT and the patient had no history of travel to an area outside the United States where WNV infection is endemic.

Blood specimens were obtained at 6-month intervals starting at 6 months through 18 months after illness onset, until WNV-specific IgM, indicative of recent infection, was undetectable. Serum samples were tested for anti-WNV IgM (capture ELISA) and IgG (indirect ELISA) (11,12). Results from the ELISA testing were expressed as a WNV-positive to WNV-negative control (P/N) ratio of observed A450 nm (MAC-ELISA) or A405 nm (IgG ELISA) as described. In these tests, P/N ratios >3.0 were considered positive and P/N ratios >2.0 and <3.0 were considered equivocal. Detailed information on WNV serologic features in this cohort study has been previously published (13).

Study Population

Of the 59 surviving patients hospitalized with WNV infection in 1999, 40 were NYC residents and eligible for inclusion in the follow-up study. During the course of the study, two additional patients with laboratory-confirmed WNV infection who had fever and headache were identified and enrolled. Thirty-eight (90.5%) of the 42 case-patients completed the first interview (6 months postonset), 35 (83.3%) participated in the second interview (12 months postonset), and 36 (85.7%) participated in the third interview (18 months postonset). Forty (95%) case-patients participated in at least one of the three interviews; 32 (76%) completed all three interviews. The proportion of interviews that were completed by proxy was 39% at the first interview to 25% at the third interview.

Recovery Assessment at 12 Months After Infection

A recovery assessment was completed for the 35 case-patients who participated in the 12-month interview. At the 12-month interview, patients responded to questions on the frequency of occurrence (never, sometimes, or often) of selected symptoms during the month before the interview and during the month before illness onset (baseline). A symptom was counted as being present if it was experienced sometimes or often. Current and baseline composite scores were calculated within each health status domain by tabulating the responses for each outcome in that domain. Difficulty walking was weighted double in computing the physical health domain score, because it appears to be a very specific symptom of severe WNV infection (1,3,4). If a response of not applicable or unknown was given for a particular outcome, then that outcome was not included in

calculating that case-patient's baseline or 12-month composite score.

Within each health status domain, the ratio of the 12-month composite score to the baseline composite score was calculated and used as a measure of recovery for that domain. Persons with a 12-month composite score ≥85% of baseline for a given health status domain were considered to be recovered in that domain. Those persons with 12-month composite ≥85% of baseline in all three health status domains were considered fully recovered.

Statistical Methods

Prevalence ratios were calculated for all outcomes at each interval relative to baseline; p values associated with prevalence ratios were calculated by using a matched analysis with McNemar test for correlated proportions. Crude and adjusted relative risks (RRs) were calculated to examine the relationships of clinical syndrome (i.e., encephalitis, meningitis, and mild illness), age, and underlying medical conditions with recovery in each health status domain at 12 months postonset. RRs were adjusted by using the method of Mantel and Haenszel. Data were analyzed by using the SPSS System for Windows, version 10.0 and SAS Version 8 (SAS Institute, Cary, NC).

Consent and Human Subjects Review

Verbal consent was obtained from participants during telephone interviews, and written consent was obtained before each follow-up blood specimen collection. The study protocol underwent human subjects review and was approved by institutional review boards of both the New York City Department of Health and Centers for Disease Control and Prevention.

Results

Table 2 shows the patients who were ill with WNV infection in 1999 (N = 59) and the 40 surviving NYC residents who were eligible for participation in the follow-up study, plus 2 additional patients with West Nile virus disease who were not hospitalized. Of the 40 surviving NYC case-patients participating in one or more interviews, the median age of the participants at illness onset was 68 years (range: 16 to 90 years), and all patients resided in their own homes before illness. At the time of diagnosis, 22 (55%) patients had encephalitis, 11 (27.5%) had meningitis, and 7 (17.5%) had illness characterized by fever and headache. Of 33 hospitalized patients with known disposition at discharge, those who had diagnoses of encephalitis were more likely to have discharge placements outside their homes ($p < 0.05$) and more likely to be >65 years of age ($p < 0.001$).

Physical, Cognitive, and Functional Health Status

Table 3 shows the prevalence of physical, cognitive, and functional sequelae reported at 6, 12, and 18 months postonset. At the 12-month interview, patients were also asked to recall the prevalence of those symptoms before

Table 2. Characteristics of participating and nonparticipating patients who survived clinical West Nile virus infection, New York City, 1999

Characteristic	All hospitalized patients, N = 59 (%)	Enrolled patients, N = 42 (%)	Participants in 12-month interview, N = 35 (%)	Nonparticipants in 12-month interview, N = 7 (%)
Age				
<65	23 (39)	16 (38)	13 (37)	3 (43)
≥65	36 (61)	26 (62)	22 (63)	4 (57)
Sex				
Female	28 (47)	20 (48)	18 (51)	2 (29)
Male	31 (53)	22 (52)	17 (49)	5 (71)
Underlying illness before infection				
Hypertension	25 (42)	17 (40)	14 (40)	3 (43)
Diabetes	12 (20)	6 (14)	5 (14)	1 (14)
Hypertension or diabetes	31 (53)	19 (45)	16 (46)	3 (43)
Clinical syndrome				
Encephalitis	37 (63)	22 (52)	19 (54)	3 (43)
Meningitis or milder illness	22 (37)	20 (48)	16 (46)	4 (57)
Discharge status [a]				
Dead	7 (12)	NA	NA	NA
Home	22 (37)	20 (50) [b]	20 (61) [c]	NA
Home of family or friend	3 (5)	3 (8) [b]	3 (9) [c]	NA
Skilled nursing facility	4 (7)	4 (10) [b]	4 (12) [c]	NA
Rehabilitation	6 (10)	6 (15) [b]	6 (18) [c]	NA
Unknown but alive	17 (29)	7 (18) [b]	0	7 (100)
Required physical therapy	NA	NA	18 (51)	NA

[a] Includes hospitalized patients only.
[b] N = 40 for these calculations.
[c] N = 33 for these calculations.

illness onset. All participants interviewed with a clinical diagnosis of encephalitis with weakness (n = 10) reported difficulty walking 6 months after illness. Those who had an initial diagnosis of encephalitis were more likely to require a wheelchair at the first follow-up interview than those with meningitis or mild illness.

The prevalence of cognitive symptoms was higher 1 year after illness compared with baseline for all cognitive outcomes. All cognitive symptoms were more common after illness onset in case-patients at intervals extending up to 18 months after acute illness (Table 3), and some symptoms did not diminish over time. Prevalence ratios of functional disabilities were also significantly elevated compared with baseline.

Analysis of Recovery Outcomes

The mean domain-specific health status score was significantly lower at 12 months compared with baseline for all three domains (data not shown). Overall, 54%, 59%, and 57% of patients were physically, cognitively, or functionally recovered, respectively (Table 4). Case-patients ≥65 years achieved recovery rates of 50%, 52%, and 45% in the respective domains of physical recovery, cognitive recovery, and functional recovery (Table 5). Only 37% of patients were considered fully recovered. Diagnosis (encephalitis versus meningitis or other mild illness) was not predictive of physical or cognitive recovery (Table 4), even after adjusting for age. Age was a positive predictor of recovery in each domain, with younger persons more likely to achieve physical, cognitive, and functional recovery (Table 5). The absence of an underlying health condition was associated with an increased likelihood of recovery in all domains (Table 6). After adjusting for baseline clinical status (Mantel-Haenszel method), younger persons (<65 years) were significantly more likely to achieve a full recovery than older persons (≥65 years) (relative risk [RR] = 3.3, 95% confidence interval [CI] 1.1–9.9). After adjusting for underlying illness, younger persons were also more likely to recover fully than older persons (RR = 2.3, 95% CI 0.97–5.5).

Discussion

We report that WNV infection can result in a protracted convalescent period with long-term physical, cognitive, and functional impairments lasting ≥18 months after acute illness. Approximately 40% of patients hospitalized in 1999 did not return to their own homes immediately after discharge, and physical therapy was required by 47% of patients after hospitalization. Comparing the prevalence of symptoms before illness with that at 12 months after WNV illness onset, physical, functional, and cognitive symptoms persisted. We estimate that 37% achieved full recovery by 12 months. Younger age (<65 years) was the only significant predictor of achieving a full recovery.

Table 3. Prevalence of signs and symptoms at intervals of follow-up in patients with clinical West Nile virus infection, New York City, 1999

Sign or symptom	Before illness onset [a] (baseline), n/N (%)	Interview 1 (6 months), n/N (%)	Interview 2 (12 months), n/N (%)	Interview 3 (18 months), n/N (%)	p value for 12 months vs. baseline [b]
Physical sequelae					
Difficulty walking	7/35 (20.0)	30/38 (78.9)	17/35 (48.6)	15/36 (41.6)	0.002
Muscle weakness	4/35 (11.5)	25/38 (65.8)	15/34 (44.1)	20/36 (55.5)	< 0.001
Fatigue	12/35 (34.3)	20/37 (54.1)	22/33 (66.7)	23/36 (63.8)	0.002
Insomnia	7/35 (20.0)	17/38 (44.7)	16/34 (47.1)	17/36 (47.2)	0.007
Muscle pain	12/35 (34.3)	14/37 (37.8)	19/34 (55.9)	14/36 (38.8)	0.035
Headache	9/35 (25.7)	13/37 (35.1)	15/34 (44.1)	13/36 (36.1)	0.014
Joint pain	7/35 (20.0)	12/38 (31.6)	11/34 (32.3)	11/36 (30.6)	0.157
Cognitive symptoms					
Memory loss	7/35 (20.0)	21/38 (55.3)	17/34 (50.0)	16/36 (44.5)	0.002
Loss of concentration	3/35 (8.6)	16/37 (42.2)	14/34 (41.2)	12/36 (33.3)	< 0.001
Depressed	5/35 (14.3)	15/38 (39.5)	13/34 (38.2)	16/36 (44.4)	0.005
Irritable	8/35 (22.9)	14/38 (36.8)	14/34 (41.2)	14/36 (38.9)	0.008
Lightheaded	4/35 (11.5)	13/38 (34.2)	17/33 (51.5)	13/35 (37.1)	< 0.001
Confusion	2/35 (5.7)	17/38 (44.8)	9/34 (26.5)	11/36 (30.6)	0.008
Functional sequelae					
Shopping	4/33 (12.1)	17/36 (47.2)	14/33 (42.4)	14/35 (40.0)	0.002
Meal preparation	2/32 (6.3)	22/31 (71.0)	12/32 (37.5)	12/34 (35.3)	< 0.001
Laundry	1/25 (4.0)	14/29 (48.3)	10/25 (40.0)	10/33 (30.3)	0.003
Light housekeeping	1/28 (3.6)	19/35 (54.3)	12/28 (42.9)	12/35 (34.3)	< 0.001
Heavy chores	5/30 (11.9)	19/33 (57.6)	19/30 (63.3)	19/34 (55.9)	0.003
Transportation	3/29 (10.3)	23/37 (62.2)	10/28 (35.7)	14/36 (38.9)	0.008

[a]Assessed by recall at the 12-month follow-up interview.
[b]Based on McNemar's test for agreement in a matched analysis.

Table 4. Recovery at 12 months post-onset by health status domain and clinical syndrome at diagnosis in patients with clinical West Nile virus infection, New York City, 1999

Recovery	Total	Recovered, n (%) [a]	Not recovered, n (%) [a]	Risk ratio	95% confidence interval
Physical recovery					
Meningitis or mild illness	16	8 (50.0)	8 (50.0)	0.86	0.46–1.6
Encephalitis	19	11 (57.9)	8 (42.1)	Referent	
Total	35	19 (54.3)	16 (45.7)		
Cognitive recovery					
Meningitis or mild illness	16	10 (62.5)	6 (37.5)	1.1	0.64–2.0
Encephalitis	18	10 (55.5)	8 (44.4)	Referent	
Total	34	20 (58.8)	14 (41.2)		
Functional recovery					
Meningitis or mild illness	16	10 (62.6)	6 (37.5)	1.2	0.67–2.1
Encephalitis	19	10 (52.6)	9 (47.4)	Referent	
Total	35	20 (57.1)	15 (42.9)		
Total recovery					
Meningitis or mild illness	16	7 (43.8)	9 (56.3)	1.4	0.58–3.3
Encephalitis	19	6 (31.6)	13 (68.4)	Referent	
Total	35	13 (37.1)	22 (62.9)		

[a]Due to rounding, not all values add up to 100%.

WNV is clinically, serologically, and epidemiologically similar to St. Louis encephalitis virus (SLEV) (14–18), and recovery after WNV infection might be comparable to that of patients recovering from SLEV-associated encephalitis (SLE). Information on sequelae from SLE has been documented after U.S. outbreaks occurring from the 1930s to the 1970s. Various methods assessed recovery from SLE, including medical examinations (with neurologic assessments) and patient or proxy interviews (19). Follow-up times varied from 6 months to 5 years after acute illness (15–23). In general, studies of recovering patients with SLE have documented generalized susceptibility to fatigue, headaches, nervousness, inability to concentrate, depression, and problems with gait and balance throughout convalescent periods of 6 months to 3 years after acute SLEV infection; on average, ≈30% of case-patients were not fully recovered 1 year after acute illness (19,20,24).

Different approaches to defining recovery were used by researchers who characterized the experience of patients after SLEV infection. After the first SLE epidemic in St. Louis in 1933, researchers defined overall recovery based on the ability to return to work. Of 331 patients, 141 (66%) reported that they felt completely recovered 12–18 months after acute illness, whereas 22 (6.7%) felt they were physically unable to return to their jobs. Although none of the patients <20 years of age was incapacitated, >10% of patients >20 years could not return to work (20). After an SLE epidemic in Mississippi in 1975, researchers conducted follow-up interviews 6 months after illness onset. Of the 175 patients contacted, 87 (49.7 %) achieved full recovery, 24 (13.7%) reported minor symptoms, and 29

Table 5. Recovery at 12 months post-onset by health status domain and age at illness onset in patients with clinical West Nile virus infection, New York City, 1999

Recovery	Total	Recovered, n (%) [a]	Not recovered, n (%) [a]	Risk ratio	95% confidence interval
Physical recovery					
<65	13	8 (61.5)	5 (38.5)	1.2	0.68–2.2
≥65	22	11 (50.0)	11 (50.0)	Referent	
Total	35	19 (54.3)	16 (45.7)		
Cognitive recovery					
<65	13	9 (69.2)	4 (30.8)	1.3	0.77–2.3
≥65	21	11 (52.4)	10 (47.6)	Referent	
Total	34	20 (58.8)	14 (41.2)		
Functional re covery					
<65	13	10 (76.9)	3 (23.1)	1.7	0.98–2.9
≥65	22	10 (45.5)	12 (54.5)	Referent	
Total	35	20 (57.1)	15 (42.9)		
Total recovery					
<65	13	8 (61.5)	5 (38.5)	2.7	1.1–6.5
≥65	22	5 (22.7)	17 (77.3)	Referent	
Total	35	13 (37.1)	22 (62.9)		

[a]Due to rounding, not all values add up to 100%.

Table 6. Recovery at 12 months postonset by health status domain and underlying health condition in patients with clinical West Nile virus infection, New York City, 1999

Recovery	Total	Recovered, n (%) [a]	Not recovered, n (%) [a]	Risk ratio	95% confidence inte rval
Physical recovery					
No underlying condition	18	11 (61.1)	7 (38.9)	1.3	0.70–2.4
Hypertension or diabetes	17	8 (47.1)	9 (52.9)	Referent	
Total	35	19 (54.3)	16 (45.7)		
Cognitive recovery					
No underlying condition	17	11 (64.7)	6 (35.3)	1.2	0.70–2.2
Hypertension or diabetes	17	9 (52.9)	8 (47.1)	Referent	
Total	34	20 (58.8)	14 (41.2)		
Functional recovery					
No underlying condition	18	12 (66.7)	6 (33.3)	1.4	0.78–2.6
Hypertension or diabetes	17	8 (47.1)	9 (52.9)	Referent	
Total	35	20 (57.1)	15 (42.9)		
Total recovery					
No underlying condition	18	9 (50.0)	9 (50.0)	2.1	0.80–5.6
Hypertension or diabetes	17	4 (23.5)	13 (76.5)	Referent	
Total	35	13 (37.1)	22 (62.9)		

[a]Due to rounding, not all values add up to 100%

(16.6 %) reported that they resumed previous activities but not at the same level. SLE patients from the Tampa Bay, Florida, outbreaks occurring from 1959 to 1962 (N = 160) had more difficulty completing tests that evaluated balance and equilibrium than controls. In particular, SLE patients had difficulty walking in straight lines and widening their lateral base of support (25). Predominant cognitive problems included nervousness, irritability, depression, and forgetfulness (15–23).

Our findings are similar to those reported in these SLE studies. Regardless of acute clinical symptoms, WNVcase-patients in this study continued to report difficulty walking, muscle weakness, fatigue, and insomnia, with >40% reporting a combination of these difficulties, and 30% continued to report persistence of memory loss, confusion, depression, and irritability at 18 months after acute illness. Eighteen months after illness, 30% of case-patients reported needing assistance with activities of daily living, mostly those requiring increased strength. Although average functional ability from 6 months to 1 year post-onset improved significantly, functional ability reached a plateau and did not improve further during the 12- to 18-month period.

Our results suggest that WNV has more severe long-term sequelae in older persons than in younger persons. These sequelae may be attributable to the severity of the patients' WNV infection, to the more general effects of serious illness and hospitalization, or to the aging process itself; regardless, WNV causes severe neurologic illness and might be associated with lasting sequelae in persons ≥65 years.

The presence of underlying disease at the time of onset of illness was not significantly associated with recovery at 12 months (RR = 1.4, 95% CI 0.58–3.3), even after adjusting for age (adjusted RR = 1.3, 95% CI 0.70–2.5). However, the lack of significance of this association could be a result of the small number of patients in our study or misclassification.

Several aspects of our investigation might limit the generalizability of these findings. Although participation was high, our estimates may be imprecise because of the small sample size. Furthermore, the ages of the study participants span a wide range (16–90 years), making adequate adjusting for age difficult. We used a structured interview questionnaire, the content and format of which, when possible, was similar across interviews to maximize comparability of data obtained over time. Proxies were used when case-patients could not be interviewed because of poor health, hearing difficulties, or a language barrier. Data were based on subjective report, either by the patient or their proxy. Subjective accounts provided by persons who are cognitively impaired might overattribute or underattribute certain dysfunctions to their WNV illness, and recall bias might have caused case-patients to selectively suppress or exaggerate information about their health status, either current or past.

Baseline information regarding physical, cognitive, and functional health before WNV disease was collected during the second follow-up interview at 1 year (i.e., by recall). Participants may have had problems recalling baseline health status over a 12-month period, limiting our ability to accurately ascertain actual baseline level of functioning. Sequelae could not be verified by objective physical examination, physician interview, or medical record review. Future studies of recovery in WNV patients should attempt to obtain more objective measurements of sequelae, such as provider interviews, medical chart review, or neurologic examination. As WNV continues to affect older age groups, further research should consider ways to control for declines in functioning associated with the aging process and to obtaining objective data regarding

baseline status. Finally, future studies should try to assess the baseline health status of WNV patients closer to the time of onset to reduce the impact of recall bias on long-term measures of recovery.

Our study documents that, in addition to causing severe acute illness, WNV meningitis ore ncephalitis results in a prolonged recuperation and rehabilitation period, especially in older persons. As WNV continues to establish itself as a national public health concern, these findings reinforce the need for local governments in affected areas to institute widespread public health measures to safeguard against WNV transmission and for persons—especially those age 65 and over —to take precautions to avoid exposure to mosquitoes and reduce mosquito breeding sites on their properties. More studies are needed to document the long-term sequelae of this increasingly common infection.

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Dr. Labowitz Klee worked on this study while she was an epidemiologist at the New York City Department of Health and completing her graduate work in clinical health psychology. She is the clinical director of the Psychosocial Rehabilitation Fellowship Program at the Errera Community Care Center of the Veterans Administration. Her professional interests include psychiatric services, specifically those involving recovery from severe mental illnesses, and housing for chronically homeless populations.

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Serotype III *Streptococcus agalactiae* from Bovine Milk and Human Neonatal Infections[1]

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Streptococcus agalactiae (group B streptococcus [GBS]) causes invasive human infections and bovine mastitis. This study examined the genetic relationship between bovine and human serotype III GBS by using molecular techniques that classify human serotype III GBS into four distinct phylogenetic lineages. Bovine serotype III GBS were largely contained in two lineages, which are distinct from the two major lineages (restriction digest types III-2 and III-3) that infect human neonates. One of the bovine lineages closely resembles the human III-1 lineage, whose members occasionally cause human neonatal infections. The bovine strains in the other lineage characteristically have an initiation factor IF2 gene (*inf*B) H allele and multilocus sequence types that are not found in human GBS strains. Evidence suggests that this "H allele" lineage is related to the human III-3 lineage. These results support the assertion that human and bovine GBS are largely unrelated and provide further insight into the genetic relation between human and bovine GBS.

Streptococcus agalactiae (group B streptococcus [GBS]) is the major etiologic agent of invasive neonatal infections in humans in industrialized countries, causing sepsis, pneumonia, meningitis, osteomyelitis, and soft tissue infections (1). GBS has also been increasingly recognized as an important pathogen in immunocompromised and elderly persons (2,3). GBS emerged as an important cause of neonatal infections in the 1960s; before this time, it was mainly recognized as a cause of bovine mastitis (4). Most data suggest that GBS strains that infect humans are distinct from strains isolated from bovine sources, since bovine strains frequently cannot be typed with antisera to determine capsular polysaccharide serotype, often express protein antigens not found on human isolates, and tend to have different biochemical properties (5–8). The possibility remains, however, that subgroups of GBS infect both humans and cows. If so, these two closely associated hosts could act as reservoirs for each other and sites for the emergence of novel pathogens.

A number of molecular methods, including multilocus enzyme electrophoresis, pulsed-field gel electrophoresis (PFGE) of restriction enzyme digest products of genomic DNA, randomly amplified polymorphic DNA (RAPD) analysis, and multilocus sequence typing (MLST), have been used to demonstrate that the population of GBS that infects humans is highly clonal and limited to a relatively small number of phylogenetic lineages (9–13). Martinez and colleagues reported in 2000 that a large sample of GBS isolated from cows in Quebec Province in Canada could be classified into five major RAPD groups, which indicates that this sample of bovine GBS also comprised a limited number of lineages (7).

The Quebec sample is particularly useful for further investigating the relationship between bovine and human GBS because most typeable bovine isolates in the sample were serotype III. Serotype III GBS strains account for a substantial proportion of early-onset neonatal human GBS infections and almost all late-onset neonatal infections (2). Four distinct phylogenetic lineages of human serotype III GBS have been identified by PFGE of restriction digest patterns and designated restriction digest patterns type III-1, III-2, III-3, and III-4 (11). Human GBS strains can also

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be assigned to each restriction digest pattern type by a distinct set of molecular markers, which include analysis of nucleotide substitutions in the centrally conserved region of the translation *infB*, the presence or absence of the inserted sequences GBSi1 and IS*1548* in three chromosomal loci, and MLST (9,11).

RAPD analysis of human GBS isolates collected with the Quebec bovine isolates suggested that the serotype III bovine strains and human GBS strains were largely unrelated, although a definite conclusion was hindered by the small number of human isolates in the study. The obstacle presented by the small human sample size can now be circumvented by the use of the molecular markers described above that identify human phylogenetic lineages of GBS, but which have not yet been applied to the study of GBS from nonhuman sources. We reexamined the Quebec sample with these molecular markers to better understand the genetic relationship between bovine serotype III GBS and human serotype III GBS.

Methods

Bacterial Isolates

The serotype III GBS were isolated from bovine milk or from vaginal and rectal swab specimens from asymptomatic pregnant women in Quebec Province, Canada, during 1996 and 1997, as previously described (7). RAPD analysis of the 224 bovine GBS isolates had assigned 210 of the isolates to four RAPD groups (I–IV); the remaining 14 isolates were ungrouped (Figure). A total of 70 of the 82 original serotype III GBS strains were recovered for this study. The remaining GBS in this collection were not serotype III or were nontypeable. Bovine isolates were studied from all RAPD groups except RAPD group I, which contained a single bovine serotype III GBS isolate that could not be recovered. Genomic DNA was extracted with the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) from individual colonies grown overnight in broth.

Molecular Analyses

The central portion of *infB* was amplified from bacterial DNA by polymerase chain reaction (PCR) with oligonucleotide primers, as described by Hedegaard et al. (14). Each amplicon was purified and sequenced.

The presence or absence of the inserted sequences GBSi1 and IS*1548* in the respective chromosomal loci was determined by using PCR of bacterial DNA with primer pairs flanking each of three sites, as previously described (11). In human serotype III GBS, the first locus is an internal region of *hylB*, the gene encoding hyaluronidase, which is either interrupted by IS*1548* or is intact. The second locus is the region between *scpB* and *lmb*, the genes encoding streptococcal C5a-ase and a laminin-binding protein, which either contains GBSi1, IS1548, or no insert. The third locus lies between *ftsY* and *sag0728*, two open reading frames (ORFs) with gene products of unknown function, which contains GBSi1 or no insert. The latter locus is either referred to as the AW-10 locus (as we refer to it) or the Y locus, according to Luan et al. (15).

MLST was carried out on 46 selected strains as previously described, and a sequence type was assigned to each strain (9). The sequences of the alleles that make up the novel MLST sequence types described in this manuscript can be found at http://sagalactiae.mlst.net (9). DNA dot blots were performed by using a 96-chamber vacuum manifold and [^{32}P]-dCTP labeled probes as previously described (16).

The complete sequences of the *infB* gene H allele and the novel IS*1563*-like insertion sequence described in this article are available in the GenBank database under accession nos. AY429663 and AY437915, respectively.

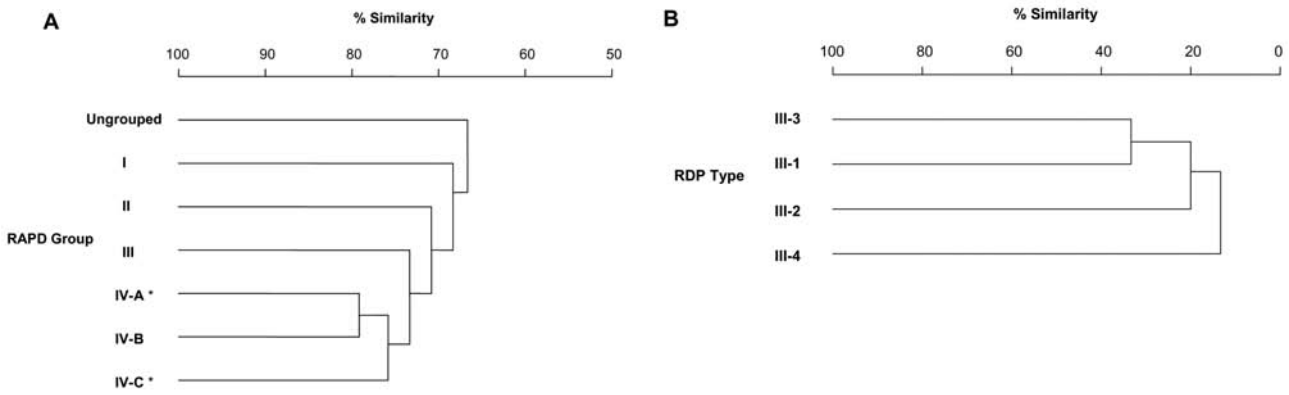

Figure. Simplified dendrograms illustrating the genetic relationship between human and bovine group B streptococcus. A) Dendrogram of Quebec sample derived by randomly amplified polymorphic DNA (RAPD) analysis; adapted from (7). All RAPD groups contain bovine serotype III GBS, but those marked by an asterisk also contain human isolates (see Table 2). B) Dendrogram of human restriction digest pattern types (RDP) of serotype III GBS derived by analysis of RDP of genomic DNA created by digestion with restriction enzyme Sse83871; adapted from (11).

Results

Analysis of *infB* Alleles in Bovine GBS

All human serotype III GBS have either the A or C allele of the gene encoding translation-initiation factor IF2 (*infB* allele, Table 1). Twenty-five of the 62 Quebec bovine isolates carried the A allele, and 2 carried the C allele, which raises the possibility that these 27 bovine GBS could be closely related to human GBS (Table 2). No strains bearing the B allele were identified, and only one strain carried the D allele. Serotype III GBS that bear the D allele are unlikely to be isolated from humans because all human GBS strains bearing the D allele have thus far been serotype Ia. The remaining 34 strains contained a previously unidentified *infB* allele, which we have designated the H allele. The *infB* H allele differs from other previously identified *infB* alleles (designated A–G) by two, three, or five nucleotide substitutions within the central conserved portion of the *infB* gene and most closely resembles the *infB* A allele (11,14). All 34 of the strains bearing the H alleles are bovine in origin (Table 2). The *infB* H allele has not been previously encountered in approximately 700 GBS isolates isolated from human sources studied in our laboratory, including over 150 serotype III strains (unpub. data; [11]). These observations suggest that GBS bearing the *infB* H allele rarely, if ever, colonize or infect humans.

Relationship between infB Allele and RAPD Group

The possibility that the A or C allele bovine strains could be related to human GBS was investigated by examining the distribution of the bovine A or C allele isolates among the groups previously determined by RAPD analysis (Figure) (7). Of the 25 bovine A allele isolates, 19 were found in RAPD group IV-A, and 2 were found in RAPD group IV-C, which suggests that at least some of the bovine A allele isolates could be genetically related to human GBS since human A allele isolates are also found in these two RAPD clusters (Table 2, Figure). The two bovine C allele isolates are in RAPD group IV-C clustered with two human C allele isolates and thus appear closely related to human C allele isolates based on RAPD analysis. As expected, neither the single bovine D allele isolate nor the 34 bovine H allele isolates cluster with human isolates by RAPD analysis (Table 2).

Identification of Bovine Serotype III GBS Related to Type III-1 GBS

The bovine A and C allele isolates were further examined by using techniques that distinguish the four known human serotype III GBS lineages. These techniques include determining the presence or absence of inserted sequences in three previously identified loci and determining the strains' sequence types by MLST (Table 1). Twenty-three of the 25 A allele bovine isolates appear closely related to human restriction digest pattern type III-1 strains because the isolates lack inserted sequences at any of the three loci examined and had a sequence type (ST) identical to those of previously studied III-1 strains (ST-23) or that was different by only one allele (ST-90, ST-92, or ST-94) (Table 3). Thus, a substantial proportion of the serotype III bovine GBS in this sample appear to come from a lineage that is associated with invasive neonatal disease, albeit rarely (9,17,18). Bovine III-1 strains appear to be genetically heterogeneous since they are found in RAPD groups III, IV-A, and IV-C, but no human III-1 strains were found in this sample; thus, the bovine III-1 strains most closely related to human III-1 strains could not be identified in this sample. Nineteen of the III-1 bovine isolates appear to lack the *scpb-lmb* locus because PCR with primers flanking the intergenic locus of *lmb* and *scpB* did not produce an amplicon.

Seven of the A allele strains appear to be restriction digest pattern type III-2 strains on the basis of an analysis of inserted sequences and MLST (Table 3). Only one of these seven isolates, NI-96-2836, is of bovine origin, however, and is found in RAPD group III, whereas the six human A allele isolates are found in RAPD groups IV-A and IV-C. These data suggest that the bovine NI-96-2836 strain is genetically divergent from human III-2 strains, despite sharing the same *infB* allele, inserted sequences, and ST with human III-2 strains. These data also suggest

Table 1. Characteristics of human serotype III GBS lineages [a]

		Inserted sequence sites[b]			
RDP type[c]	*infB* allele[d]	AW-10	*hylB*	*scpb-lmb*	ST[e]
III-1	A	No insert	No insert	No insert	23, 25
III-2	A	No insert	IS*1548*	IS*1548*	19, 21
III-3	C	GBSi1	No insert	GBSi1	17, 29
III-4	A	No insert	No insert	GBSi1	1

[a]GBS, group B streptococcus.
[b]The presence of inserted sequences is determined at the three chromosomal locations (see Methods).
[c]RDP type refers to phylogenetic lineages originally defined by analysis of restriction digest patterns of chromosomal DNA.
[d]*infB* is the highly conserved gene encoding initiation-translation factor IF2.
[e]ST refers to the isolates' sequence type, based on the alleles of seven housekeeping genes identified by multilocus sequence typing.

Table 2. Distribution of *infB* alleles in serotype III GBS from different RAPD groups[a]

RAPD group[b]	Species of origin	Isolates studied	*infB* allele				
			A	B	C	D	H
II	Bovine	31	1	0	0	0	30
III	Bovine	4	3	0	0	1	0
IV-A	Bovine	19	19	0	0	0	0
	Human	1	1	0	0	0	0
IV-B	Bovine	2	0	0	0	0	2
IV-C	Bovine	4	2	0	2	0	0
	Human	7	5	0	2	0	0
Ungrouped	Bovine	2	0	0	0	0	2
	Total	70	31	0	4	1	34

[a]GBS, group B streptococcus.
[b]RAPD group identified by randomly amplified polymorphic DNA analysis.

that restriction digest pattern type III-2 strains, a major cause of human neonatal infections, are rarely, if ever, isolated from bovine milk.

One of the two remaining A allele strains, SH-96-4807, appears indistinguishable from III-3 strains on the basis of MLST because it has ST-17, which is characteristic of III-3 strains. Unlike III-3 strains, however, SH-96-4807 has no inserted sequences in any of the three sites and contains an *infB* A allele instead of the C allele typical of III-3 strains. The remaining A allele isolate, SH-96-3696, appears to be most closely related to H allele strains, on the basis of its RAPD group, inserted sequences, and ST. No bovine isolate related to restriction digest pattern type III-4 was found in this sample.

The four C allele isolates have the typical inserted sequences and ST (ST-17) found in restriction digest pattern type III-3 strains, with the exception of human isolate 1004A, which has a truncated form of IS*1548* in the *scpb-lmb* intergenic region (Table 4). These strains, which cluster together in RAPD Group IV-C, were isolated from both human and bovine sources. These data indicate that strains from the restriction digest pattern type III-3 lineage infect both humans and bovine udders but that bovine III-3 strains are rare. The only D allele strain has ST-93, an ST which differs by at least three alleles from all the STs previously described for human GBS, which again indicates that D allele serotype III GBS rarely or never colonize humans.

Analysis of H Allele Isolates

Ten H allele isolates were selected to represent strains from RAPD Group II, Group III, and the ungrouped isolates and analyzed in the same fashion. As shown in Table 5, all of the isolates studied have large inserts in the AW-10 site. The 1,700-bp inserts would be the correct size for the GBSi1 insert found in III-3 strains, and sequencing demonstrated an intact copy of GBSi1 in a 1,700-bp amplicon from one of the strains. A 3,000-bp insert from one strain was amplified and sequenced and found to comprise GBSi1, interrupted by an IS*3*-like insertion sequence identical to sequences found in both the genome M1 strain of *S. pyogenes* and the genome serotype V strain of *S. agalactiae* (19,20).

Table 3. Inserted sequences and sequence type (ST) of bovine strains containing the *infB* A allele[a]

RAPD group	Isolate	Origin	Inserted sequence site			ST	RDP type
			AW-10	*hylB*	*scpb-lmb*		
II	SH-96-3696	Bovine	3,400[b]	None	None	67	Unknown[c]
III	NI-96-2836	Bovine	None	IS*1548*	IS*1548*	19	III-2-like
	SH-96-4807	Bovine	None	None	None	17	III-3-like
	RF-96-2997	Bovine	None	None	None	94	III-1
IV-A	1003A	Human	None	IS*1548*	IS*1548*	19	III-2
	AL-97-0498	Bovine	None	None	None	23	III-1
	SH-96-3417						
	All 17 others	Bovine	None	None	No product[d]	23,90,92	III-1-like
IV-C	NI-96-3213	Bovine	None	None	No product[d]	23	III-1-like
	ASS-96-666	Bovine	None	None	No product[d]	23	III-1-like
	1007B	Human	None	IS*1548*	IS*1548*/IS*1381*[e]	86	III-2
	1009A, 15888	Human	None	IS*1548*	IS*1548*	19	III-2
	13228, 1009B	Human	None	IS*1548* (truncated)[f]	IS*1548*	19	III-2

[a]RAPD, randomly amplified polymorphic DNA; RDP, restriction digest pattern.
[b]A 3,400-bp insert is also found in other strains from randomly amplified polymorphic DNA (RAPD) group II (see text and Table 5).
[c]This strain appears to be related to the H allele strains described in Table 5.
[d]No polymerase chain reaction product was produced from these strains in multiple attempts.
[e]In this strain, the insertion sequence IS *1381* is upstream from IS *1548* in the *scpb-lmb* locus.
[f]S*1548* in the *hylB* locus is missing 657 bp from the 3′ end of its open reading frame in this strain.

Table 4. Analysis of inserted sequences of strains containing the *infB* C allele[a]

RAPD	Isolate	Origin	Inserted sequence site			ST	RDP type
			AW-10	*hylB*	*scpb-lmb*		
IV-C	SF 96-5547	Bovine	GBSi1	700	GBSi1	17	III-3
	1004A	Human	GBSi1	700	IS*1548*[b]	17	III-3
	1000B	Human	GBSi1	700	GBSi1	17	III-3
	SF-96-4054	Bovine	GBSi1	700	GBSi1	17	III-3

[a]RAPD, randomly amplified polymorphic DNA; ST, sequence type; RDP, restriction digest pattern.
[b]An insert highly homologous to IS *1548* but lacking bp 603–1,301 was found in th is strain. IS *1548* has not previously been found in this locus in III-3 strains.

A 3,400-bp insert was sequenced and found to consist of an inserted sequence that has identical direct and inverted repeat sequences to the insertion sequence IS*1563*, but it has a predicted amino acid sequence that is 75% identical to that of IS*1563*. This IS*1563*-like insertion sequence is located upstream of GBSi1 exactly as IS*1563* is found upstream of GBSi1 in this locus in restriction digest pattern type II-2 strains (11).

The AW-10, *hylB*, and *scpb-lmb* loci were studied in the remaining H allele strains not shown in Table 5. No inserted sequences were found in the AW-10 site in seven isolates, while the remaining strains have either the 1,700-bp, 3,000-bp, or 3,400-bp inserts at AW-10. No inserted sequences were found in the *hylB* and *scpb-lmb* sites in any of the H allele strains. Two of the H allele strains, both of which are in RAPD group IV-B, appear to lack the *scpb-lmb* locus.

MLST shows that 8 of these 10 H allele isolates are ST-61. ST-61 isolates are found in RAPD groups II, IV, and in ungrouped isolates, which indicates genetic divergence among H allele strains that is detected by RAPD but not by MLST. ST-61 differs from ST-67 by one allele, which confirms that SH-96-3696, the A allele strain found in RAPD II that has ST-67, is in the same clonal complex as the H allele serotype III strains. SH-96-3696 also has a 3,400-bp insert at AW-10, and no insert at the other two sites, which provides further evidence that this isolate is related to H allele strains. The other two STs found in the H allele strains, ST-91 and ST-105, differ from ST-61 by two alleles. Thus, the H allele strains are clonally related.

III-3–specific Sequence Tags in H Allele Serotype III GBS

ST-61, the most common ST of the H allele strains, differs by two alleles from ST-17, the most common ST of III-3 strains. This two-allele difference in ST and the observation that isolates from these two lineages both have GBSi1 in the AW-10 site led us to search for other evidence that the two lineages are related.

We previously described 10 short sequence tags that are found in all III-3 strains but not in III-1 or III-2 strains (16). We therefore performed dot-blot hybridization with eight of these probes on a selection of A, H, and C allele strains from this sample to determine the distribution of the III-3–specific sequences among these various lineages. As expected, all of the III-3–specific probes hybridized with every C allele strain tested, whereas the III-3 probes hybridized rarely or never with the A allele strains. In contrast, seven of the eight III-3 probes hybridized with almost all the H allele strains (Table 6).

Table 5. Inserted sequences and sequence types of *infB* H allele strains[a]

RAPD	Isolate	Size of PCR poroduct from inserted sequence sites (bp)			ST
		AW-10	*hylB*	*scpb-lmb*	
II	RF-96-2834	3,000[b]	700[c]	650[d]	61
	ASS-97-0701	3,000	700	650	61
	AL-96-1653	1,700[e]	700	650	61
	SF-96-6312	3,400[f]	700	650	61[g]
	SF-96-4396	1,700	700	650	91
	NI-96-2521	3,000	700	650	105
IV-B	ASS-96-659	3,000	700	No product	61[g]
	SH-96-5461	1,700	700	No product	61
Ungrouped	AL-96-2049	3,000	700	650	61
	NI-96-3329	3,000	700	650	61

[a]RAPD, randomly amplified polymorphic DNA; ST, sequence type.
[b]A 3,000-bp amplicon was sequenced and found to contain an IS*3*-like insertion sequence interrupting GBSi1.
[c]A 700-bp product from the *hylB* site indicates that there is no inserted sequence in the si te.
[d]A 650-bp product from the *scpb-lmb* site indicates that there is no inserted sequence in the site.
[e]A 1,700-bp product was amplified and found to contain an intact copy of GBSi1.
[f]A 3,400-bp amplicon was sequenced and found to contain GBSi1 and an IS *1563*-like insertion sequence .
[g]The *glcK* (glucose kinase) gene in these two strains contains a mobile genetic element in the portion of *glcK* used to determine the ST of GBS strains. These two strains were found to be sequence type 61 (ST-61) after the inser ted sequence was removed. The identical inserted sequence in the *glcK* gene has been found in other ST-61 bovine strains and is described elsewhere (21).

Table 6. Distribution of III-3–specific sequence tags

III-3–specific sequence	*infB* allele[a]		
	H	A	C
DY-1	10/10	0/10	3/3
DY-3	2/10	1/10	3/3
DY-11	7/10	0/10	3/3
AA 3.8	10/10	2/10	3/3
AA 3.14	10/10	0/10	3/3
AA 3.16	9/10	1/10	3/3
AA 4.1	10/10	2/10	3/3
AA 4.13	10/10	0/10	3/3

[a]Number of strains with sequence tag by DNA hybridization/number of strains tested.

Discussion

A key finding of this investigation is that the serotype III GBS strains isolated from bovine milk in this sample are largely genetically distinct from the serotype III GBS strains that commonly infect humans. The two most common lineages of serotype III GBS that colonize women and infect infants in the United States and Japan are restriction digest pattern types III-2 and III-3, whereas III-1 and III-4 strains are rarely isolated from the genitourinary tract of women and are rarely associated with invasive disease (11,16).

Only one bovine strain that resembled RDP type III-2 GBS was identified in the 67 isolates, and RAPD analysis indicates that this isolate is distinct from the 7 human III-2 strains in the sample. Thus, III-2 strains that infect humans are unlikely to infect bovine udders. Only two bovine strains were identified that had the genotypic characteristics of restriction digest pattern type III-3 strains. These two strains appear closely related to two human III-3 isolates by RAPD analysis, which makes it likely that III-3 strains can infect bovine udders, but they appear to do so infrequently.

We also found that the vast majority of bovine serotype III GBS in this sample belong to one of two major phylogenetic lineages. A separate study of bovine GBS isolates collected in the United Kingdom used MLST to identify two clonal complexes that are similar, if not identical, to the two lineages identified here; this finding suggests that two clones of GBS may predominate among bovine mastitis caused by GBS in North America and England (21).

The data presented here show that the first bovine lineage is closely related to restriction digest pattern type III-1 GBS (Table 7). The lack of human III-1 strains in this sample makes it difficult to use the RAPD analysis to determine how closely these III-1–like bovine strains resemble human III-1 strains. That no III-1 human isolates were found in this small sample is not surprising because human III-1 isolates rarely colonize the female genitourinary tract (although they occasionally cause neonatal infections) (9,17,18). Most III-1 bovine isolates in the sample studied here appear to lack the *scpb-lmb* locus, a finding consistent with that reported by Francken et al., who showed that absence of a putative composite transposon that contains the *scpB* and *lmb* genes is a feature of bovine GBS (22). Possibly only a few of the III-1 bovine GBS are capable of infecting both humans and cows, since all human GBS appear to have the *scpb-lmb* locus.

The other major bovine serotype III GBS lineage in this sample is composed of strains that possess an *infB* H allele, with the exception of a single A allele strain that, on the basis of RAPD and MLST, appears closely related to the H allele strains. The H allele strains have STs that differ from each other by no more than two alleles, which indicates that these strains are likely to have a recent common ancestor.

We believe that strains in this lineage are unlikely to colonize or infect humans since we have not identified an *infB* H allele strain in >160 human serotype III GBS isolates, and since the STs of the bacteria in this lineage were not found in a large sample of human GBS obtained from diverse geographic areas (9). However, the major ST (ST-61) found in this lineage differs from the major ST of III-3 strains by only two alleles, which suggests that strains from this group may share a relatively recent common ancestor. H allele strains were found to contain previously identified III-3–specific sequence tags, which supports this hypothesis.

The identification of two III-3 strains of bovine origin and a third bovine strain (SH-96-4807) that is genetically distinct from III-3 strains but with the ST typical of III-3 strains (ST-17) supports the concept that III-3 strains share a recent common ancestor with H allele strains and retain

Table 7. Similarities and differences between the two major bovine lineages and related human lineages

Bovine lineage	Human lineage	Similarities	Differences
III-1	III-1	Same *infB* allele (A)	Many bovine III -1 strains lack *scpb-lmb*
		No inserted sequences in AW -10, *hylb* or *scpb-lmb* sites	
		Sequence types (STs) the same or differ by <1 allele by multilocus sequence typing (MLST)	
H allele	III-3	GBSi1 inserted in AW -10 site	Different *infB* allele (H vs C)
		No inserted sequence in *hylb*	BSi1 not found in *scpb-lmb*
		STs differ by only two alleles by MLST	Additional inserted sequences or none in AW -10 site with GBSi1
		III-3 specific sequence tags in genome	Some bovine H allele strains lack *scp-lmb*

some genetic traits necessary for bovine udder colonization. No bovine III-2 strains were identified, but a single bovine strain (NI-96-2836) appears to be related to strains in the III-2 lineage by all molecular markers (although RAPD analysis found it to be clearly distinct from human III-2 isolates). These III-3–like and III-2–like bovine strains are both in RAPD group III (Table 3) and thus appear by RAPD analysis to be more related to each other than to other major bovine lineages or to human isolates. RAPD analysis also suggests that human III-3 strains are more related to human III-2 strains than to the H allele strains, despite the observation that MLST puts human III-3 strains at a greater phylogenetic distance from III-2 strains than from the H allele strains.

The clustering together of human III-2 and III-3 isolates by RAPD analysis, despite their clear distinction by MLST, leads us to hypothesize that common genetic determinants that account for host tropism (human versus bovine) have been acquired by both the III-2 and III-3 lineages and strongly influence the clustering of isolates, as shown by RAPD analysis. If so, the two bovine isolates that closely resemble III-3 and III-2 strains by MLST, but which resemble each other more closely by RAPD analysis, may represent intermediate genotypes between GBS lineages that have a more clear-cut tropism for either humans or bovines. The exact relationship between these human and bovine lineages, and genes important for host tropism, could be clarified by comparative genomics. Such studies, along with further studies of bovine GBS lineages of other serotypes, could also provide insight into the exact relationship between human and bovine strains and help determine whether these hosts act as reservoirs for each other's pathogenetic lineages and for the emergence of new pathogenic clones.

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Thrombocytopenia and Acute Renal Failure in Puumala Hantavirus Infections

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Nephropathia epidemica, caused by Puumala virus (PUUV) infection, is a form of hemorrhagic fever with renal syndrome of variable severity. Early prognostic markers for the severity of renal failure have not been established. We evaluated clinical and laboratory parameters of 15 consecutive patients with acute PUUV infection, which is endemic in the Alb-Danube region, South Germany. Severe renal failure (serum creatinine >620 μmol/L) was observed in seven patients; four required hemodialysis treatment. Low platelet count (<60 x 10^9/L), but not leukocyte count, C-reactive protein, or other parameters obtained at the initial evaluation, was significantly associated with subsequent severe renal failure ($p = 0.004$). Maximum serum creatinine was preceded by platelet count nadirs by a median of 4 days. Thrombocytopenia <60 x 10^9/L appears predictive of a severe course of acute renal failure in nephropathia epidemica, with potential value for risk-adapted clinical disease management.

Worldwide, approximately 60,000–150,000 patients per year are hospitalized with hantavirus infections (1,2). *Hantavirus* spp. (*Bunyaviridae* family) are transmitted to humans by inhalation of aerosolized excreta of persistently infected rodents (3,4). Puumala virus (PUUV) is a representative of this genus responsible for most cases of hantavirus infections in northern Europe (1,5). The red bank vole (*Clethrionomys glareolus*) is the natural host reservoir of PUUV. Nephropathia epidemica attributable to acute PUUV infection is a mild form of hemorrhagic fever with renal syndrome (1,4–6). Endemic seasonal outbreaks of PUUV infections are common in Scandinavia, but outbreaks in central Europe are restricted to distinct regions, e.g., in South Germany (2,4,7).

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Nephropathia epidemica is characterized by acute fever, headache, nausea, vomiting, myalgia, abdominal and loin pain, mild hepatitis and pancreatitis, and interstitial nephritis with acute renal failure. Hemodialysis is required in 10% to 30% of hospitalized patients with acute PUUV infections (8). A complete recovery of renal function is regularly achieved after several weeks (1,9). Acute renal failure in nephropathia epidemica is frequently accompanied by thrombocytopenia, elevated leukocyte count, proteinuria, hematuria, and low serum calcium (9–11), but early prognostic markers have not yet been established to identify patients at high risk for a severe course of acute renal impairment. We evaluated clinical and laboratory parameters that could predict the severity of acute renal failure suitable for risk-adapted disease management in patients with nephropathia epidemica.

Patients and Methods

From 1998 to 2001, all consecutive patients with nephropathia epidemica and serologically confirmed hantavirus infection were studied. Patients were admitted by physicians and regional hospitals to the nephrology division of the University Hospital of Ulm, a center of nephrology and infectious diseases in South Germany with a patient base of 100 km (radius) on both sides of the River Danube. Hemodialysis was started in patients with severe symptoms of uremia (serum creatinine >620 μmol/L, serum urea nitrogen >150 mg/dL, serum potassium >6.0 mmol/L, oliguria <500 mL/day, or progressive body weight increase with edema). After discharge, a follow-up examination of all patients was conducted in our outpatient clinic. Blood pressure, leukocyte count, hemoglobin, platelet count, prothrombin time, activated partial thromboplastin time, C-reactive protein, serum electrolytes, alanine amino- transferase (ALT), serum protein, serum creatinine, urea nitrogen, proteinuria, and microscopic

qualitative and quantitative (Addis count) urine analysis were evaluated. Clinical and laboratory data obtained before admission were also evaluated. The study protocol was approved by the Ethics Committee, University of Ulm, and written informed consent was obtained from all patients. Studies were conducted in accordance with the Declaration of Helsinki.

In all patients, acute hantavirus infection was serologically diagnosed by PUUV-specific immunoglobulin (Ig) G enzyme immunoassay (EIA) (Progen, Heidelberg, Germany). In addition, Hantaan virus (HTNV)-, PUUV- and Dobrava virus (DOBV)-specific IgM and IgG antibodies were detected by in-house monoclonal antibody–capture or μ-capture EIAs (Charité, Berlin), as previously described (12); by immunofluorescence assays (IFA) using HTNV-, PUUV-, and DOBV-infected Vero E6 cells; or by an immunoblot (IB) using recombinant hantavirus antigens (Mikrogen, Martinsried, Germany). In nine patients, hantavirus serotyping was performed by chemiluminescence focus reduction neutralization assays (c-FRNTs), as described recently (13).

Nonparametric tests were used for statistical analysis (Fisher exact test, Mann-Whitney U test), and significance was set at a level of $p < 0.05$. No adjustment for multiple comparisons was made, and results were interpreted in an exploratory manner. All statistical analyses were conducted with SPSS 8.0 software package (SPSS Inc., Chicago, IL). If not indicated otherwise, data are given as median values with range (minimum to maximum).

Results

Fifteen patients (mean age 37 ± 8 years; male:female ratio12:3) with nephropathia epidemica were treated at the University Hospital of Ulm from January 1998 to December 2001 (Table 1). PUUV infections varied in different years by number of patients and seasons with a high incidence (Figure 1: November to December 1998: 2 patients; 1999 none; January to May 2000: 9 patients; September to November 2001: 4 patients). All but one patient lived on the north side of the River Danube. No patient was working in agriculture, forestry, or other professions considered at high risk for contact with infected rodent excreta. Acute or chronic use of nonsteroidal antiinflammatory drugs was denied.

Patients were admitted to the hospital 5 days (range 2–10 days) after acute onset of clinical symptoms, but the first laboratory examination had already been performed 3 days (median, range 1–9 days) after onset of clinical symptoms by general practitioners. Prominent clinical and laboratory findings of acute nephropathia epidemica were fever (100%), abdominal or loin pain (80%), fatigue (67%), myalgia (60%), hepatitis (60%), headache (47%), nausea (40%), and conjunctival bleeding (20%) (Table 1). Dyspnea and pulmonary infiltrations were not observed on x-ray images. Ultrasound examination showed intact kidney forms in all patients.

PUUV-reactive IgG and IgM antibodies were detected in all patients (Table 2). EIA-positive results were confirmed by at least one independent test (in-house EIA,

Table 1. Characteristics of 15 patients with acute PUUV infection associated with mild or severe acute renal failure[a,b]

	Mild acute renal failure (n = 8)	Severe acute renal failure (n = 7)	p value
Age (y)	38 (25–53)	35 (22–53)	n.s.[c,d]
Sex (male:female r atio)	5:3	7:0	n.s.[e]
Fever (>38.5°C)	8	7	n.s.[e]
Abdominal or loin pain	7	5	n.s.[e]
Fatigue	4	6	n.s.[e]
Myalgia	5	4	n.s.[e]
Hepatitis (ALT >20 U/mL)	4	5	n.s.[e]
Headache	4	3	n.s.[e]
Nausea/vomiting	1	5	0.041[e]
Conjunctival bleeding	1	2	n.s.[e]
Purpura	0	2	n.s.[e]
Highest C-reactive protein (mg/L)	50 (27–112)	90 (7–122)	n.s.[d]
Lowest platelet count (x 10^9/L)[b]	113 (26–250)	34 (18–122)	0.016[d]
Highest leukocyte count (x 10^9/L)	9.9 (8.0–15.3)	15.1 (12.0–22.7)	0.029[d]
Lowest serum calcium (mmol/L)	2.22 (1.85–2.27)	2.02 (1.98–2.21)	0.029[d]
Hematuri a (cells/min)	10.2 (2.6–27)	76.4 (21.2–129.0)	0.009[d]
Leukocyturia (cells/min)	16.2 (10.4–22)	41.0 (17.5–191.0)	0.0017[d]
Proteinuria (>1.5 g/day)	2	4	n.s.[e]
Tubular cell casts	1	5	0.001[e]

[a]PUUV, Puumala virus; mild and severe acute renal failure was defined as serum creatinine ≤620 μmol/L and >620 μmol/L, respectively; ALT, alanine aminotransferase .
[b]Of all parameters, only thrombocytopenia predicted subsequent severe renal failure. Medians (range) and actual number of patients are given.
[c]n.s., not significant.
[d]Mann-Whitney U test.
[e]Fisher exact test.

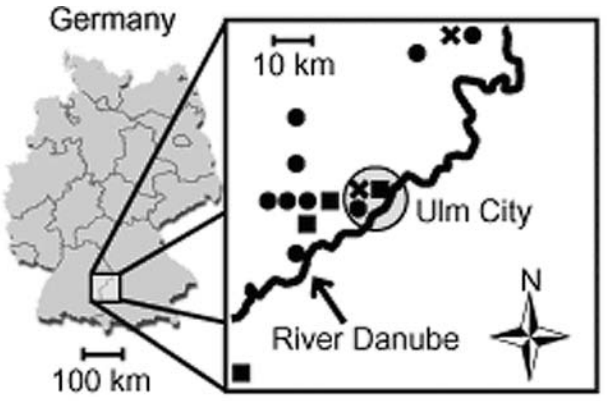

Figure 1. Residence of 15 study patients with hantavirus infection and nephropathia epidemica, according to year of diagnosis (X, November–December 1998; black circle, January–May 2000; black square, September–November 2001). Note the River Danube as a potential natural barrier of Puumala virus hantavirus infection. Shaded circle indicates Ulm city.

IFA, IB). During the acute phase of PUUV infection, cross-reactive neutralizing antibodies to other hantavirus serotypes (HTNV, Tula virus) were detected in two patients (c-FRNT), but PUUV infection was confirmed in the convalescent-phase infection by high endpoint titers for PUUV antibodies compared to other hantavirus serotypes. Because of the limited quantity of serum specimens, the complete array of virologic tests was not performed in all samples, but available serologic data demonstrated acute PUUV infections in all patients (Table 2).

In all patients, a combination of low platelet count (<150 x 10^9/L), elevated serum creatinine (>150 μmol/L), or elevated C-reactive protein (>10 mg/L) was detected. In seven patients (47%), severe acute renal impairment with a serum creatinine >620 μmol/L developed; four (27%) of these patients required hemodialysis as an acute intervention for 3 days (range 2–9 days) because of symptoms of uremia. During hospital stay, polyuria (>3 L/day) was present in only three patients (20%), and no patient became anuric (<500 mL/day). Serum protein was ≥60 g/L in all patients, plasma coagulation parameters were not altered, and mild bleeding signs (subconjunctival bleeding, petechiae) were present in only three patients with a platelet count <60 x 10^9/L. Patients remained in the hospital for 10 days (range 5–19 days), and follow-up examination was conducted at our outpatient clinic for 5 months (range 0.2–12.5 months).

We found that lower platelet count, higher leukocyte count, the presence of tubular cell casts in urine, quantitative hematuria and leukocyturia (Addis count), and nausea were significantly associated with severe acute renal failure (serum creatinine >620 μmol/L) (Table 1). In contrast, age, sex, blood pressure, fever, abdominal or loin pain, myalgia, headaches, fatigue, bleeding signs, hemoglobin, prothrombin time, activated partial thromboplastin time, C-reactive protein, sodium, potassium, ALT, serum protein, and proteinuria were not associated with serum creatinine >620 μmol/L (Table 1).

The platelet count nadir was observed 4 days (range 1–6 days) after the acute onset of clinical symptoms. Seven days after initial symptoms (range 3–12 days), platelet counts had already returned to normal in all patients, with values >150 x 10^9/L (Figure 2). Maximum serum

Table 2. Virologic results of 15 patients with acute PUUV infection [a]

Patient	IgG-EIA (Progen) index	IgG titers			IgM titers	c-FRNT titers				
	PUUV	PUUV	HTNV	DOBV	PUUV	PUUV	HTNV	DOBV	SEOV	TULV
1	9	6,400	Neg	Neg	3,200	640	<40	160	<40	160
2	2.1	2,560	640	–	2,560	–	–	–	–	–
3	2.2	6,400	400	400	1,600	2,560	<40	<40	<40	40
4	3.3	51,200	6,400	400	12,800	640	160	40	<40	160
5	3.8	51,200	3,200	Neg	Pos[b]	2,560	160	40	<40	40
6	3.1	6,400	800	Neg	6,400	2,560	640	40	<40	160
7	3.1	12,800	800	Neg	1,280	640	<40	<40	–	40
8	4.3	12,800	3,200	800	3,200	2,560	160	40	<40	160
9	1.8	Pos[c]	–	–	Pos[c]	–	–	–	–	–
10	2.9	12,800	3,200	3,200	400	2,560	160	40	<40	160
11	7.1	12,800	1,600	400	1,600	–	–	–	–	–
12	2.9	Pos[c]	–	–	Pos[c]	–	–	–	–	–
13	4.9	51,200	400	Neg	3,200	–	–	–	–	
14	2.5	Pos[c]	–	–	Pos[c]	–	–	–	–	–
15	3.3	12,800	1,600	400	1,600	2,560	40	<40	40	40

[a]PUUV, Puumala virus; Ig, immunoglobulin; EIA, enzyme immunoassay; c-FRNT, chemiluminescence focus reduction neutralization assays; HTNV, Hantaan virus; DOBV, Dobrava virus; SEOV, Seoul virus; TULV, Tula virus; –, not available.
[b]IgM-EIA (Progen, Heidelberg, Germany).
[c]Immunoblot (Mikrogene, Martinsried, Germany).

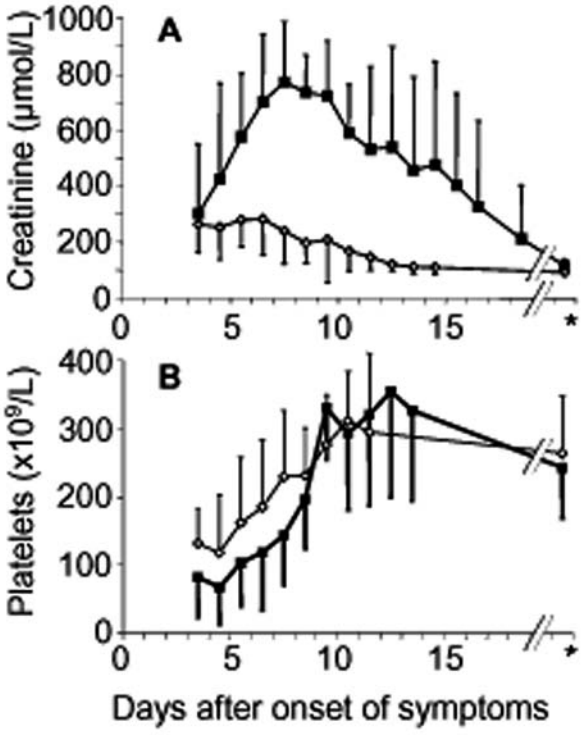

Figure 2. Course of serum creatinine (A) and platelet count (B) in patients with mild (diamond, serum creatinine <620 µmol/L, n = 8) or severe acute renal failure (black square, serum creatinine >620 µmol/L, n = 7). Mean values and SD are shown. *Denotes evaluation at end of followup (median, 5 months).

creatinine values were observed 7 days (range 3–14 days) after onset of disease, and renal function returned to normal within 14 days (range 7–38 days) after onset of symptoms, as defined by serum creatinine <150 µmol/L.

In patients with severe renal failure, platelet count nadirs preceded maximum serum creatinine values by 4 days (range 2–10 days). In patients with a mild or severe course of acute renal failure, the platelet count was different at initial evaluation ($p = 0.04$), but serum creatinine values at initial evaluation did not differ ($p = 0.15$). We analyzed the potential prognostic value of low (<60 x 10^9/L) and moderately decreased (≥60 x 10^9/L) platelet count for the severity of subsequent renal failure. This value represented the median of all patients at first evaluation, which generated two equally sized patient cohorts.

The time interval between initial clinical symptoms and first laboratory evaluation did not differ in the two groups (2 days, range 0–6 days). A platelet count of <60 x 10^9/L preceded severe renal failure in six of seven patients, and all patients with hemodialysis had a platelet count <60 x 10^9/L in the early phases of infection. Acute severe renal impairment (sensitivity 0.86, specificity 0.88, positive predictive value 0.86) developed in one of eight patients with a platelet count ≥60 x 10^9/L. The initial serum creatinine value was not different in the two groups ($p = 0.54$, Mann-Whitney U test), but the maximum value was significantly higher in patients with a platelet count of <60 x 10^9/L during the early phase of infection (Figure 3A). Although associated with a severe course of nephropathia epidemica (Table 1), an increased leukocyte count at first examination was not predictive of the severity of subsequent renal failure (Figure 3B).

Discussion

Early prognostic parameters for the course of renal impairment might be beneficial for risk-adapted disease management of nephropathia epidemica, but they have not been established to date (1,14). Thrombocytopenia is a well-known transient symptom of this condition (15), and we found that severe thrombocytopenia (<60 x 10^9/L) was a prognostic parameter predictive for consecutive severe acute renal failure (serum creatinine >620 µmol/L).

In Germany, the hantavirus seroprevalence in humans is 1%–2%, but higher seroprevalences are found in disease-endemic regions, such as the Alb-Danube Region, South Germany (16). Most of our patients might have acquired PUUV infection during leisure activities, as occupations were not found to be risk factors in this study. Fluctuations of infected rodent populations may be responsible for the seasonal and endemic outbreaks of PUUV infections (4,6–18). Most patients with nephropathia epidemica resided to the north of the River Danube (Figure 1), which raises the possibility that the river acts as a geographic barrier for the migration of PUUV-infected rodent populations. However, direct evidence for this assumption was not provided in this study.

The diagnosis of an acute PUUV infection is established by detecting PUUV-specific IgM antibodies (19). Cross-reactive neutralizing antibodies to closely related hantavirus serotypes are common in the early but not in the convalescent phase of infection (20,21), as observed in two patients. In all patients, acute PUUV infection was proven by the different serologic tests, which were equally able to discriminate various hantavirus serotypes (Table 2).

Infections of endothelial and tubular cells, in conjunction with various immunologic mechanisms, are responsible for acute renal failure and thrombocytopenia in nephropathia epidemica. The maturation of hantavirus-infected dendritic cells may efficiently stimulate a proinflammatory immune response of specific T cells (22). β3 integrins are adhesive receptors on platelets and endothelial cells and may contribute to capillary leak and platelet activation in PUUV infection (3,23,24). Endothelial cell damage, circulating immune complexes, complement activation, T-cell activation, and cytokine response with high levels of tumor necrosis factor-α and interleukin-6 might cause acute renal failure and peripheral consumption of

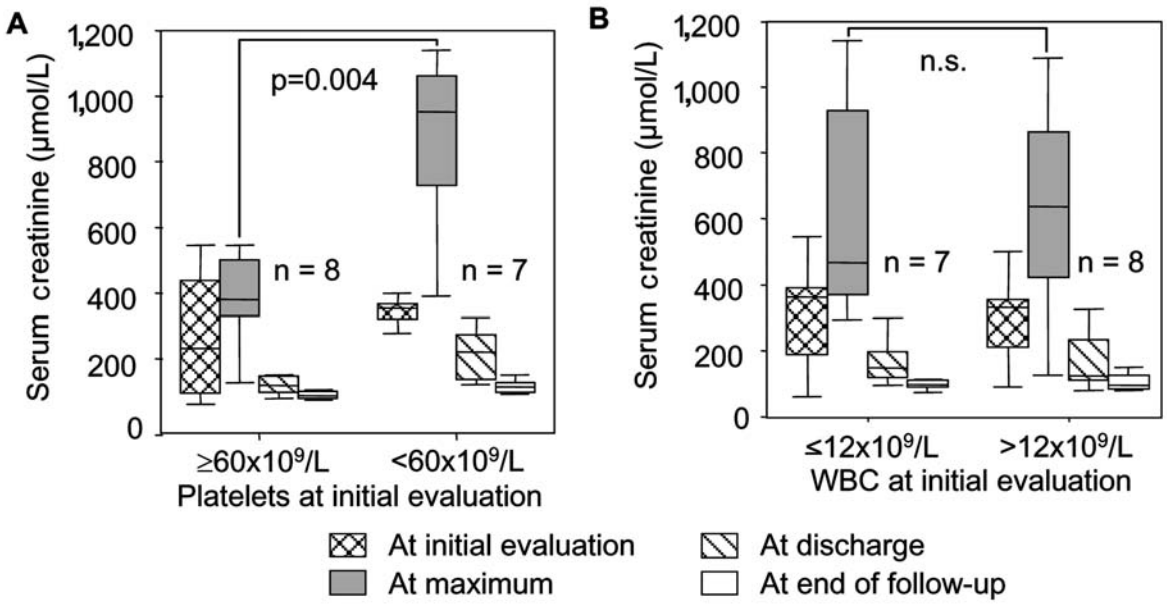

Figure 3. Temporal course of serum creatinine in patients with hantavirus infection, stratified according to A) platelet count and B) leukocyte count at initial evaluation. Platelet count, but not leukocyte count, is a significant predictor of subsequent renal failure (p = 0.004, Mann-Whitney). Box plots with median, interquartile range, minimum and maximum values are shown. n.s., not significant; WBC, leukocyte count.

platelets (9,25,26). Similar to PUUV infections, severe thrombocytopenia may predict severe organ failure in other infectious diseases, including Rocky Mountain spotted fever (27).

In our patients, acute PUUV infection was a highly dynamic process, characterized by a short transient thrombocytopenia followed by mild-to-severe acute renal failure. We found statistical evidence that severe thrombocytopenia (<60 x 10^9/L) is a significant early prognostic parameter for subsequent severe acute renal failure (serum creatinine >620 µmol/L). In other studies, thrombocytopenia was not identified as a predictive marker for severe acute renal failure (9,28); in these studies, laboratory data before hospital admission were not reported (9,28). These parameters were included to evaluate the earliest phase of infection. Leukocyte count was elevated in patients with a severe course of renal failure but was not predictive at first examination (Figure 3B). Similarly, quantitative hematuria, quantitative leukocyturia, and tubular cell casts obtained 1 day (range 0–12 days) after admission to hospital were associated with, but not predictive for, severe renal failure.

A specific therapy for nephropathia epidemica is not generally applicable, but a symptomatic therapy including hemodialysis may be individually required. If the platelet count is <60 x 10^9/L in the early phase of infection, acute renal failure with serum creatinine >620 µmol/L is impending, and a tight control of clinical and laboratory parameters, including hospitalization, is mandatory. A platelet count ≥60 x 10^9/L in the early phase of infection is predictive for mild renal failure, and outpatient treatment might be possible in absence of uremia, anuria, pulmonary symptoms, or other signs of severe infection. If a patient's platelet count has not dropped below 60 x 10^9/L before day 6 after initial symptoms, the clinician may feel relatively comfortable predicting that severe renal failure will not occur. Later phases of infection are characterized by increased serum creatinine values but normalizing platelet counts (Figure 2). Early discharge from hospital may be justified in all patients at low risk for consecutive severe acute renal failure and in all patients with ongoing reconstitution of renal function. Thus, platelet counts obtained during the early phase of infection are suggested as a promising parameter for a risk-adapted, cost-effective disease management.

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Pharmacy Data for Tuberculosis Surveillance and Assessment of Patient Management

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Underreporting tuberculosis (TB) cases can compromise surveillance. We evaluated the contribution of pharmacy data in three different managed-care settings and geographic areas. Persons with more than two anti-TB medications were identified by using pharmacy databases. Active TB was confirmed by using state TB registries, medical record review, or questionnaires from prescribing physicians. We identified 207 active TB cases, including 13 (6%) missed by traditional surveillance. Pharmacy screening identified 80% of persons with TB who had received their medications through health plan–reimbursed sources, but missed those treated solely in public health clinics. The positive predictive value of receiving more than two anti-TB medications was 33%. Pharmacy data also provided useful information about physicians' management of TB and patients' adherence to prescribed therapy. Pharmacy data can help public health officials to find TB cases and assess their management in populations that receive care in the private sector.

Controlling and preventing tuberculosis (TB) continue to be major public health challenges in the United States (1). Information obtained through TB surveillance ensures that TB-control activities are appropriate and can be used to evaluate the effectiveness of public health programs (2). Because TB surveillance relies heavily on laboratories and providers to report cases to local health departments, surveillance data can be compromised by underreporting, particularly by private-sector clinicians who treat TB infrequently. Pharmacy data, often available in automated form, may supplement traditional TB reporting, especially because anti-TB medications are rarely used to treat other conditions.

A Massachusetts study found that persons with TB who were identified through pharmacy dispensing records and who had not been previously reported to the state health department represented 16% of all new cases (3). In that study, receipt of two or more anti-TB drugs identified most cases of active TB. These results suggested that pharmacy dispensing information could supplement traditional TB surveillance. In addition, pharmacy dispensing information for persons with active TB provided useful information about appropriateness of prescribed treatment regimens and adherence to therapy (4).

We therefore evaluated the contribution of pharmacy data to overall TB surveillance and to assessing the quality of TB management. We performed this study through health plans to facilitate access to pharmacy dispensing data and medical records.

Methods

Study Population

Members of three different health plans in Michigan (1993–1999), Missouri (1996–1998), and Tennessee (1998) were included in the study population. Study periods were based on availability of pharmacy data.

All health plans met our basic criteria of providing most of the medical care to defined populations, providing prescription drug benefits, having automated pharmacy

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claims files, and having accessible full-text medical records. The health plans differed in some ways. Most importantly, plan C (see below) routinely delegated care of recognized TB patients to local health departments or had members obtain their anti-TB drugs from public health programs separate from the plan's regular pharmacy programs and data systems. Additionally, the structure of the three health plans and their populations differed; they consisted of the following: a mixed staff and group model that included a large urban population (plan A); an independent practice association (IPA) health plan affiliated with a managed-care organization, principally serving an employed population (plan B); and a mixed IPA and staff model, principally serving Medicaid enrollees (plan C). Staff-model health plans employ providers who practice in common facilities. IPA-model health plans contract with providers who practice in their own offices (5). Prior institutional review board approval was obtained from participating health plans.

Pharmacy Screening

Health plan pharmacy dispensing data were screened to identify all members who received two or more anti-TB medications during the study period. For plans A, B, and C, we screened, respectively, approximately 1.3 million, 1.0 million, and 1.6 million health plan person-years. The anti-TB medications included in the screening were isoniazid, rifampin, pyrazinamide, ethambutol, streptomycin, ethionamide, kanamycin, cycloserine, capreomycin, para-aminosalicylic acid (PAS), and drugs containing any combination of these medications. Although at least 90% of health plan members had some form of pharmacy benefit, the plans varied considerably in directing their members to public health facilities to obtain anti-TB medications.

Identifying TB Cases

Reporting confirmed or clinically suspected TB to local or state health departments by providers, laboratories, boards of health, or administrators of hospitals is mandatory in Michigan, Missouri, and Tennessee, which maintain registries of all verified cases. State health department staff in all three states determined whether health plan members identified as having received two or more anti-TB medications had been reported previously to the health departments by matching to the state TB registries by using previously described methods (6).

For all plan members who received two or more anti-TB medications and who were not previously reported to the state health departments, information was obtained through review of medical records. A case of TB was defined according to the Centers for Disease Control and Prevention (CDC) surveillance definition (7). In a culture-positive case, *Mycobacterium tuberculosis* was isolated from a clinical specimen. In a smear-positive case, acid-fast bacilli (AFB) were demonstrated in a specimen in the absence of a culture. A clinical case-patient met all of the following criteria: a positive tuberculin skin test, signs and symptoms compatible with TB, and treatment with two or more anti-TB drugs. Case-patients without a positive culture for *M. tuberculosis* that were not known to the health departments were verified by review with clinicians experienced in diagnosing and treating TB.

To estimate the number of TB cases not detected by using pharmacy data, each health plan's membership during the study period was matched to the state health department's TB registry entries during the same period by using minimal disclosure methods (6). Potential matches were confirmed with full identifiers. To determine the source of care for patients not identified through pharmacy screening, health department records of all such patients in plans A and B and a random sample in plan C were reviewed.

Assessing TB Management

Automated pharmacy dispensing records were used to characterize TB therapy for persons with active TB who met pharmacy screening criteria in plans A and B. Plan C did not participate in the assessment of TB management because members were routinely referred to public health clinics for treatment, and information about medications was unavailable from the health plan pharmacy database. In addition, pharmacy dispensing records from two additional health plans affiliated with plan B were screened, and TB cases verified through medical record review were included in the analysis.

All filled prescriptions were identified for isoniazid, rifampin, pyrazinamide, ethambutol, streptomycin, ethionamide, kanamycin, cycloserine, capreomycin, PAS, and drugs containing a combination of these medications. Initial regimens, i.e., those dispensed at the start of therapy before susceptibility results were known, and final treatment regimens were graded for consistency with American Thoracic Society (ATS) and CDC guidelines in effect at the time of diagnosis (8). The appropriateness of doses based on patient weight was not evaluated.

Two measures were calculated for therapeutic adequacy. The standard regimen dispensed is a percentage calculated by comparing the cumulative dose of each drug dispensed with the total recommended. Each drug received equal weight to a maximum of 100% per drug, as noted in the following formula for a three-drug regimen: percent standard regimen = $([D_1/SR_1] + [D_2/SR_2] + [D_3/SR_3]) \times (100/3)$, where D_X is the cumulative dose for drug X and SR_X is the recommended total dose. Patients with a score ≥80% were considered to have received an appropriate amount of anti-TB medication. The days without medication for isoniazid or another drug required for the duration

of treatment are calculated by dividing the total number of days without medication (based on medication refill intervals and quantities dispensed) by the number of days between the first and last dispensing (4,9).

Analysis

The sensitivity of pharmacy data was defined as the number of verified TB cases detected by pharmacy screening divided by the total number of verified TB cases identified through the TB registry, pharmacy data, or both methods. The positive predictive value (PPV) of pharmacy screening was defined as the number of verified TB cases detected by pharmacy data divided by the total number of persons meeting pharmacy screening criteria; persons with undetermined case status were excluded. Exact binomial confidence intervals were calculated for sensitivity and PPV (8).

Results

Dispensing Anti-TB Drugs

A total of 244 patients received two or more anti-TB drugs (Table 1). Of these, 13 (5%) met the TB case definition and had not been previously reported to their respective state health departments. Another 61 (25%) were active TB case-patients. Sixty-three percent did not meet the TB case definition, and the status of the remaining 7% could not be determined because the medical records were either unavailable or insufficient.

Of 153 patients who received at least two anti-TB medications but did not meet the CDC TB case definition, 62 (41%) were treated for suspected active TB. Of these, 15 (24%) received a full course of therapy for suspected active TB. Twenty-one (14%) received more than one drug during treatment for latent TB infection, 63 (41%) were treated for non-TB mycobacterial infections, and 7 (4%) were treated for noninfectious conditions or for unknown reasons (Table 2).

The overall rate of initiating two or more anti-TB drugs was 6 per 100,000 person-years, ranging from 3 to 11 per 100,000 person-years in the three health plans. Confirmed case rates ranged from 0.9 to 4.3 per 100,000 person-years screened. The 1998 TB incidence for the three states ranged from 3.4 to 8.1 cases per 100,000 persons (9). For persons meeting pharmacy screening criteria, the proportion confirmed as new case-patients did not vary significantly among the three plans (Table 1).

Newly Identified Cases of TB

A total of 207 health plan members meeting TB case definitions (53 in plan A, 22 in plan B, and 132 in plan C) were identified through pharmacy data or health department records (Table 3). Among these, 13 case-patients (6%) were unknown to the respective state health departments. Two persons with TB unknown to one health department had been reported to Mississippi State Department of Health. None of the 13 were culture-positive for *M. tuberculosis*; one lacked a microbiology culture but met the smear-positive case definition, and the remaining 12 met the CDC TB clinical case definition. All except one involved active pulmonary disease.

One hundred thirty-three TB cases were known to the state health departments but were not identified through pharmacy databases. We reviewed the records of 81 of these patients, of whom 61 (75%) received their anti-TB medications from public health clinics; this proportion ranged from 58% (22/38) in plan A to 93% in plan C (26/28). An additional 3 (4%) were treated at Veterans Administration (VA) facilities or were diagnosed with TB during hospitalization and died before discharge. Health plan medical records did not include information about TB diagnosis and treatment for 17 (21%) patients. Reasons for this may include TB treatment exclusively by other providers and incomplete documentation in accessible medical records.

The overall sensitivity of the pharmacy screening method to identify persons with active TB was 36% (28% in plan A, 32% in plan B, and 39% in plan C). However, the overall sensitivity was 80% after the extrapolated number of persons who received their TB medication from public health clinics rather than the health plans was excluded (Figure 1). The positive predictive value of the pharmacy screening method to identify persons with active TB was 33% (21% in plan A, 50% in plan B, and 36% in plan C) (Figure 1).

Assessing Management of TB

Of the 29 plan A (n = 15) and plan B (n = 14) members with active TB identified through pharmacy screening, health plan and health department records indicated that 17

Table 1. Identification of tuberculosis (TB) cases by using pharmacy screening

Cases	Plan A (%)	Plan B (%)	Plan C (%)	Total (%)
Total no. dispensed 2 or more anti-TB drugs	73	28	143	244
Matched to TB registry (previously reported TB cases)	12 (17)	6 (21)	43 (30)	61 (25)
Previously unreported TB cases (verified by record review)	3 (4)	1 (4)	9 (6)[a]	13 (5)
Not a TB case (verified by record review)	55 (75)	7 (25)	91 (64)	153 (63)
Case status not determined	3 (4)	14 (50)	0	17 (7)

[a]Includes two cases not found in the state health department's TB registry but reported to other state health departments.

Table 2. Reasons for meeting pharmacy screening criteria among persons without active tuberculosis (TB)

Reasons why non -TB cases met screening criteria	Plan A (%)	Plan B (%)	Plan C (%)	Total (%)
Suspected active TB, full course of therapy	7 (13)	0	8 (9)	15 (10)
Suspected active TB, empiric therapy discontinued	12 (22)	0	35 (38)	47 (31)
Treatment of latent TB infection	8 (14)	3 (43)	10 (11)	21 (14)
Other mycobacterial infections	26 (47)	4 (57)	33 (36)	63 (41)
Other or unknown	2 (4)	0	5 (5)	7 (4)
Total	55	7	91	153

(59%) did not receive treatment in public health clinics and were likely to have received their anti-TB medications through health plan–reimbursed pharmacies. Twenty-eight (97%) patients received initial regimens through pharmacies reimbursed by the health plan. In all instances, the initial regimen dispensed was appropriate. For all 17 patients not treated in public health clinics, the final regimen described in the medical record was adequate with regard to the agents used, doses prescribed, and intended duration of treatment.

Fifteen of the 17 health plan–treated patients received anti-TB medications for at least 70 days (compared to 3 of 13 who were treated outside the health plans [relative risk = 3.8, $p < 0.01$]), with a median dispensing duration of 180 days (interquartile range 150–324 days). The median standard-regimen-dispensed score was 100% (interquartile range 93%–100%) (Figure 2). Based on health plan pharmacy data, one patient received an inadequate treatment regimen, with a standard-regimen-dispensed percentage of only 48%. Another health plan–treated patient received a standard-regimen-dispensed score of 100% but had a days-without-medication score of 51%, because of a gap in anti-TB therapy of 143 days. One additional patient with culture-positive *M. tuberculosis* infection received a standard-regime-dispensed score of 68% and a 60-day duration of dispensing. In all of these cases, the treating physician did not describe noncompliance or document a non–health plan source of anti-TB medications.

Discussion

TB surveillance has traditionally depended on reporting by laboratories, public health clinics, hospitals, and private practitioners. Several retrospective studies (3,10–13) indicate that TB cases may be underreported, particularly those without positive cultures. In this study, we found that 6% of all TB cases in the three participating health plans had not been reported to state health departments. Most cases missed by traditional surveillance were culture- and smear-negative; however, nearly all patients with missed cases had clinical evidence of pulmonary disease and were therefore of public health interest.

The recent shift of populations at risk for TB, including Medicaid recipients, into managed care raises concerns about reporting. As the proportion of patients with TB who are cared for outside traditional public health–funded clinics grows, the benefit of adjunct surveillance methods based on pharmacy data is likely to increase, since these data are available for a large segment of the U.S. population.

Although we used health plan data for this study, health departments could more efficiently obtain this information directly from pharmacy benefits management companies (PBMs) that act as intermediaries between managed-care organizations and pharmacies, because they administer and manage the prescription drug benefit programs for these organizations. Working directly with PBMs has two advantages. First, PBM information is accessible in real time. Second, since the three largest PBMs in the United States manage the pharmacy claims of approximately 200 million persons, information available from a small number of PBMs could provide a rich resource for public health screening (14–17).

The percentage of cases in these three health plans that were missed by traditional surveillance (6%) was lower than the 16% missed in the Massachusetts study (3). This difference may reflect the fact that public health clinics cared for more patients in these health plans than in Massachusetts, where 60% of patients were treated solely by health plan providers, compared to about 40% for these health plans.

Patients who received their anti-TB medications from public health clinics were not identified through health

Table 3. Detecting tuberculosis (TB) cases by using pharmacy screening and state health department TB registries

Case identification	Plan A (%)	Plan B (%)	Plan C (%)	Total (%)
Pharmacy screening only	3 (6)	1 (5)	9 (7)	13 (6)
State health department only (all cases)	38 (72)	15 (68)	80 (61)	133 (64)
State health department only (health plan–treated patients)	16 (52)	2 (22)	0[a]	18 (19)
Both methods	12 (22)	6 (27)	43 (32)	61 (30)
Total (all cases)	53	22	132	207
Total (health plan–treated patients[b])	31	9	52	92

[a]Extrapolated from review of a random sample of 28 of the 80 TB cases identified by the state health department and not by pharmacy screening.
[b]Excludes TB patients receiving anti-TB medication from public health clinics; these medications are not included in the health plan pharmacy databases.

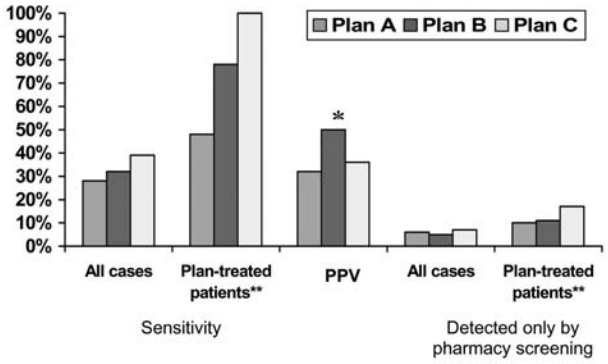

Figure 1. Sensitivity and positive predictive value (PPV) of pharmacy screening and percentage of tuberculosis (TB) cases detected only by pharmacy screening. *Of 28 members who met pharmacy screening criteria, TB case status was verified for 14. PPV calculation based on total of 14 with verified status. **Health plan–treated patients excludes patients receiving anti-TB medication from public health clinics.

plans' pharmacy data. However, because these patients are already known to the public health system, supplemental surveillance methods are unnecessary. Pharmacy screening identified 80% of the patients with TB who were not treated outside the health plan. This estimate is conservative; we probably underestimated the number of persons receiving anti-TB medications from public health clinics or other healthcare systems because we based this assessment on the private providers' records. Intermittent enrollment may also have compromised the sensitivity of pharmacy-based screening. Larger databases that include pharmacy information from multiple health plans within a geographic area, such as those maintained by PBMs, are likely to improve case-finding.

The most common reasons for dispensing two or more anti-TB medications to persons who did not meet the case definition were 1) more than one drug used to treat latent TB infection; 2) suspected active TB; 3) treatment of other mycobacterial infections; and 4) treatment for suspected active TB and receiving full courses of therapy, despite not meeting the CDC surveillance definition for TB, based on information available from their medical records. Persons in the last category may warrant additional evaluation by health departments because the case definition may not detect all patients who meet clinical standards for treatment.

The PPV of pharmacy screening criteria may be lower in clinical settings where treatment of non-TB mycobacterial infections is common. One strategy to increase the efficiency of pharmacy-based screening would be to use microbiologic culture information to quickly identify and exclude from further follow-up any persons with results indicating mycobacterial species other than *M. tuberculosis*. Complete laboratory reporting for *M. tuberculosis* is an important prerequisite for efficiently implementing this surveillance strategy.

In routine practice, pharmacy data might be used for active TB case-finding, with direct reporting from organizations dispensing drug information, such as health plans or PBMs, to local or state health departments. These data are typically available from health plans within 1 or 2 months and from PBMs within a day. Such reporting would require verifying case status by health department personnel.

Obtaining and reviewing medical records for this study were labor-intensive, but collecting this information from providers in real time should be more efficient. The cost of reporting would be relatively small for health plans or pharmacy benefits managers, and the cost per person identified would be small for large organizations. The additional costs for health departments to evaluate the status of persons not already identified will vary considerably across health departments. Despite the increased emphasis on privacy, current laws specifically allow reports of protected health information to support public health activities.

Although pharmacy data may be useful, they will not replace traditional surveillance of suspected and confirmed TB cases. Because rapidly following-up suspected TB cases is essential to prevent the spread of *M. tuberculosis*, educating providers to report suspected cases promptly to public health officials will continue to be important.

Automated pharmacy data also provided useful information about physicians' management of TB and about patients' adherence to prescribed therapy. Monitoring these aspects of TB care is particularly important when care is decentralized or when patients receive care from

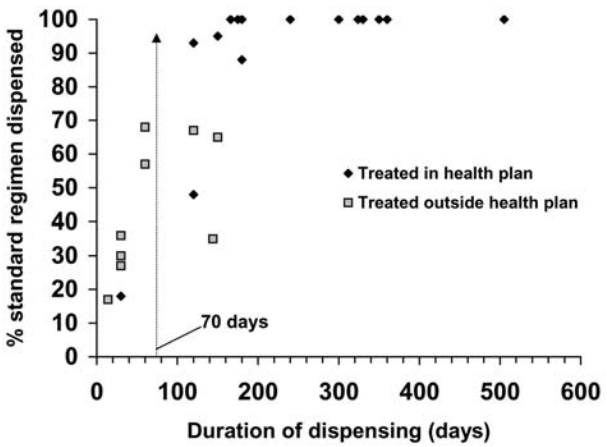

Figure 2. Pharmacy-dispensing profiles of tuberculosis (TB) case-patients treated in the health plans and at least partially outside the health plan. Percentage of standard regimen dispensed is plotted against duration of dispensing anti-TB medications for the two groups. A cutoff value of ≥70 days of medication dispensed from health plan–reimbursed pharmacies identifies all but one of the health plan–treated TB case-patients.

more than one provider. Pharmacy information demonstrated that, in nearly all cases, appropriate empiric regimens were prescribed. In most cases managed by health plan providers, full ATS/CDC-recommended regimens were dispensed. Consistent with the Massachusetts study results, using a cutoff value of at least 70 days of therapy identified most patients treated solely within the health plan. This practice is important in monitoring adherence to therapy, since automated pharmacy information is complete only for these patients. Pharmacy data also identified several persons with evidence of suboptimal adherence to therapy. Pharmacy information on anti-TB drugs could thus be used for monitoring the appropriateness of case management and to evaluate the program.

This study and our earlier work demonstrate that pharmacy data may be useful in settings where TB care is provided by the private healthcare system. Centralized repositories of pharmacy data, such as those maintained by PBMs, may facilitate even more efficient application of this surveillance strategy to find TB cases and assess TB management for large patient populations. Similar studies in other settings could expand our understanding of current surveillance limitations and provide better estimates of the true burden of TB in the United States. Similar strategies could also be considered to augment traditional surveillance for other diseases of public health importance.

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Antimicrobial Drug Use and Methicillin-resistant *Staphylococcus aureus*, Aberdeen, 1996–2000

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Similar to many hospitals worldwide, Aberdeen Royal Infirmary has had an outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA). In this setting, the outbreak is attributable to two major clones. The relationships between antimicrobial use and MRSA prevalence were analyzed by time-series analysis. From June 1997 to December 2000, dynamic, temporal relationships were found between monthly %MRSA and previous %MRSA, macrolide use, third-generation cephalosporin use, and fluoroquinolone use. This study suggests that use of antimicrobial drugs to which the MRSA outbreak strains are resistant may be an important factor in perpetuating the outbreak. Moreover, this study confirmed the ecologic effect of antimicrobial drug use (i.e., current antimicrobial use) may have an effect on resistance in future patients. Although these results may not be generalized to other hospitals, they suggest new directions for control of MRSA, which has thus far proved difficult and expensive.

Antimicrobial drug resistance occurs in hospitals worldwide. One of the most globally important microorganisms is methicillin-resistant *Staphylococcus aureus* (MRSA), which now causes more than 40% of all *S. aureus* bacteremias in the United Kingdom (1). Measures to control MRSA outbreaks have concentrated on transmission of the organism and prospective screening for carriage, in combination with general infection control measures such as patient isolation, use of barrier precautions, and environmental decontamination (2). Eradicating MRSA colonization has also been used to curb the spread of MRSA. Despite these measures, incidences of MRSA continue to rise (2,3). Guidelines for controlling MRSA in hospitals rarely include information on controlling antimicrobial use, possibly because relatively little data quantify the relationships between antimicrobial use and MRSA rates, especially in outbreak situations (4–8). To date, mathematical modeling has predicted that the effect of antimicrobial prescribing patterns in an outbreak situation is likely to be slight (9).

Epidemic MRSA type 15 (EMRSA-15) is presently the most common clone in the United Kingdom, followed by EMRSA-16, both of which are termed "super-clones" because of their potential for spreading nationally and internationally (10). Compared to other MRSA in the United Kingdom, EMRSA-15 and EMRSA-16 are more successful at surviving, colonizing, and spreading in the hospital environment (11). Both clones are typically resistant to all β-lactams, macrolides, and fluoroquinolones (10). The northeast of Scotland has seen a rapid spread of EMRSA-16 and, to a lesser extent, of EMRSA-15 during the last 7 years after they first emerged in the area's main teaching hospital, Aberdeen Royal Infirmary.

We investigate the dynamics of the MRSA outbreak at Aberdeen Royal Infirmary and possible relationships between MRSA prevalence and antimicrobial drug use, by time-series analysis. Time-series analysis creates a mathematical model to fit a series of dynamic observations to forecast future behavior on the basis of retrospective behavior. Unlike other statistical methods that assume observed data to be independent, time-series analysis takes into account the stochastic dependence of consecutive observations or autocorrelation (12,13). This method is appropriate when data are measured repeatedly at equal intervals for long periods and when these intervals are much shorter than the study period. Time-series analysis has been applied in medical specialties such as endocrinology, cardiology, environmental medicine, and the study of chronic diseases

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(13). The analysis of interrupted time-series or intervention analysis is considered the strongest quasi-experimental method to ascertain the longitudinal effect of healthcare interventions (13–15). Additionally, extensions of this method, e.g., transfer function modeling and econometric dynamic modeling, can take into account external factors that may influence the target series over time and can demonstrate a temporal relationship between these external factors and the target series (13–15). Because series of antimicrobial drug use data and resistance data always show an autocorrelation, this method has been proposed by López-Lozano et al. to study the relationship between antimicrobial drug use and resistance (16).

Materials and Methods

Aberdeen Royal Infirmary is a 1,200-bed tertiary referral hospital covering a population of approximately 500,000. It comprises various medical and surgical specialties and is close to other specialized hospitals. For each month of the study period, January 1, 1996, to December 31, 2000, numbers of inpatient-days per ward were obtained from the hospital's admission department. During the study period, all *S. aureus* isolated were screened for susceptibility to methicillin by the comparative disc susceptibility test method on nutrient agar at 30°C with 48 h incubation (17). Methicillin resistance was confirmed by carrying out an Etest MIC. Susceptibility of the *S. aureus* isolates to a range of additional antimicrobial drugs was established by the comparative disc test method (17). Additionally, the Aberdeen MRSA outbreak was investigated by the Scottish MRSA Reference Laboratory, which conducted independent confirmation and genotyping. The Reference Laboratory carried out multiplex polymerase chain reaction (PCR) with primers to *mec*A, *nuc*, rRNA, 16S rRNA, (18–20) and pulsed-field gel electrophoresis (PFGE) typing of *Sma*I digested DNA (21).

Monthly data for all *S. aureus* on which antimicrobial drug susceptibility tests were carried out were exported from the clinical microbiology information system into a database. Information stored included patient identifier, hospital, ward, specimen type, and antimicrobial drug–susceptibility pattern. Because we did not systematically and uniformly search for MRSA carriers, isolates obtained from surveillance screening were excluded. Only the first *S. aureus* isolate from each patient within 7 days was exported from the clinical microbiology laboratory information system into an Access (Microsoft, Redmond, WA) database. Variations in the antimicrobial susceptibility pattern of *S. aureus* isolates from the same patient within the 7-day period were not considered. From these data, the monthly prevalence of MRSA isolates was calculated as a percentage, where the denominator was the total number of *S. aureus* tested for methicillin resistance.

Monthly quantities of all antimicrobial drugs delivered to each hospital ward during the study period were exported from the pharmacy information system and stored both at the individual antimicrobial drug and class level in an Access (Microsoft) database. Antimicrobial drug use was expressed as a number of defined daily doses (DDDs) per 1,000 patient days, where the DDD for each antimicrobial drug was defined by the World Health Organization (WHO) (22). As in most hospitals, data on patient exposure to antimicrobial drugs were not available at Aberdeen Royal Infirmary. For a specific antimicrobial drug class, however, the number of DDDs approximates the average number of patients exposed to an antimicrobial drug from this class each day. This measurement is the unit WHO recommends to express ecologic pressure attributable to antimicrobial drugs (23).

Time-series analysis was carried out to explore the relationships between each antimicrobial drug use series and the %MRSA series. For this purpose, linear transfer function models were built according to the identification method proposed by Pankratz (15). This analysis was completed by a graphic exploration of the series. Line plots at monthly time intervals were produced for the %MRSA and for use of each antimicrobial drug class to visualize their evolution over time and to confirm the relationships between %MRSA and antimicrobial drug use.

Once the basic characteristics (i.e., autocorrelation, seasonality, and general trend) of each of the %MRSA and antimicrobial drug use series were established, a multivariate analysis was performed to quantify the relationships between use of several antimicrobial classes and %MRSA through the use of econometric dynamic time-series modeling techniques (14,24,25). Specifically, polynomial distributed lag (PDL) modeling was used to detect and quantify lagged effects of antimicrobial drug use on %MRSA. The details of the modeling technique are presented in the Appendix. For the purposes of this study, data were analyzed with Eviews 4.0 (Quantitative Micro Software, Irvine, California, USA).

Results

From January 1996 through December 2000, the clinical microbiology laboratory isolated 9,441 nonduplicate, nonsurveillance *S. aureus*, including MRSA and methicillin-susceptible *S. aureus* (MSSA), from 6,412 hospitalized patients. Numbers ranged from 97 to 241 *S. aureus* isolates per month and demonstrated no seasonal patterns (Figure 1). The annual %MRSA from 1996 to 2000 were 0.6, 5.0, 14.9, 24.1, and 31.9, respectively. MRSA were rarely isolated before December 1996; after that date, a sustained increase was observed, with marked peaks of %MRSA observed in April 1998 (22%), April 1999 (30.5%), and February 2000 (38.2%) (Figure 1). Basic

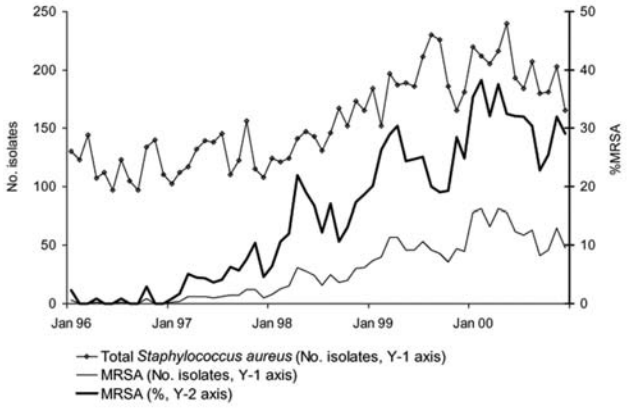

Figure 1. Evolution of the monthly number of clinical nonduplicate *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) isolates and monthly %MRSA, Aberdeen Royal Infirmary, January 1996–December 2000.

time-series analysis techniques and graphic exploration showed a spring seasonal variation of MRSA but no such seasonal variation for MSSA (Figure 1). From 1997 to 2000, the epidemic clones, EMRSA-16 and EMRSA-15, represented 80.0% and 15.4%, respectively, of 584 MRSA strains submitted for genotyping to the Scottish MRSA Reference Laboratory. Both clones were typically resistant to all β-lactams, macrolides, and fluoroquinolones but otherwise susceptible to other agents tested. The percentage of co-resistance to other antimicrobial drugs in all nonduplicate, nonsurveillance MRSAs (EMRSA-16, EMRSA-15, and other MRSA) isolated at Aberdeen Royal Infirmary during the outbreak is presented in Table 1. From 1996 to 2000, the annual use of systemic antibacterial agents showed little variation: 837, 953, 919, 963, and 938 DDD/1,000 patient-days, respectively. However, major variations occurred in the monthly use and seasonality of individual classes of antimicrobial drugs (Table 2).

Time-series analysis showed that %MRSA had a relationship with the use of many antimicrobial drug classes. The relationship was strongest for macrolides, fluoroquinolones, and penicillins with β-lactamase inhibitors, whereas other classes showed a significant but weaker relationship (Table 3). Graphic exploration confirmed these findings and pointed at third-generation cephalosporin use as another series to be introduced in the initial multivariate model (Figure 2). We also examined scatter plots and correlations of %MRSA with use of individual classes of antimicrobial drugs with up to 8-month delays (online Appendix Figure, available at http://www.cdc.gov/ncidod/eid/vol10no8/02-0694_app.htm). However, this last approach proved less useful than time-series analysis, and graphic exploration of the time series in identifying relationships and optimal delays between antimicrobial drug use and %MRSA and could be misleading. For example, scatter-plots and correlations showed an inverse correlation between MRSA and tetracycline use. However, graphic exploration showed that this correlation reflected opposite general trends rather than monthly parallel variations between these two variables (Figure 2).

A multivariate PDL model was built to relate %MRSA with use of these classes of antimicrobial drugs. The final model included previous monthly %MRSA as well as use of macrolides, third-generation cephalosporins, and fluoroquinolones as independent variables responsible for variations in %MRSA (Table 4). The greatest total effect of antimicrobial drug use on the %MRSA was found within the first two or three significant lag periods, after which the effect progressively decreased to reach nonsignificant values a few months after the end of the direct effect.

The sum of the direct and indirect effects of 10 DDD/1,000 patient-days or 30 more patients treated with a macrolide (Table 4) was an increase in %MRSA by the value 2.84 after 8 months. This change in antimicrobial drug use had more effect on the %MRSA in 1997 than in 2000. For example, in June 1997 the %MRSA was 3.6%. According to our model, an increase in macrolide use of 10 DDD/1,000 patient-days, or 30 more treated patients, made the %MRSA rise to 3.6 + 2.84 = 6.4% after 8 months or an 81% increase over June 1997. In June 2000, the

Table 1. Antimicrobial drug coresistance in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates and in methicillin-susceptible *S. aureus* (MSSA), Aberdeen Royal Infirmary, 1997–2000

	MRSA isolates		MSSA isolates			
Antimicrobial drug	No. tested for coresistance	No. resistant (%)	No. tested for coresistance	No. resistant (%)	Risk ratio	p value
Ciprofloxacin	1,218	1,195 (98.1)	515	183 (35.5)	13.4	< 0.0001
Clindamycin	2,722	2,666 (97.9)	7,715	956 (12.4)	89.6	< 0.0001
Erythromycin	2,721	2,669 (98.1)	7,701	1,115 (14.5)	90.0	< 0.0001
Fusidic acid	2,736	36 (1.3)	7,798	636 (8.2)	0.20	< 0.0001
Gentamicin	1,350	11 (0.8)	3,276	44 (1.3)	0.68	NS[a]
Mupirocin	2,514	154 (6.1)	5,180	99 (1.9)	1.92	< 0.0001
Rifampin	1,005	62 (6.2)	72	8 (11.1)	0.95	NS
Tetracycline	997	109 (10.9)	468	94 (20.1)	0.76	< 0.0001
Trimethoprim	1,060	18 (1.7)	0	–	–	–

[a]NS, nonsignificant.

Table 2. Characteristics of the monthly antimicrobial use time series, January 1996–December 2000.

Antimicrobial drug class	Average monthly use[a] (minimum–maximum)	Trend[b]	Seasonality[c]
Combinations of penicillins with β-lactamase inhibitors	228.6 (119.9–334.9)	Upward	Yes (0.294)
β -lactamase resistant penicillins	116.1 (49.1–202.1)	No	No
Macrolides	90.2 (32.7–177.9)	Upward	Yes (0.371)
Penicillins with extended spectrum	90.1 (43.9–177.4)	No	No
Third-generation cephalosporins	62.5 (43.8–103.1)	Upward	Yes (0.226)
β -lactamase-sensitive penicillins	54.6 (0–110.5)	No	No
Combinations of sulfonamides and trimethoprim, including derivatives	52.9 (0–86.8)	No	No
Fluoroquinolones	51.9 (19.4–87.5)	Upward	No
Second-generation cephalosporins	32.9 (5.3–87.1)	Downward	No
Other antibacterial drugs[d]	32.7 (16.3–45.9)	Upward	No
Tetracyclines	30.9 (0–63.4)	Downward	No
Aminoglycosides	24.8 (11.8–44.1)	Upward	Yes (0.236)
Glycopeptides	13.5 (4.6–25.5)	Upward	No
Lincosamides	6.1 (0–15.7)	Upward	Yes (0.208)
First-generation cephalosporins	5.2 (0.7–14.5)	No	No
Carbapenems	4.0 (0–8.5)	No	No

[a]Defined daily doses (DDD) per 1,000 mean patient-days.
[b]Based on regression of the series on time (according to the results of Dickey-Fuller unit root tests, none of the series needed to be differenced).
[c]Autocorrelation of order 12, based on the correlogram and the partial correlogram. When seasonality was present, the figure in parenthesis indicates the estimated autocorrelation of order 12, i.e., the correlation between antimicrobial use on a given month and use on the same month 1 year before.
[d]Amphenicols, monobactams, other quinolones, imidazoles, fusidic acid, and nitrofurantoin derivatives.

%MRSA had reached 32.1%. An increase in macrolide use of 10 DDD/1,000 patient-days, or 30 more treated patients, made the %MRSA rise to 32.1 + 2.84 = 34.9% after 8 months or a 9% increase over June 2000. This observation suggests that antimicrobial drug use was a more important ecologic risk factor at the start of the outbreak than once MRSA had become endemic in the hospital. However, macrolide use kept increasing during the study period (Figure 2), which compensated for the decrease in the size of the effect of antimicrobial drug use on %MRSA. Similar effects were observed for third-generation cephalosporin and fluoroquinolone use, i.e., an increase of 10 DDD per 1,000 patient-days on a certain month or 30 more treated patients, resulted in an increase in %MRSA by 4.99 after

Table 3. Summary of transfer function models explaining the monthly %MRSA by use of each antimicrobial drug class[a]

Antimicrobial class[b]	Average delay (months)	Direction of effect[c]	p value	R^{2}[d]
Combinations of penicillins with β-lactamase inhibitors	2	Positive	0.04	0.92
	4	Positive	0.01	
β–lactamase-resistant penicillins	0	Negative	0.02	0.90
	6	Positive	0.002	
Macrolides	1	Positive	0.0001	0.93
Penicillins with extended spectrum	1	Positive	0.03	0.91
Third-generation cephalosporins	1	Positive	0.04	0.90
β –lactamase sensitive penicillins	6	Positive	0.04	0.89
Combinations of sulfonamides and trimethoprim, including derivatives	4	Positive	0.02	0.90
Fluoroquinolones	4	Positive	0.0004	0.92
Second-generation cephalosporins		No relationship		
Other antibacterials[e]	0	Positive	0.002	0.91
Tetracyclines	4	Positive	0.03	0.91
	7	Negative	0.0007	
Aminoglycosides		No relationship		
Lincosamides	7	Positive	0.02	0.89
First-generation cephalosporins		No relationship		
Carbapenems	3	Positive	0.03	0.90

[a]MRSA, methicillin-resistant *Staphylococcus aureus*.
[b]Glycopeptide use is not presented in this table because it showed an inverse relationship with %MRSA. In other words, %MRSA explained the monthly variations of glycopeptide use and not the reverse (Discussion).
[c]Positive direction of effect: increase in antimicrobial use results in increase in %MRSA and inversely. Negative direction of effect: increase in antimicrobial use results in decrease in %MRSA and inversely.
[d]All models include the variable %MRSA with a 1-month delay and a p value < 0.0001.
[e]Amphenicols, monobactams, other quinolones, imidazoles, fusidic acid, and nitrofurantoin derivatives.

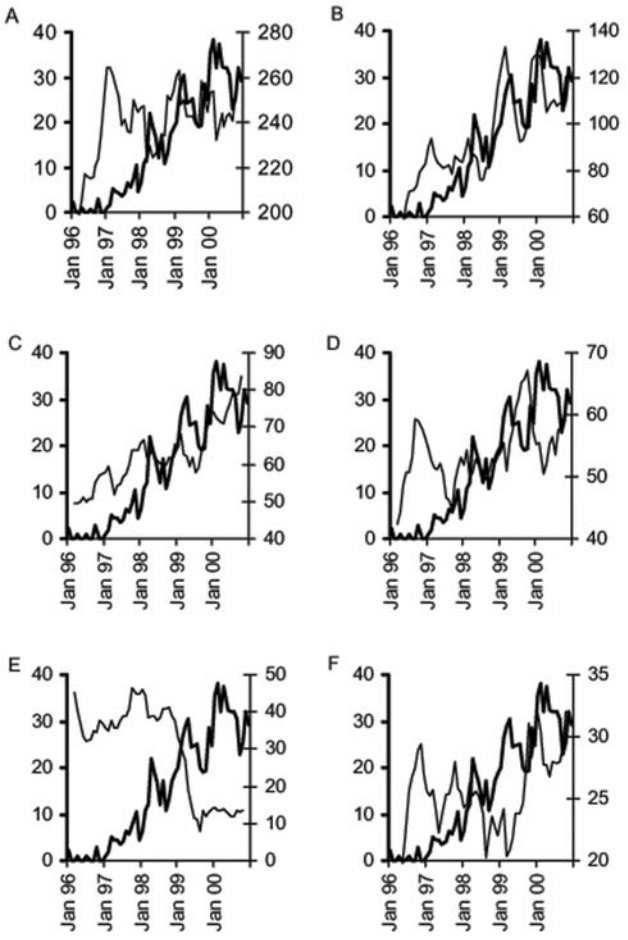

Figure 2. Examples of graphic exploration of the relationship between the monthly % methicillin-resistant *Staphylococcus aureus* (%MRSA) and the monthly use of individual classes of antimicrobials, Aberdeen Royal Infirmary, January 1996-December 2000 (━, %MRSA; –, Antimicrobial use, 5-month moving average, right Y-axis); A) penicillins with β-lactamase inhibitors, B) macrolides, C) third-generation cephalosporins, D) fluoroquinolones, E) tetracyclines, and F) aminoglycosides.

12 months for third-generation cephalosporins and by 4.40 after 11 months for fluoroquinolones.

The determination coefficient (R^2) of the final model was 0.902, i.e., 90.2% of the variations of the monthly %MRSA from June 1997 to December 2000 were explained by the model. The model that did not take antimicrobial drug use into account (i.e., considered previous monthly %MRSA) had a lower determination coefficient (0.811) and over- or underestimated the monthly %MRSA by 7.93%. The model that took into account both previous monthly %MRSA and previous use of the three key classes of antimicrobial drugs, with a determination coefficient of 0.902, produced an average discrepancy of 2.84 percentage points with the observed %MRSA. Therefore, taking antimicrobial drug use into account helped to improve the precision in forecasting the monthly %MRSA by 64%, which is a clear indication that antimicrobial drug use has a substantial causal effect on the %MRSA.

We compared coresistance patterns of MRSA isolates from the outbreak (i.e., 1997–2000) and of MSSA from the same period (Table 1), which confirmed the consistency of the antimicrobial drug use included in the model. MRSA isolates from the outbreak period were almost always resistant to erythromycin, clindamycin, and ciprofloxacin, whereas MSSA isolates from the same period were resistant in 14.5%, 12.4%, and 35.5% of cases, respectively. Resistance of MRSA isolates to the other antimicrobial drugs tested never exceeded 11% and was lower than in MSSA isolates with the exception of mupirocin (6.1% in MRSA isolates, 1.9% in MSSA isolates).

Finally, a curve of the summed monthly use of macrolides, third-generation cephalosporins, and fluoroquinolones, which took into account their respective lags for direct effects, was constructed and plotted on the same graph as monthly %MRSA (Figure 3). This figure shows the striking parallel nature of the relationship between the lagged use of these specific antimicrobial classes and the %MRSA at Aberdeen Royal Infirmary, which confirms the findings visually.

Discussion

For the first time, a powerful statistical model provides evidence of a strong temporal relationship between antimicrobial drug use and the varying prevalence of MRSA over time during an outbreak in a single hospital. The fact that only three classes of antimicrobial drugs, namely third-generation cephalosporins, fluoroquinolones, and macrolides, showed this relationship is not surprising. In the past, exposures to cephalosporins (26,27), fluoroquinolones (27–32), and macrolides (30) have been reported as patient risk factors for MRSA infection or colonization. And cephalosporin (4,8,33,34), fluoroquinolone (5,8,33), and macrolide use (8) have been reported as ecologic risk factors for high, or parallel variations of, MRSA prevalence or incidence. At Aberdeen Royal Infirmary, MRSA isolates were typically resistant to macrolides and fluoroquinolones (Table 1). Additionally, third-generation cephalosporins have poor activity against MRSA. At the same time, macrolides (clarithromycin and erythromycin), third-generation cephalosporins (mainly cefotaxime), and fluoroquinolones (essentially ciprofloxacin) were among the most used antimicrobial drugs at Aberdeen Royal Infirmary (Table 2), thus providing MRSA isolates with an ecologic advantage over other bacteria. Although the Aberdeen Royal Infirmary MRSA isolates were almost always resistant to clindamycin, use of lincosamides was among the lowest, which might

Table 4.[a] Estimated multivariate polynomial distributed lag (PDL) model for monthly %MRSA (R^2=0.902)

		Direct effect[b]			Indirect effect[c]	Sum of both effects[d]		
Explaining variable	Lag (mo.)	Coeff	T-stat	p	Coeff	Coeff[e]	T-stat	p
%MRSA	1	0.420	3.96	0.0003				
Macrolide use								
Each month	1	0.083				0.083	4.02	0.0003
	2	0.055			0.035	0.090	5.34	<0.0001
	3	0.027			0.038	0.065	6.02	<0.0001
	4				0.027	0.027	3.16	0.003
Overall	1–3	0.165	4.02	0.0003				
	2–4				0.100			
	1–4					0.265		
Third-generation cephalosporin use								
Each month	4	0.116				0.116	2.75	0.009
	5	0.087			0.049	0.136	3.27	0.002
	6	0.058			0.057	0.115	3.70	0.0007
	7	0.029			0.048	0.077	3.91	0.0004
	8				0.032	0.032	2.75	0.009
Overall	4–7	0.290	2.75	0.009				
	5–8				0.186			
	4–8					0.476		
Fluoroquinolone use								
Each month	4	0.170				0.170	3.43	0.002
	5	0.085			0.071	0.156	3.37	0.002
	6				0.066	0.066	2.31	0.03
Overall	4–5	0.255	3.43	0.002				
	5–6				0.137			
	4–6					0.392		
Constant		–36.7	–4.42	0.0001				

[a]MRSA, methicillin-resistant *Staphylococcus aureus*.
[b]Past %MRSA as well as past use of these three antimicrobial drug classes had direct effects on %MRSA. These direct effects diminished the longer the lag time.
[c]Because every increase in %MRSA by the value 1 was followed the next month by a significant increase in %MRSA by the value 0.420, use of the three antimicrobial drug classes also had indirect effects on the %MRSA. As 0.420 is <1, these indirect effects necessarily vanished over time. As an example, decreasing indirect effects are only presented for a few months. There were substantial indirect effects of macrolide use up to month 8 (final coefficient for sum of both effects = 0.284), of third -generation cephalosporin use up to month 12 (final coefficient for sum of both effects = 0.499), and of fluoroquinolone use up to month 11 (final coefficient for sum of both effects = 0.440).
[d]Each month, the total effect of each class of antimicrobial on the %MRSA resulted from the sum of the direct and indirect effects.
[e]The estimated coefficients indicate the values by which the %MRSA would increase in response to an increase in 1 DDD per 1,000 patient-days for each of the three significant antimicrobial classes, when all other variables remain constant. Since the average figure for monthly patient-days at Aberdeen Royal Infirmary is 22,800, 10 DDD per 1,000 patient-days correspond to approximately 230 DDD per month or thirty 7- to 8-day antimicrobial courses. For example, an increase in macrolide use by 10 DD D per 1,000 patient -days on a certain month, or 30 more patients treated with a macrolide as compared with the previous month, would lead to a direct increase in %MRSA by 0.83, 1 month later, by 0.55, 2 months later and by 0.27, 3 months later. The total direct effect would therefore be evident after 3 months, amounting to an increase in %MRSA by the value 1.65. Additionally, %MRSA indirectly attributable to macrolide use would increase by the value 0.35 (i.e., 0.83 x 0.42) after 2 months and by 0.38 (i.e.. [0.83 x 0.42] + [0.55 x 0.42]) after 3 months. From the 4th month onwards, there would be no direct effect of macrolide use on the %MRSA, only ever-decreasing indirect effects that would practically disappear after 8 months (decreasing effects in months 5 to 8 not shown).

explain why it did not appear as a risk factor in the multivariate model.

In addition to antimicrobial drug use, the final model also included the %MRSA observed 1 month before. As mentioned, we did not uniformly look for MRSA colonization. The pressure attributable to MRSA-colonized patients is a known risk factor for MRSA acquisition (8,35), which in turn affects the number of MRSA infections and the %MRSA in *S. aureus* from clinical samples. We therefore think that the %MRSA observed 1 month before is a surrogate for the pressure attributable to MRSA-positive patients during the past month.

The study was an ecologic and uncontrolled observational study in a single hospital. Selection bias was unlikely because data represented all hospitalized patients. Information bias was unlikely because data were not specifically collected for our study but for other purposes, i.e., routine clinical microbiologic diagnosis for *S. aureus* data and pharmacy accounting for antimicrobial drug use data. Confounding factors cannot be excluded but are unlikely for two reasons. First, as a result of the applied modeling strategy, the monthly variation in %MRSA not explained by the model (9.8%) was random. Therefore, the role of any possible unidentified confounding variable is thought to be minimal. Second, infection control policies, including measures such as barrier nursing, single room isolation, and eradication of carriage have consistently been applied to all MRSA patients during the study period,

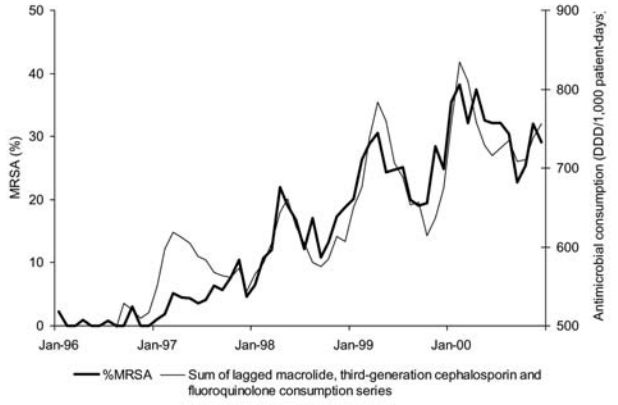

Figure 3. Evolution of the monthly % methicillin-resistant *Staphylococcus aureus* (MRSA) and monthly sum of lagged antimicrobial use as identified in polynomial distributed lag (PDL) model: macrolides (lags of 1 to 3 months), third-generation cephalosporins (lags of 4 to 7 months), and fluoroquinolones (lags of 4 and 5 months), Aberdeen Royal Infirmary, January 1996-December 2000.

although a shortage of single rooms often necessitated several MRSA-positive patients being assigned to a single nurse. Staff MRSA carriers were not actively sought, but use of gloves and hand washing, as appropriate, were constantly emphasized. Active patient contact tracing was applied, when possible, but environmental cleaning relied on standard cleaning schedules rather than environmental screening and targeted interventions. This policy was in line with national guidelines (36). The relationships between antimicrobial drug use and the %MRSA were unlikely to be attributable to chance because p values in the model were low. Additionally, the cause-effect relationships in the model were validated by their temporal nature (i.e., use of macrolides, third-generation cephalosporins, and fluoroquinolones always preceded %MRSA). Additionally, for each of these antimicrobial drug classes, the effect of antimicrobial drug use on the %MRSA was directional (i.e., an increase in use resulted in increased %MRSA and a decrease in use resulted in decreased %MRSA). In contrast, variations in glycopeptide use followed variations in %MRSA with an average delay of 1 month (coefficient = 0.45).

The relative importance of antimicrobial drug use compared to cross-transmission or changes in the patient case-mix could not be assessed. In ecologic analyses with aggregated data, additional data, such as volumes of medicated soaps or alcoholic solutions used for hand hygiene, could be used as surrogates for infection control practices; however, these data were not available. As in many hospitals, patient-level data were not available, which is why we modeled aggregated microbiology and pharmacy data. Models that use patient-level data on both antimicrobial drug exposure and MRSA may reach different conclusions. For example, the risk period for a patient for acquiring MRSA then developing an infection would be limited to hospital stay, which is generally short and rarely longer than 1 month. However, our model showed that a delay of several months was sometimes necessary to observe an ecologic effect of antimicrobial drug use on the %MRSA. This result is difficult to interpret since it means that antimicrobial drug exposure of some patients on a certain month has an impact on MRSA infections in other patients several months later. Since antimicrobial drug use data are based on dispensations to the wards, antimicrobial drugs can be stocked in the wards and used over several months. However, pharmacy data showed that antimicrobial agents were dispensed several times per ward each month, making this explanation unlikely. Another explanation could be that the increase in antimicrobial drug use would contribute to increasing the size of the reservoir of MRSA carriers. First, MRSA clones would be selected in antimicrobial drug-exposed patients. Then, the size of the reservoir of MRSA carriers would gradually increase through the spread of these MRSA clones to other patients, hospital staff, and the environment. This increase would become evident in clinical samples after several months when the MRSA reservoir reached a certain size. For fluoroquinolones, this hypothesis is supported by the results of Bisognano et al. (37) and Harbarth et al. (31). These authors showed that sub-MIC levels of ciprofloxacin increase adhesion of quinolone-resistant MRSA, which could explain persistent MRSA carriage and failure of mupirocin treatment in patients who received a fluoroquinolone. Antimicrobial drug use and cross-transmission probably work together to influence the %MRSA, and if all cross-transmission were to stop after implementing a very successful control program, the relationship between fluoroquinolone use and %MRSA would most probably disappear. Further research is needed to confirm this hypothesis and, more generally, to understand why long delays are also observed for other antimicrobial drugs, e.g., third-generation cephalosporins.

At Aberdeen Royal Infirmary, antimicrobial drug prescribing is overseen by an antibiotic committee, which provides and regularly updates a joint hospital-community antibiotic policy and stewardship program (38). Antimicrobial prescribing audits are performed periodically, but changing prescribing practices to control MRSA has not been attempted. Third-generation cephalosporin prescribing was addressed previously during an outbreak of *Klebsiella pneumoniae* displaying extended-spectrum β-lactamase activity (39). With the implementation of the British Thoracic Society guidelines for treatment of community-acquired pneumonia (40), macrolide and third-generation cephalosporin (mainly cefotaxime) prescribing has increased, which has been paralleled by the increase in

MRSA. As the Aberdeen MRSA clones are relatively susceptible, a policy of therapeutic substitution has been implemented in MRSA problem areas, starting with the replacement of cephalosporins by non–β-lactam antimicrobial drugs in surgical prophylaxis. The increase in fluoroquinolone prescribing has not been explained, but audits indicate that it is commonly used to treat serious nosocomial gram-negative infection (38).

Our study showed a quantifiable, temporal relationship between use of three classes of antimicrobial drugs (macrolides, third-generation cephalosporins, and fluoroquinolones) and the %MRSA. Because the study was performed in one hospital during an outbreak in which two predominant strains were circulating, it might not apply to other hospitals. Nevertheless, the use of antimicrobial drugs other than anti-staphylococcal penicillins and to which the MRSA outbreak strains are resistant might be a factor that would promote the outbreak. Moreover, the ecologic effect of antimicrobial drug use was confirmed (i.e., current antimicrobial drug use might have an effect on resistance in future patients). The effect of antimicrobial use on the %MRSA was greatest when the outbreak started and decreased when the %MRSA increased. Large decreases in antimicrobial drug use would have been needed to affect MRSA once it had become endemic. However, programs to control prescriptions of selected antimicrobial drug classes could represent an adjunct measure to active surveillance cultures and barrier precautions for the control of clonal outbreaks of MRSA, which has proved difficult and expensive.

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Appendix

Polynomial Distributed Lag (PDL) model

A PDL model was built to detect and quantify the lagged effects of antimicrobial use on the % methicillin-resistant *Staphylococcus aureus* (MRSA). In a PDL model, the relationship between the dependent variable (resistance) and the independent variables (past resistance and antimicrobial use) should evolve smoothly over time, through the use of "polynomial lags." The optimum PDL model was arrived at by the "general-to-specific" econometric methodologic characteristics. This meant that, initially, many possible independent variables were included in the model, some of which were ultimately found to be irrelevant. Additionally, for all the independent variables, lags of up to 8 months were initially included to identify direct effects. The initial dynamic regression model with PDLs considering %MRSA series as the dependent variable and several antimicrobial drug use series as explanatory series was the following:

$$\%MRSA_t = \alpha + \sum_{i=1}^{8} \beta_{0i} \%MRSA_{t-i} + \sum_{i=0}^{8} \beta_{1i} MAC_{t-i} + \sum_{i=0}^{8} \beta_{2i} 3GC_{t-i} + \sum_{i=0}^{8} \beta_{3i} FQ_{t-i} + \sum_{i=0}^{8} \beta_{3i} PIB_{t-i} + \varepsilon_t$$

with PDL restrictions on the coefficients of antimicrobial use and where MAC means macrolide use, 3GC third-generation cephalosporin use, FQ fluoroquinolone use and PIB use of penicillins with β-lactamase inhibitors. The model was initially estimated on the full study period, i.e., January 1996–December 2000, using a degree q_j of the polynomial equal to 3. The estimated model was compatible with normal white noise errors (absence of autocorrelation and absence of heteroskedasticity), and no signs of nonmodeled nonlinearities were seen.

This initial model was then simplified to eliminate irrelevant antimicrobial drug uses and unnecessary lags. In the first steps of the simplification, all antimicrobial drugs were kept in the model, and the simplification took the form of reducing the order of the polynomial and eliminating unnecessary lags. Along this process, use of penicillins with β-lactamase inhibitors did not appear to play a significant role and was eliminated from the model. We also tried to introduce use of each of the other antimicrobial drug classes that showed a relationship in Table 3; however, none appeared to play an important role, and they were not included in the model. Further simplification of the distributed lags of macrolide use, third-generation cephalosporin use, and fluoroquinolone use of the %MRSA itself led to a model in which, through CUSUM and CUSUMSQ statistics, a structural change was detected around the middle of 1997. Application of the Chow test located the change in June 1997. The %MRSA was virtually zero in 1996 and started to increase at the beginning of 1997, which was consistent with the fact that the MRSA epidemic strain, resistant to macrolides and fluoroquinolones, only became predominant in 1997. In 1996, 56% and 50% of MRSA isolates were resistant to erythromycin and ciprofloxacin, respectively, whereas these percentages suddenly rose to 92% and 89%, respectively, in 1997. Data before June 1997 were considered as not being part of the outbreak and were therefore not included in the final model. The validity of the simplified, final model from June 1997 onwards was checked by a battery of specification and diagnostic tests to verify the absence of autocorrelation of residuals, absence of heteroskedasticity, normality of residuals, absence of nonmodeled nonlinearities and absence of structural change.

The basic measure of forecasting quality, Root Mean Squared Error of Forecast (RMSEF) was also computed, which provided an average measurement of the amount by which the model over- or underestimated the %MRSA. RMSEF was calculated for a model without antimicrobial drug use (based on past %MRSA only) and compared with that of the final model, which included antimicrobial drug use.

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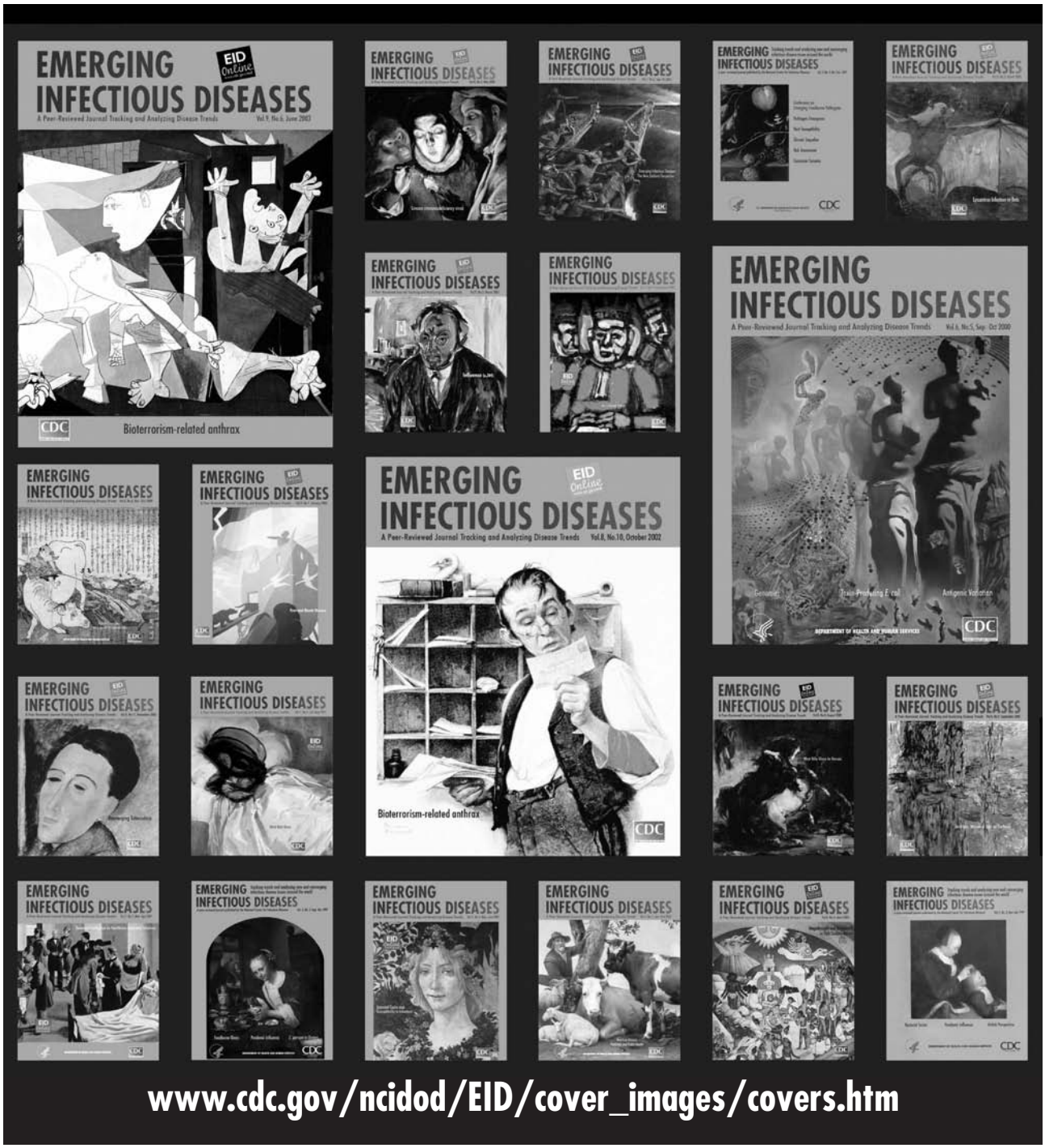

Acute Encephalitis Hospitalizations, California, 1990–1999: Unrecognized Arboviral Encephalitis?

Rosalie T. Trevejo*

Historically, Western equine encephalomyelitis and St. Louis encephalitis caused substantial human and equine illness and death in California. This study describes the epidemiology of encephalitis with data from 13,807 patients hospitalized in California with acute encephalitis from 1990 through 1999. The incidence of encephalitis hospitalizations decreased over this period. The greatest proportion of case-patients was hospitalized in the winter. Encephalitis of unspecified origin was the most common diagnosis, and arboviral encephalitis was the least common. Some California counties had concurrent increases in encephalitis rates and in detected arboviral activity in sentinel chickens and mosquito vectors. However, a diagnosis of arboviral encephalitis was made for few hospitalized patients in these counties during these periods. Although some arboviral encephalitis cases may have been undiagnosed, such cases probably did not occur frequently. Active hospital-based surveillance during periods of heightened sylvatic arboviral activity could increase detection of arboviral encephalitis.

Encephalitis, or inflammation of the brain, is a serious clinical syndrome with many potential infectious, postinfectious, and postimmunization causes (1,2). Recognized causes of infectious encephalitis in humans include, but are not limited to, herpes simplex viruses, arboviruses, lymphocytic choriomeningitis, mumps, cytomegalovirus, Epstein-Barr virus, human herpesvirus 6, and enteroviruses. The epidemiology of encephalitis in the United States is characterized by the predominance of cases with unknown origin (3–6).

Historically, Western equine encephalomyelitis (WEE) and St. Louis encephalitis (SLE) viruses (WEEV and SLEV, respectively) were important causes of encephalitis in California residents, particularly in the Central Valley and southern California (7). Since the 1960s, the incidence of WEE and SLE has decreased dramatically, although sporadic cases are still reported (7). Most recently, two small SLE epidemics were reported in Los Angeles and the Central Valley in 1984 and 1989, respectively (8,9). Reports of arboviral encephalitis cases are uncommon in southern California, despite evidence of endemic WEEV and SLEV activity in birds and mosquito vectors in that area (10). Sylvatic West Nile virus (WNV) activity was recently detected for the first time in California, which may contribute to changes in the epidemiology of central nervous system disease (11). The 1999 appearance of WNV in New York and adjacent states produced illness and death among humans, horses, and several avian species (12).

Healthcare providers and diagnostic laboratories in California are required to report human encephalitis cases to the California Department of Health Services (CDHS) under Title 17 of the California Code of Regulations. The code stipulates that the reporter identify the cause as viral, bacterial, fungal, or parasitic. Because this surveillance system is passive, human encephalitis cases may be underreported, even when the cause of encephalitis is identified (5). In this study, hospital discharge data were used to estimate the incidence of acute encephalitis and to provide a basis for comparison with the number of reported cases of encephalitis. In addition, the encephalitis hospitalization rates of districts with differing levels of sylvatic arboviral activity were compared. These data may provide a useful baseline to evaluate trends in encephalitis hospitalizations in California, including unusual occurrences of arboviral encephalitis. Such baseline data may prove useful given the recent detection of WNV in California and the potential for introduction of other arboviral agents.

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Materials and Methods

Data Sources

Hospital discharge data (public use version A) include information on approximately 3.5 million yearly discharges from all California hospitals that serve the civilian population; federal facilities or state hospitals for patients with mental disorders or developmental disabilities are excluded (13). The data do not contain patient names or other personal identifiers. Patients discharged from acute-care hospitals in California from 1990 through 1999 were the source population for the present study. Patients with acute infectious or unspecified encephalitis as the principal diagnosis or one of the 24 additional diagnoses were selected by using the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM codes listed in Table 1 [14]). Only data on the first hospitalization of patients with more than one encephalitis-related hospitalization were included in the analysis. When available, the record linkage number, based on an encrypted Social Security number, was used to identify patients with multiple hospitalizations. Patients with no record linkage number were assumed to have been hospitalized only once.

The annual number of reported encephalitis cases from 1990 through 1999 was obtained from CDHS, Division of Communicable Disease Control. Arboviral surveillance data were obtained from published reports (15–17).

Data Analysis

The average age- and sex-specific incidence rates of encephalitis (cases per 10^5 person-years) were calculated by using the California population projections for 1995 (18). These estimates were adjusted to account for the proportion of cases with missing demographic information by assuming that the proportion with missing data is the same within each subgroup. A 95% confidence interval was calculated for each group-specific rate. Such rates were

Table 1. Diagnoses for acute infectious or unspecified encephalitis among hospitalized patients in California, 1990–1999[a]

Diagnosis (ICD-9-CM[b])	No. of encephalitis diagnoses (%)[c]
Encephalitis of unspecified cause	
Unspecified cause of encephalitis (323.9)	4,841 (34.7)
Unspecified non-arthropod-borne viral diseases of CNS (049.9)	2,932 (21.0)
Viral encephalitis with specified cause, not arboviral	
Acute paralytic poliomyelitis specified as bulbar (045.0)	44 (0.3)
Other specified nonarthropodborne viral diseases of central nervous system (049.8)	288 (2.1)
Herpetic meningoencephalitis (054.3)	2,007 (14.4)
Encephalomyelitis due to rubella (056.01)	6 (0.04)
Rabies (071)	13 (0.1)
Mumps encephalitis (072.2)	14 (0.1)
Encephalitis in viral diseases classified elsewhere (323.0)	76 (0.5)
Other causes of encephalitis	
Other encephalitis due to infection classified elsewhere (323.4)	117 (0.8)
Other causes of encephalitis (323.8)	2,196 (15.8)
Postinfectious causes of encephalitis	
Postvaricella encephalitis (052.0)	421 (3.0)
Post measles encephalitis (055.0)	41 (0.3)
Postinfectious encephalitis (323.6)	595 (4.3)
Bacterial/rickettsial causes of encephalitis	
Tuberculous encephalitis or myelitis (013.6)	30 (0.2)
Meningococcal encephalitis (036.1)	128 (0.9)
Syphilitic encephalitis (094.81)	11 (0.1)
Encephalitis in rickettsial diseases classified elsewhere (323.1)	0 (0)
Parasitic/protozoal causes of encephalitis	
Meningoencephalitis attributable to toxoplasmosis (130.0)	82 (0.6)
Meningoencephalitis attributable to *Naegleria* (136.2)	10 (0.1)
Encephalitis in protozoal diseases classified elsewhere (323.2)	4 (0.03)
Arthropodborne viral encephalitis	
Mosquitoborne viral encephalitis (062.0–062.9)	63 (0.5)
Tickborne viral encephalitis (063.0–063.9)	6 (0.04)
Viral encephalitis transmitted by other and unspecified arthropods (064)	14 (0.1)
Total no. of encephalitis diagnoses[d]	13,939

[a]Patients with a concurrent diagnosis of AIDS are excluded.
[b]International Classification of Diseases, 9th Revision, Clinical Modification.
[c]Source: Office of Statewide Health Planning and Development, Patient Discharge Data, Public Version A.
[d]Total number of encephalitis diagnoses is greater than the number of encephalitis patients (n = 13,807) because some patients had two or more encephalitis diagnoses.

compared by using a chi-square test for proportions. Annual incidence rates were calculated by using annual population estimates for California (19). Linear trends in proportions were evaluated by using a chi-square test for trend (20). Significance probabilities <0.05 (p values) were considered a strong indication of systematic influence (i.e., not chance variation).

Encephalitis hospitalization rates were evaluated separately for Sacramento and Yolo, Sutter and Yuba, and Riverside and Imperial Counties. These counties had a sufficient population size to allow calculation of meaningful rates and had reported sylvatic arboviral activity during the study period. The number of encephalitis cases was insufficient to calculate county-specific rates for each category of encephalitis and therefore the rates are for all encephalitis hospitalizations. Data were combined for Sacramento-Yolo and Sutter-Yuba to reflect the collection of arboviral surveillance data by a single bi-county agency for each of these districts, and for Riverside-Imperial to reflect their proximity and similarity in sylvatic arboviral activity. Temporal trends in encephalitis rates from 1991 through 1999 were examined in conjunction with arboviral surveillance data on sentinel chicken seroconversions to WEEV and SLEV and on virus-positive mosquito pools. The county-specific encephalitis rates for years with increased sylvatic arboviral activity were compared with the average rate for the remaining years by using a two-sample test for equality of proportions. The county of residence for patients hospitalized in 1990 was not available, precluding the inclusion of data from that year in the county-level analyses. Analyses were conducted with EpiInfo 6 (version 6.04d; Centers for Disease Control and Prevention, Atlanta, GA), SAS (version 8; SAS Institute, Cary, NC), and S-Plus 2000 (Professional Release 3; MathSoft, Inc., Seattle, WA).

Results

From 1990 through 1999, a total of 17,318 patients were hospitalized with acute encephalitis; 3,511 (20.3%) had a concurrent diagnosis of AIDS. The proportion of encephalitis patients per year with AIDS decreased from 27% in 1990 to 9.5% in 1999 (chi-square test for trend = 475.9, $p < 0.001$). Annual rates for patients with and without AIDS are shown in Figure 1. All subsequent analyses were limited to the 13,807 patients without a concurrent diagnosis of AIDS because of the distinct epidemiologic characteristics of these two populations.

Unspecified encephalitis made up most of the encephalitis diagnoses (55.7%), followed by specified viral encephalitis (not arboviral) (17.6%) and "other" causes of encephalitis (16.6%) (Table 1). Arthropodborne viral disease constituted <1% of the encephalitis diagnoses, with a total of 83 diagnoses among 82 patients. Some patients had more than one ICD-9-CM code for encephalitis. Thus, the total number of diagnoses was greater than the number of patients (Table 1).

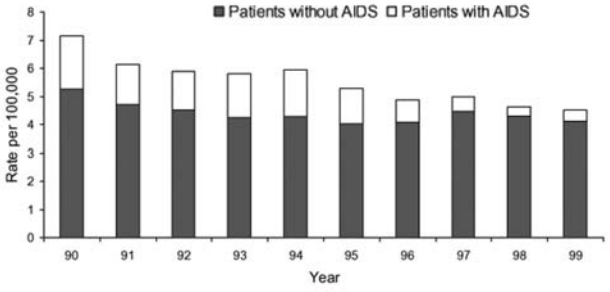

Figure 1. Cumulative incidence of encephalitis hospitalizations in California, 1990–1999 (n = 17,318).

The encephalitis rate was highest in infants (<1 year old), followed by persons ≥65 years of age (Table 2). The lowest rate was in persons 20–44 years of age. The chi-square test for proportions indicated that the difference in the rates between each age group was significant. Female patients had a rate that was significantly higher than that of male patients.

A comparison of the annual number of patients hospitalized with encephalitis with the number of encephalitis cases reported to CDHS is shown in Figure 2. On average, the number of patients hospitalized with acute infectious or unspecified encephalitis was 10-fold higher than the number of encephalitis cases reported to CDHS.

The epidemiology of encephalitis in specific counties with sylvatic arboviral activity over the study period was further examined to evaluate the potential role of undiagnosed arboviral encephalitis. Most patients hospitalized with encephalitis in Sacramento-Yolo, Sutter-Yuba, and Imperial-Riverside Counties were diagnosed with unspecified encephalitis (Table 3). Only 10 patients were diagnosed with arthropodborne viral encephalitis, 2 from Sacramento-Yolo, 1 from Sutter-Yuba, and 7 from Riverside-Imperial. The number of admissions per quarter was distributed fairly evenly for each bi-county area when the data from 1991 through 1999 were combined (Table 3). In no case did the proportion of hospitalizations for any given quarter differ significantly from the null value of 25% (one-sample test for proportions: $p > 0.05$).

Annual encephalitis rates are shown for Sacramento-Yolo (Figure 3), Sutter-Yuba (Figure 4), and Imperial-Riverside (Figure 5) Counties from 1991 through 1999. The encephalitis rates in these areas increased during some years when increased arboviral activity was detected in sentinel chickens flocks and mosquito pools (Table 4). For instance, the encephalitis rate in Sutter-Yuba increased in 1997 (two-sample test for proportions: $p = 0.041$), when 41 sentinel chickens seroconverted to WEEV. A smaller increase in the encephalitis rate was observed in

Table 2. Characteristics of patients hospitalized with acute infectious or unspecified encephalitis, California, 1990–1999[a]

	No. of patients[b]	1995 population[c]	Rate (95% CI)[d]
Sex			
Male	6,684	16,062,552	4.2 (4.1–4.3)
Female	7,123	16,000,360	4.5 (4.3–4.6)
Age group, y			
<1	868	552,649	15.7 (14.7–16.8)
1–4	973	2,356,048	4.1 (3.9–4.4)
5–19	2,350	6,801,354	3.5 (3.3–3.6)
20–44	4,157	12,964,498	3.2 (3.1–3.3)
45–64	2,707	5,958,743	4.5 (4.4–4.7
65+	2,752	3,429,620	8.0 (7.7–8.3)
Overall	13,807	32,062,912	4.3 (4.2–4.4)

[a]Patients with a concurrent diagnosis of AIDS are excluded.
[b]Source: Office of Statewide Health Planning and Development, Patient Discharge Data, Public Version A.
[c]Population projections, State of California, Department of Finance.
[d]Cases per 10^5 person-years = (frequency/[1995 population x 10]) x 100,000; CI = confidence interval.

Sacramento-Yolo in 1996 and 1997 (two-sample test for proportions: p = 0.028), when 20 and 18 sentinel chickens seroconverted to WEEV, respectively. In contrast, the encephalitis rates did not increase in Sacramento-Yolo and Sutter-Yuba in 1993, when increased WEEV activity was detected in sentinel chickens and mosquito pools. In Riverside-Imperial, a small increase in the encephalitis rate in 1991 (two-sample test for proportions, p = 0.004) corresponded with an increase in detected WEEV and SLEV activity in sentinel chickens and mosquito pools. The proportion of encephalitis hospital admissions was higher than expected (>25%) during the summer (July–September) in Sutter-Yuba in 1997 (9/13 [69.2%]) and in Imperial-Riverside in 1991 (48/161 [29.8%]) (one-sample test for proportions: p = 0.008 and p = 0.187, respectively). No such increase occurred in Sacramento-Yolo in 1996 and 1997 (34/135 [25.2%]).

Discussion

The encephalitis rate showed an overall decrease during the study period. The high proportion of patients with unspecified encephalitis in this study (Table 1) is consistent with findings from other studies and raises questions about potential causes of these encephalitis cases (3,5). Arboviral encephalitis was diagnosed in <1% of patients hospitalized with acute encephalitis from 1990 through 1999. This finding indicates that this type of encephalitis is either exceedingly rare in California or underdiagnosed. In the absence of public health alerts during periods of epizootic arboviral activity, clinicians may be disinclined to pursue laboratory testing for arboviral agents because of a low index of suspicion. Furthermore, outside of academic interest, clinicians may not have much incentive to request laboratory testing for specific agents for patients with viral encephalitis if a specific diagnosis will not change the course of treatment.

ICD-9-CM codes used in the hospital discharge database provide a standardized means of comparing data between hospitals. A previous study used ICD-9-CM codes in the National Hospital Discharge Survey to describe the epidemiology of encephalitis (4). In both studies, the age-specific encephalitis rates were highest among infants (<1 year) and the elderly (≥65 years) (Table 2). This finding may be due in part to infection with herpes simplex, which is a common cause of nonepidemic, acute encephalitis that occurs most frequently in children and the elderly (6). Patients with AIDS, 92.5% of whom were men, were excluded in the present study, which likely resulted in a higher proportion of females compared to the national study.

An advantage of using hospital discharge data to study the epidemiology of encephalitis is that most patients with encephalitis are likely to be hospitalized because of the severity of the illness. Accordingly, these findings are more readily generalized to the population of California, unlike the passive surveillance data, which are limited by underreporting (21). In the present study, the annual number of hospitalized encephalitis patients was approximately 10-fold greater than the annual number of reported cases (Figure 2). The actual degree of underreporting may be less, as not all of the hospitalizations for encephalitis may have been due to reportable causes. However, evidence exists that arboviral encephalitis was underreported in

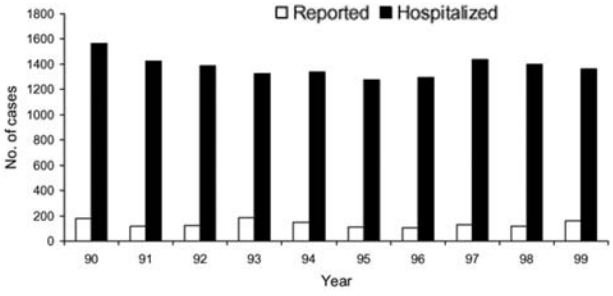

Figure 2. Comparison of hospitalized versus reported encephalitis in California, 1990–1999. Hospitalized patients with a concurrent diagnosis of AIDS were excluded.

Table 3. Diagnoses and month of admission for patients hospitalized with acute infectious or unspecified encephalitis in selected California counties, 1991–1999

	Sacramento-Yolo	Sutter-Yuba	Imperial-Riverside
1995 Population estimates	1,271,500	135,400	1,500,300
Diagnosis (ICD-9-CM[a])	No. of encephalitis diagnoses (%) [b]		
Encephalitis of unspecified cause	305 (60.2)	45 (70.3)	338 (58.2)
Viral encephalitis with specified cause, not arboviral	74 (14.6)	11 (17.2)	112 (19.4)
Other causes of encephalitis	76 (15.0)	5 (7.8)	64 (11.1)
Postinfectious causes of encephalitis	38 (7.5)	1 (1.6)	45 (7.8)
Bacterial/rickettsial causes of encephalitis	10 (2.0)	0 (0)	7 (1.2)
Parasitic/protozoal causes of encephalitis	2 (0.4)	1 (1.6)	5 (0.09)
Arthropodborne viral encephalitis[c]	2 (0.4)	1 (1.6)	7 (1.2)
Total no. of encephalitis diagnoses [d]	507	64	578
Month of hospital admission	No. of admissions (%)		
January–March	112 (22.1)	17 (26.6)	160 (28.0)
April–June	126 (24.9)	12 (18.8)	127 (22.2)
July–September	139 (27.5)	15 (23.4)	140 (24.5)
October–December	129 (25.5)	20 (31.3)	145 (25.3)
Overall	506 (100)	64 (100)	572 (100)

[a]International Classification of Diseases, 9th Revision, Clinical Modification.
[b]Source: Office of Statewide Health Planning and Development, Patient Discharge Data, Public Version A.
[c]Arboviral encephalitis was diagnosed in Sacramento in 1992 (n = 1) and 1997 (n = 1), in Yuba in 1999 (n = 1), in Imperial in 1997 (n = 1), and in Riverside in 1991 (n = 3), 1994 (n = 2), and 1997 (n = 1).
[d]Total number of encephalitis diagnoses in Sacramento-Yolo and Imperial-Riverside is greater than the number of encephalitis patients because some patients had two or more encephalitis diagnoses.

California from 1990 through 1999, with 82 patients hospitalized with arthropodborne viral encephalitis but only 7 arboviral encephalitis cases reported. Encephalitis cases with an unspecified cause may also be disproportionately unreported, since encephalitis cases reported under the current passive surveillance system request that the reporter specify the cause as viral, bacterial, fungal, or parasitic.

A disadvantage of relying on the public use hospital discharge dataset to describe the epidemiology of encephalitis is the lack of patient identifiers. This fact may have resulted in multiple hospitalizations for individual patients being included, as evidenced by the number of hospitalizations for poliomyelitis and rabies (Table 1). Another disadvantage is the lack of information on laboratory test results used to make diagnoses. While the high proportion of unspecified encephalitis cases in the present study possibly resulted from underuse of appropriate diagnostic tests, other studies do not support this hypothesis. For instance, from 1956 through 1958, a total of 1,595 encephalitis patients were identified in Kern County through active hospital-based surveillance and evaluated by using a standard battery of tests (5). No cause was identified for 569 (36%) patients, and WEE and SLE accounted for ≤5% of cases per year. When advanced diagnostic methods were used, the cause of encephalitis was identified for only 126 (38%) of 334 patients referred to the California Encephalitis Program from June 1998 through December 2000; no patients with arboviral encephalitis were identified (3). These findings raise the possibility that current diagnostic tests may simply be inadequate for identifying all possible causes of encephalitis.

Given the limited number of encephalitis hospitalizations in any given county, all encephalitis diagnoses were combined to provide a meaningful examination of county-specific trends. Combining these diagnoses may have obscured trends in specific disease agents, although most of the patients in Sacramento-Yolo, Sutter-Yuba, and Riverside-Imperial had a diagnosis of unspecified encephalitis (Table 3). In Sacramento-Yolo and Sutter-Yuba, concurrent increases in sylvatic WEEV transmission and in the rates of encephalitis hospitalizations in 1997 occurred (Table 4; Figures 3 and 4), increasing the likelihood that a proportion of the unspecified encephalitis cases may have been due to arboviral encephalitis. A similar pattern was observed in Imperial and Riverside Counties in 1991, when levels of sylvatic WEEV and SLEV transmission were particularly high (Table 4 and Figure 5). The proportion of hospital admissions for

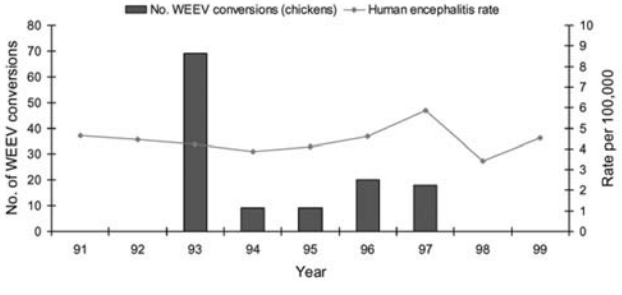

Figure 3. Annual rate of encephalitis hospitalizations and annual number of sentinel chicken seroconversions to Western equine encephalomyelitis virus (WEEV) infection, Sacramento and Yolo Counties, California, 1991–1999. Hospitalized patients with a concurrent diagnosis of AIDS were excluded.

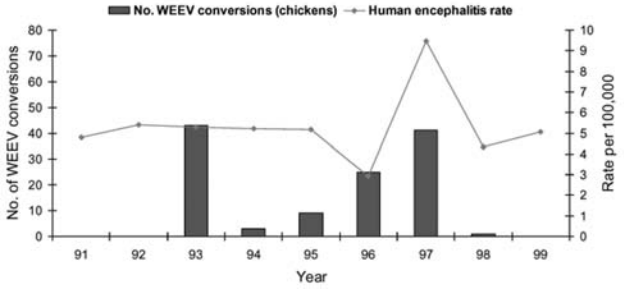

Figure 4. Annual rate of encephalitis hospitalizations and annual number of sentinel chicken seroconversions to Western equine encephalomyelitis virus (WEEV) infection, Sutter and Yuba Counties, California, 1991–1999. Hospitalized patients with a concurrent diagnosis of AIDS were excluded.

encephalitis in Sutter-Yuba in 1997 and Imperial-Riverside in 1991 increased during the summer months, when arboviral and enteroviral transmission most commonly occur (1). In contrast, statewide hospital admissions for encephalitis were significantly higher during the winter months, which indicates that arboviral encephalitis is typically an unimportant contributor to encephalitis hospitalizations. A study of encephalitis patients in California from 1956 through 1958 also found an unexplained increase in the proportion of cases with undetermined origin during the winter (5). One possible cause, lymphocytic choriomeningitis, occurs most commonly in the winter, although it is thought to be rare (1).

The arboviral surveillance data used in the present study lacked denominator data on mosquito pools and sentinel chicken specimens tested for most years. In addition, mosquito pool and sentinel chicken surveillance is not uniform across mosquito control districts. In spite of these limitations, notable increases in arboviral activity in mosquito pools (Table 4) and sentinel chickens (Figures 3–5) were observed in some years. Many potential reasons exist for the lack of consistent correlation between sylvatic arboviral activity and encephalitis rates. One possibility is that mosquito population indices, sylvatic arboviral transmission levels, or both, were not always sufficient to increase the risk for human infection. For instance, *Culex tarsalis* population indices are correlated with sentinel chickens seroconversion rates for WEEV and SLEV (22). A retrospective study of a 1989 SLE epizootic in the Central Valley, with high *Cx. tarsalis* abundance, 70 virus-positive mosquito pools, and seroconversion of 71% of sentinel chickens, identified 28 (43%) of 65 aseptic meningitis and encephalitis patients as SLE patients (9,23). In the present study, the encephalitis rate in Imperial-Riverside increased slightly in 1991, when WEEV and SLEV activity was detected in many mosquito pools and sentinel chickens, but remained relatively unchanged in 1994 and 1995, when many sentinel chickens seroconverted but few mosquito pools were virus-positive. Another possible contributor to the study findings is variation in the virulence of circulating arboviruses over the study period. Three phenotypes of WEEV, which differed in their virulence properties in adult mice, were isolated from mosquito pools collected in California from 1991 through 1995 (24). Lastly, sylvatic arboviral activity and encephalitis rates may not be correlated. For instance, no encephalitis cases were detected during an intense WNV epizootic in Connecticut (25). In fact, an aseptic meningitis epidemic attributable to enteroviruses was detected during an avian epizootic of WNV, while no WNV meningitis cases were detected (26).

Many factors could explain the observed decrease in the incidence of clinical WEE and SLE cases since the 1960s. Mosquito control and water management programs have been effective at reducing mosquito vector populations (27). Changes in human behavior may also have coincided with the decrease in the incidence of arboviral illness (28). With the advent of television and air-conditioning, people are more likely to remain indoors during twilight hours, when peak feeding by vector species takes place. Earlier research showed rural residents to be at higher risk for arboviral illness than urban residents (29). With changes in land use over the past century, a greater proportion of the human population now resides in urban and suburban settings. A 1995 California study of outpatients attending county health department clinics found a significantly higher seroprevalence of WEEV among residents of rural Imperial and Sutter Counties than Sacramento County residents; the seroprevalence for SLEV was significantly higher in Imperial than in both Sacramento and Sutter Counties (30). However, although this study was conducted in areas with both sporadic and enzootic WEEV and SLEV transmission, the overall seroprevalence levels for both viruses were low.

The methods used in the present study are useful for evaluating trends in the incidence of emerging or potentially emerging diseases. The epidemiology of arboviral

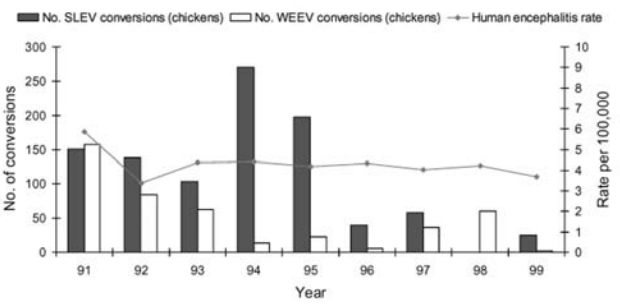

Figure 5. Annual rate of encephalitis hospitalizations and annual number of sentinel chicken seroconversions, Imperial and Riverside Counties, California, 1991–1999. Hospitalized patients with a concurrent diagnosis of AIDS were excluded. SLEV, St. Louis encephalitis virus; WEEV, Western equine encephalitis virus.

Table 4. SLEV- and WEEV-positive *Culex tarsalis* pools in selected California counties, 1991–1999[a,b]

	No. of virus-positive mosquito pools (no. pools tested [c]			
	Sacramento-Yolo	Sutter-Yuba	Imperial-Riverside	
Year	WEEV	WEEV	WEEV	SLEV
1991	0	0	73	44
1992	0	0	20	10
1993	81	31	13	15
1994	4	0	1	7
1995	2	1	0	0
1996	9	2	0	1
1997	10	14	0	0
1998	0 (187)	0 (172)	11 (968)	1 (968)
1999	0 (548)	0 (232)	0 (918)	0 (918)

[a]SLEV, St. Louis encephalitis virus; WEEV, Western equine encephalitis virus.
[b]Sources: Hui e t al., 1999 (1991 –1997); CDHS, Vector-Borne Disease Section, Annual Reports (1998 & 1999).
[c]The number of mosquito pools tested (denominator) was only available for 1998 and 1999.

encephalitis will likely change with the establishment of WNV in California, making active hospital-based surveillance for arboviral disease an important supplement to traditional passive reporting. These findings suggest that unrecognized arboviral encephalitis has not constituted a large proportion of the unspecified encephalitis patients who were hospitalized from 1991 through 1999. However, the study results do indicate the potential utility of intensified surveillance efforts during periods of increased sylvatic arboviral activity. During such periods, implementing active hospital-based surveillance for encephalitis, acute flaccid paralysis, and aseptic meningitis, with collection and testing of diagnostic specimens, may result in detecting cases of arboviral disease that would otherwise go undiagnosed and unreported.

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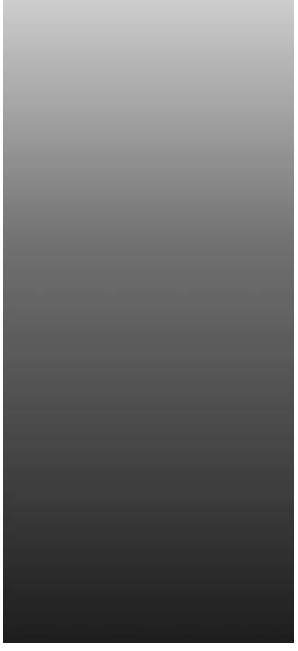

Human Herpesviruses 6 and 7 and Central Nervous System Infection in Children[1]

Asad Ansari,*†[2] Shaobing Li,* Mark J. Abzug,*† and Adriana Weinberg*†

The role and frequency of human herpesviruses (HHV)-6 and -7 in central nervous system (CNS) diseases of children are unclear. Cerebrospinal fluid samples from 245 pediatric patients (median age 43 days), submitted for evaluations of possible sepsis or of neurologic symptoms, were tested for HHV-6 and HHV-7 DNA by polymerase chain reaction. HHV-6 DNA was found in 3 of 245 samples, and HHV-7 was found in 0 of 245 samples. The three patients with HHV-6 DNA were <2 months of age. HHV-6 was likely pathogenic in two patients with meningitis, who lacked evidence of another microbiologic cause. HHV-6 and HHV-7 are uncommon causes of CNS infection in children. HHV-6 may occasionally cause meningitis in young infants.

Human herpesvirus (HHV)-6 and HHV-7 are ubiquitous T-lymphotropic viruses that infect most humans. Infections with either agent occur primarily during childhood. Seroprevalence of HHV-6 reaches >80% in children >2 years (1–3). Antibody prevalence for HHV-7 reaches 75% in 3- to 6-year-old children and 98% in adults (3–5). HHV-6 and HHH-7 have been associated with a variety of clinical manifestations, including fever, rash, and seizures (6–10). Immunocompromised hosts, particularly transplant recipients, are at increased risk for symptomatic primary or reactivation disease associated with HHV-6 or HHV-7 (11–13).

The role of HHV-6 and 7 in central nervous system (CNS) disease is an area of ongoing investigation. The range of CNS manifestations ascribed to these viruses includes asymptomatic infection, febrile convulsions, seizure disorders, meningitis, meningoencephalitis, facial palsy, vestibular neuritis, demyelinating disorders, hemiplegia, and, rarely, fatal encephalitis (14–18). Investigators have been unable to culture HHV-6 or HHV-7 from cerebrospinal fluid (CSF) (14). However, HHV-6 and HHV-7 DNA have been detected in CSF and other body fluids by polymerase chain reaction (PCR), which implicates these viruses in neurologic disorders. HHV-6 DNA was identified in CSF of 14.8% of children evaluated for fever, sepsis, or seizures, with higher prevalence found among children with seizures (16). HHV-6 DNA was also detected in CSF of 70% to 90% of children who had neurologic symptoms during their primary HHV-6 infection, with a disproportionate association with recurrent febrile seizures (17). In a case-control study, HHV-6 DNA was found in CSF of 23% of patients who received an allogeneic bone marrow transplant who had CNS symptoms; it was found in <1% of patients with hematologic malignancies without neurologic symptoms (18). Other investigators have found a much lower prevalence (0%–4%) of HHV-6 DNA in CSF of AIDS patients with neurologic symptoms and in CSF of children with febrile seizures (19,20). Similarly, although HHV-7 DNA has been detected in CSF of as many as 8.8%–14% of children with neurologic symptoms (21,22), other studies have found a lower prevalence (0%–2%) in CSF of AIDS patients with neurologic symptoms and in children with febrile seizures (19,20).

Because of the conflicting results in the medical literature, the frequency at which HHV-6 and HHV-7 are associated with neurologic disease is unclear. The goal of this study was to further define the role of HHV-6 and HHV-7 as causes of CNS disease in children.

Materials and Methods

Study Design

The study, approved by the University of Colorado Multiple Institutional Review Board, was conducted with all CSF clinical samples from pediatric patients submitted for herpes simplex virus (HSV) PCR to the Clinical Virology Laboratory at the University of Colorado from December 1998 through February 2000. When multiple specimens were submitted for one patient, only the first one was tested. Specimens positive for other microorganisms were not excluded. Peripheral blood specimens from

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these patients were not available to study. Information regarding demographics, clinical manifestations, diagnostic studies, management, discharge diagnosis, and outcome was gathered by retrospective chart review for patients seen at The Children's Hospital, Denver. CNS diagnoses and classification of seizures were based on the assessments of the primary treating physicians.

Definitions

Infectious and postinfectious encephalitis were defined as the presence of encephalopathy or focal neurologic abnormalities, an abnormal CSF profile but negative CSF microbiologic studies, and a history or serologic result consistent with a current or preceding acute infectious illness. CSF pleocytosis was defined as >25 leukocytes x 10^6/L for preterm neonates, >22 leukocytes x 10^6/L for term neonates, and >7 leukocytes x 10^6/L for all other patients. Infections not involving the CNS were classified as other infections.

HHV-6 PCR

HHV-6 PCR was performed (23) with the following primers and probes (24): 5′ AAG CTT GCA CAA TGC CAA AAA ACA G (17627–17603), 5′ AAC TGT CTG ACT GGC AAA AAC TTT T (17405–17429), and 5′ AAC TGT CTG GCA AAA ACT TTT (17516–17492). DNA was extracted from 50-µL aliquots of CSF previously stored at –70°C with Chelex purification matrix (Bio-Rad Laboratories, Inc, Hercules, CA). PCR was carried out in 50-µL mixtures containing 20 µL of extracted DNA, 1.25 units of PfuI (Stratagene, La Jolla, CA), 200 µmol/L of each of four deoxynucleoside triphosphates, and 0.5 µmol/L of each primer in PfuI buffer (Stratagene). The samples were amplified in duplicate for 45 cycles. The amplified DNA was separated according to molecular weight by using 3% agarose gel electrophoresis and transferred to nylon membranes. The identity of the DNA band (223 bp) was confirmed by detection with a digoxigenin-conjugated probe, antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim Biohemicals, Indianopolis, IN), and chemiluminescent substrate (Tropix, Bedford, MA). Each patient sample was run in duplicate. Each assay included two negative controls (water) and two positive controls (100 genomic copies of HHV-6 DNA/reaction tube). The tests were considered valid if the controls yielded the expected results, and a specimen was considered positive if both replicates tested positive. Specimens with discordant replicates were reanalyzed in an independent run and considered positive if ≥50% of replicates gave positive results. The ability to detect HHV-6 DNA after at least four freeze-thaw cycles remained intact.

The analytical sensitivity of HHV-6 PCR was 8–10 copies of genomic DNA per reaction tube, as determined by serial dilutions of a sample with known DNA copy number. To rule out the presence of inhibitors in the extracted DNA, 20 HSV-negative CSF samples were spiked with the equivalent of 2 HSV DNA copies per PCR reaction tube. All samples yielded a positive result for HSV, whereas the unspiked specimens remained HSV-negative. The ability to detect HHV-6 strains from different geographic regions was confirmed by using 14 HHV-6–containing specimens obtained from diverse laboratories with recognized expertise in diagnosing HHV-6 infections as well as our laboratory. No cross-reactivity occurred with other DNA viruses, including other herpesviruses, parvovirus, adenovirus, and polyomavirus, thereby establishing the specificity of the HHV-6 PCR.

HHV-7 PCR

HHV-7 PCR was performed by using the methods described for HHV-6 with modified sample preparation. The primers were 5′ TAT CCC AGC TGT TTT CAT ATA GTA AC and 5′ GCC TTG CGG TAG CAC TAG ATT TTT TG, and the probe was 5′ AGA ATT CTG TAC CCA TGG GCA CAT TTG TAC (25; GenBank accession no. L03525). The PCR product was 186 bp long. The assay included two negative controls (water) and two positive controls (100 genomic copies of HHV-7 DNA per reaction tube).

The sensitivity of HHV-7 PCR was 8–10 copies of DNA per reaction tube, as determined by detecting HHV-7 DNA from samples extracted from infected cells. Specimens were prepared by boiling aliquots of nonhemorrhagic CSF for 55 s. If CSF contained blood, DNA was extracted with Qiagen DNA extraction kit (Qiagen, Valencia, CA). Testing with control viral DNA indicated that boiling did not affect the sensitivity of the PCR. The presence of inhibitors was ruled out by spiking 20 HSV-negative CSF samples with the equivalent of two HSV DNA copies per reaction tube and showing that all spiked samples became HSV-positive, whereas unspiked samples remained negative. The ability to detect HHV-7 DNA after at least three freeze-thaw cycles remained intact.

Results

Study Population

CSF samples obtained from 245 patients were tested for HHV-6 and HHV-7 DNA by PCR. Clinical data were available for the 218 patients hospitalized at The Children's Hospital, Denver (Table 1). The median age was 43 days; 68% were ≤2 years of age; 13% were 2–6 years, and 19% ≥6 years. Fever occurred in 56% of patients, rash in 11%, and seizures in 25%. CNS disorder diagnoses at discharge included meningitis in 61 patients (enteroviral, 21; bacterial, 4; aseptic/indeterminate etiology, 36), encephalitis in 21 patients (HSV, 4; acute dissem-

inated encephalomyelitis, 4; infectious/postinfectious, 13), febrile seizures in 7 patients, and seizure disorder in 29 patients. A non-CNS disease had been diagnosed in the remaining 100 patients, which included a diagnosis of other infections in 60. Nineteen patients were immunocompromised (leukemia, 6; posttransplant, 3; premature, 3; HIV, 2; others, 5), of whom 1 had a febrile seizure, 1 had encephalitis, 2 had meningitis, and 5 had other infections. CSF pleocytosis was found in 90 (43%) of 209 patients for whom data were available.

HHV-6 Infections of CNS

PCR identified HHV-6 DNA in CSF of 3 of 245 patients (Table 2). Patient 1, a 5-day-old full-term male infant of a diabetic mother, had a generalized seizure on the day he was born. His seizure was attributed to hypoglycemia because his concurrent glucose level was 0 mmol/L. On day 5 of life, a fever of 38.3ºC developed for which he underwent a complete workup for sepsis. Results of a physical examination were normal with no signs or symptoms of neurologic dysfunction. His blood count was remarkable for a leukocyte count of 8,600 x 10^6/L (46% neutrophils and 21% bands) and a platelet count of 40,000 x 10^6/L. His CSF showed 40 leukocytes x 10^6/L (20% neutrophils, 9% bands, 31% lymphocytes, 33% monocytes, and 6% macrophages), 4,110 erythrocytes x 10^6/L, protein 169 g x 10^{-2}/L, and glucose 2.3 mmol/L. CSF HSV PCR, viral culture, and bacterial cultures of CSF and blood and urine were negative. Diagnosis at discharge was aseptic meningitis. The patient recovered spontaneously without sequelae.

Patient 2 was a 38-day-old full-term female infant who had a fever of 39.2ºC and irritability. The physical examination showed a few vesicular lesions on the tonsillar pillar as well as a diffuse, erythematous, macular blanching rash. CSF studies found 19 leukocytes x 10^6/L (34% lymphocytes, 12% monocytes, and 55% macrophages), 1 erythrocyte x 10^6/L, protein 48 g x 10^{-2}/L, and glucose 2.3 mmol/L. PCRs of CSF for HSV and enterovirus and bacterial cultures of CSF, blood, and urine were negative. She recovered spontaneously without sequalae. Her discharge diagnosis was aseptic meningitis.

Patient 3 was a male infant of 24 weeks' gestation with respiratory distress syndrome. A blood culture was taken on day 10 of life because of worsening respiratory distress; results were positive for *Candida*. A lumbar puncture was performed when the blood grew *Candida*. The patient did not have any neurologic symptoms or signs. CSF had 1 leukocyte x 10^6/L, 1,780 erthrocytes x 10^6/L, protein 105 g x 10^{-2}/L, and glucose 8.1 mmol/L. CSF studies, including PCRs for HSV and enterovirus, as well as bacterial, viral, and fungal cultures, were negative. He subsequently died of the complications of prematurity and candidal sepsis, which were unrelated to the CNS.

Table 1. Characteristics of the study population with available clinical data [a]

Characteristic	No. patients (%)
Demographic	
Age: median (range)	43 days (0–5,948 days)
<2 y	148 (68)
2–6 y	28 (13)
>6 y	42 (19)
Male:female	1.1:1
Clinical and laborato ry	
Fever	121 (56)
Rash	24 (11)
Seizures	53 (25)
Seizure disorder	29 (55)
Febrile	7 (13)
Metabolic	5 (9)
Infectious/postinfectious	5 (9)
Intracranial bleed	4 (8)
Hypoxia	3 (6)
Meningitis	61 (28)
Encephalitis	21 (10)
CSF pleocytosis	90 (43)
Immunocompromised	19 (9)

[a]N = 218; CSF, cerebrospinal fluid.

All 3 were <2 months of age, and 2 had CSF pleocytosis. Overall, HHV-6 DNA was detected in 2 (2.2%) of 90 specimens from patients with pleocytosis and in 2 (3.3%) of 61 patients with a discharge diagnosis of meningitis. HHV-6 DNA was not found in any patients who had encephalitis, febrile seizures, or a seizure disorder. None of the 19 immunocompromised patients had HHV-6 DNA in CSF.

HHV-7 Infections of CNS

HHV-7 DNA was not found in any of the 245 CSF samples tested by PCR. Spiking random samples with HHV-7 DNA generated positive PCR results.

Discussion

HHV-6 DNA was uncommon in CSF of children (1.2%), and HHV-7 DNA was not detected in any CSF specimen in this study. HHV-6 DNA was found in a small percentage of meningitis patients (3.3%), but neither HHV-6 nor HHV-7 DNA was detected in samples from any patients with encephalitis, febrile seizures, or seizure disorders. Our limited detection of HHV-6 and HHV-7 contrasts with results of other studies, such as the report of Caserta et al. (16), in which HHV-6 DNA was found in CSF of 14.8% of children evaluated for fever, sepsis, or seizures, and studies by Pohl-Koppe et al. (21) and Yoshikawa et al. (22), in which HHV-7 DNA was found in CSF of 8.8% to 14% of children with neurologic symptoms. Our findings may have differed from previous reports for the following reasons: 1) differences in PCR sensitivity; 2) differences in degree of intactness of DNA in specimens that were thawed after being frozen for prolonged periods; 3) geographic varia-

Table 2. Characteristics of patients with HHV-6 DNA detected in cerebrospinal fluid[a]

Patient	Age (d)	Temperature maximum (°C)	Rash	Seizure	CSF leukocyte count (cells/μL)	Outcome	HHV-6 directed treatment
1	5	38.3	No	Yes[b]	40	Alive	None
2	38	38.4	Yes	No	19	Alive	None
3	10	38.7	No	No	1	Deceased	None

[a]CSF, cerebrospinal fluid; HHV-6, human herpesvirus 6.
[b]Infant of a diabetic mother who had a seizure on the day he was born. His serum glucose level was 0. Lumbar puncture was performed for a new fever on day 5 of life.

tion; or 4) differences in patient populations and, particularly, differences in age groups.

Technical laboratory problems were unlikely. Our assays are as sensitive or more sensitive than those previously reported that use plasmid-encoded DNA, and we detected a panel of samples identified by other laboratories, which use different primers or probe sets. However, these latter experiments were limited in number and could not completely rule out sensitivity differences related to the primers and probe sets. The presence of inhibitors was ruled out by spiking experiments. We tested the effect of freezing and thawing by subjecting several samples to three to four freeze-thaw cycles and found good preservation of both HHV-6 and HHV-7 DNA. Also, the PCR method used in this study detected cytomegalovirus DNA in CSF specimens stored at –70°C for up to 6 years (23). Yoshikawa et al. (22), who reported a 16% incidence of HHV-6 and HHV-7 in their pediatric patients with neurologic disorders, detected the viral DNA in the CSF cell pellet, but not in the liquid phase. Other investigators, however, who found a high incidence of HHV-6 and HHV-7 in pediatric patients with neurologic disorders (16,21), recovered viral DNA from liquid CSF.

Our results are not likely to be biased by the study population. Although lower seroprevalence rates have been found in some regions of the world, for example, Morocco (20% for HHV-6) and northern Japan (44% for HHV-7) (5,26), no evidence has been found of low seroprevalence of HHV-6 and HHV-7 infections in specific regions of the United States. Also, the demographic characteristics of our study population were similar to those of the 487 children described by Caserta et al. (16), including a median age similar to the median age of 1.5 months (range 0.1–36) in the latter series (16). The populations evaluated by Yoshikawa et al. (22) and Pohl-Koppe et al. (21) were somewhat older, with mean ages of 6 and 2.8–6 years, respectively. However, the number of patients >2 years of age included in our study population was similar to the numbers of participants enrolled in these two studies (43 and 68, respectively). Therefore, age differences do not appear to explain the discrepant findings.

The clinical features of patients 1 and 2 and the absence of another identified infectious agent suggest that HHV-6 may have been the cause of fever, pleocytosis, and the clinical aseptic meningitis diagnosed in these two infants. Other studies have not found an association between HHV-6 DNA in CSF and CSF pleocytosis. However, we note that the CSF of patient 1 was contaminated by blood. Therefore, the presence and magnitude of pleocytosis are difficult to state with certainty; moreover, we cannot be certain whether HHV-6 DNA was truly present in CSF or derived from contamination with peripheral blood. In patient 3, the identification of a well-established pathogen and the normal results of neurologic and CSF examinations suggest that HHV-6 DNA in the CSF may have represented an asymptomatic infection or contamination with peripheral blood. The cases of HHV-6 infection of CNS occurred in infants <2 months of age, and two occurred in the early neonatal period. Infections in newborns have been previously described. Although horizontal transmission through saliva is postulated to be the most common method of transmission of HHV-6 and HHV-7 (27–29), infection in neonates as well as detection of HHV-6 DNA in aborted fetuses' cord blood and in the female genital tract suggest that transplacental or perinatal transmission is possible (30–33).

Our study included 29 patients with CNS disorders caused by a known pathogen other than HHV-6 and HHV-7 (enterovirus, herpes simplex virus, bacterial pathogen); none of these patients had HHV-6 or HHV-7 DNA in CSF. This finding suggests that HHV-6 or HHV-7 DNA is not frequently detected in CSF in association with other CNS infections, as might be expected if these viruses established latency in CNS and reactivated nonspecifically with other pathogens. Conversely, this finding supports the idea that detecting HHV-6 or HHV-7 DNA in CSF of patients with neurologic disorders is consistent with a pathogenic role. Our study population included a modest number of immunocompromised patients of whom a small subset had neurologic diagnoses. These numbers are insufficient to ascertain the roles of HHV-6 and HHV-7 in neurologic disease in immunodeficient patients.

In conclusion, our data indicate that HHV-6 and HHV-7 are uncommon causes of CNS infection in children. HHV-6 may occasionally cause meningitis in young infants. Further studies are required to define the role of HHV-6 and HHV-7 in neurologic disorders in immunocompromised hosts.

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Group G Streptococcal Bacteremia in Jerusalem

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Group *G Streptococcus* (GGS) can cause severe infections, including bacteremia. These organisms often express a surface protein homologous to the *Streptococcus pyogenes* M protein. We retrospectively studied the characteristics of patients from the Hadassah Medical Center with GGS bacteremia from 1989 to 2000. Ninety-four cases of GGS bacteremia were identified in 84 patients. The median age was 62 years, 54% were males, and 92% had underlying diseases (35% had a malignancy, and 35% had diabetes mellitus). The most frequent source for bacteremia was cellulitis (61%). *emm* typing of 56 available isolates disclosed 13 different types, including 2 novel types. Six patients had recurrent bacteremia with two to four bacteremic episodes, five had chronic lymphatic disorders, and two had *emm* type *st*G840.0 in every episode. Recurrent bacteremia has not been described for invasive group *A Streptococcus*. We describe an entity of recurrent GGS bacteremia, which is associated with lymphatic disorders and possibly with *emm st*G840.0.

Large colony-forming group G β-hemolytic streptococci (GGS) were first isolated in patients with puerperal sepsis in 1935 (1). GGS are known to be commensals and pathogens in domestic animals. In humans, they may colonize the pharynx, skin, gastrointestinal and female genital tract (2). In recent years, GGS have been reported with increasing frequency as the cause of a variety of human infections, such as pharyngitis, cellulitis, meningitis, endocarditis, and sepsis (2–8). Bacteremia attributable to GGS has been related to underlying conditions, such as alcoholism, diabetes mellitus, malignancy, intravenous substance abuse, or breakdown of the skin (2–7,9,10).

The taxonomy of these organisms has been reevaluated in recent years. The Lancefield group G carbohydrate may be encountered in several β-hemolytic streptococcal species, including *Streptococcus anginosus* and *S. canis*, but mainly in *S. dysgalactiae* subsp. *equisimilis*, which is the subject of our study (11). This subspecies also hosts variants with Lancefield group A, C, and L carbohydrates. The subspecies epithet was determined by gene sequencing of the group C species previously named *Streptococcus equisimilis*, which showed it to be indistinguishable from group G *S. dysgalactiae*. This finding resulted in the description of a new taxon, *S. dysgalactiae* subsp. *equisimilis*. The true proportions of the non-G carbohydrates among members of this taxon are difficult to estimate. In a prospective, population-based study of invasive *S. pyogenes* infections (12), we found six isolates with the group A antigen to be *S. dysgalactiae* subsp. *equisimilis*. Among isolates from infections attributable to *S. dysgalactiae* subsp. *equisimilis*, the group C and G antigen was found much more commonly in human infections than group A (GAS) (11).

S. dysgalactiae subsp. *equisimilis* and *S. pyogenes* share virulence factors such as streptokinase, C5a peptidase, M protein, streptolysin S, and certain exotoxin genes (13–18). The M protein is an important virulence factor because it confers resistance to phagocytosis (19). M proteins of GAS and GGS obtained from human infections have similar biologic, immunochemical, and structural features (20). The substantial polymorphism exhibited by GAS M proteins has also been described for GGS (21). We have conducted a 12-year retrospective study to establish the incidence, clinical features, epidemiologic characteristics, and *emm* typing of GGS isolates that cause bacteremia in a large tertiary-care center in Jerusalem, Israel.

Materials and Methods

A retrospective study was conducted from 1989 through 2000 at the Hadassah Hospitals, Jerusalem, Israel. This is a 1,000-bed, tertiary-care center with all major disciplines represented, including hematology, oncology, and

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bone marrow transplantation. The mean number of annual admissions during the study period was 62,433.

Clinical Characteristics

We reviewed the records of all patients in whom a positive blood culture of GGS was reported. Demographic and clinical data were collected. Death rates were measured only during hospitalization. Descriptive statistics were performed with the SPSS (SPSS Inc., Chicago, IL) statistical package release 11.01. The Fisher exact test was used for differences in proportions. A two-sided p value of <0.05 was considered significant.

Microbiologic Methods

Patients with GGS bacteremia were retrieved from the microbiology laboratory database of bacteremia. No more than one isolate per admission was included. Clinical specimens were collected and handled according to standard protocols. During the study period, the BACTEC 460 radiometric system and the BacTAlert (Organon Teknika, Belgium) blood culture system were used.

All catalase-negative, chain-forming, gram-positive cocci that were β-hemolytic on 5% sheep blood agar were Lancefield serogrouped by using kits according to the manufacturer's instructions (PathDox Strep Grouping, DPC Diagnostic Products Corporation, Los Angeles, CA). We had 56 isolates available for further analysis. These had been stored at –70°C in the laboratory collection of blood culture isolates. All isolates displayed large colonies and did not belong to the *S. anginosus* group ("*S. milleri*").

Molecular Methods

An overnight growth was resuspended in saline and heated at 70°C for 15 min. Bacteria were then resuspended in 50 μL of 10 mmol/L Tris, 1 mmol/L EDTA, pH 8. Ten microliters of mutanolysin (3,000 U/mL) and 2 μL hyaluronidase (30 mg/mL) were added. After incubation at 37°C for 30 min and heat inactivation at 100°C for 10 min, the supernatant was subjected to polymerase chain reaction (PCR). Fifty-six GGS isolates were *emm* typed. PCR was performed as described (http://www.cdc.gov/ncidod/biotech/strep/doc.htm). Primers used for amplification of GGS DNA were G1F and G1R, previously described by Schnitzler et al. (21).

According to recommendations (http://www.cdc.gov/ncidod/biotech/strep/doc.htm), the sequence of the sense strand of the *emm* hypervariable coding region was determined by using primer 1 (5′ TATTCGCTTAGAAAATTAA 3′) by automated sequencing (Hy Laboratories Ltd., Rehovot, Israel). The sequence of base pair numbers 30–260 was submitted by using the Streptococcal Group A Subtyping Request Form, to the Blast 2.0 Core Facility (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm), where *emm* type was determined (22).

The 12 strains from recurrent infection were analyzed by pulsed-field electrophoresis (PFGE). Chromosomal DNA was digested with *Sma*I and prepared and analyzed as described with minor modifications (23).

Results

The 504 bloodstream isolates of β-hemolytic *Streptococcus* from 1989 through 2000 included the following: 232 (46%) group A, 171 (34%) group B (GBS), 94 (19%) group G, and 7 (1.4%) groups F and C. The 94 episodes of GGS bacteremia involved 84 patients, 6 of whom had recurrent infections. Patient characteristics are summarized in Tables 1 and 2. The annual incidence of GGS bacteremia was 0.0–0.2 cases/1,000 admissions during the 12-year study; the incidence ranges for GAS and GBS were 0.2–0.48 and 0.19–0.3, respectively (Figure 1).

Five patients died, but only two of the deaths were directly attributed to the GGS bacteremia. The other three deaths were related to malignancy in two patients and congestive heart failure in one.

Of the 56 GGS isolates available for *emm* typing, we found 13 *emm* types (including 2 subtypes of *stG*6: *stG*6.0 and *stG*6.1). These types included *stG*507.1, a variant of *emmLG*507.0 (GenBank accession no. X79527) and *stG*6792.0 (identical to a partial gene sequence, accession

Table 1. Summary of clinical characteristic of 94 patients with group G streptococcal bacteremia

Characteristic	No. of patients (%)
Age (y)	
<10	3 (3.1)
10–50	28 (29.8)
51–75	42 (44.7)
>75	21 (22.3)
Median (range)	62 (2–92)
Male	51 (54.2)
Median LOH[a] in days	10
Underlying disorder	
Diabetes mellitus	33 (35.1)
Malignancy	33 (35.1)
Hypertension	18 (19.1)
No disease	8 (8.5)
Type of infection	
Cellulitis	56 (59.6)
Primary bacteremia	18 (19.1)
Soft-tissue infection[b]	4 (4.3)
Bone and joint	4 (4.3)
Endocarditis	3 (3.1)
Respiratory	3 (3.1)
Postpartum	1 (1.1)
Line sepsis	1 (1.1)
Unknown	4 (4.3)
Death rate	5 (5.3)

[a]LOH, length of hospitalization.
[b]Soft tissue infection, pressure sore attributable to diabetes.

Table 2. *emm* types of 94 patients with group G streptococcal bacteremia

emm types	No. of isolates
*stG*485.0	10
*stG*840.0	7
*stG*6.1	7
*stG*166b.0	6
*stG*4222.0	5
*stG*10.0	3
*stG*5420.0	3
*stC*74a.0	3
*stG*245.0	3
*stG*480.0	3
*stC*36.0	2
*stG*6792.0[a]	2
*stG*6.0	1
*stG*507.1[a]	1

[a]New *emm* types.

no. AF485842, listed from a blood isolate of *S. dysgalactiae* subsp. *equisimilis*), which were not in the database. The patient with the *stG*507.1 bacteremia had cellulitis, and two patients had the *stG*6792.0 strain: one was a 17-year-old patient with venous malformations of the leg and pelvis who had GGS sepsis and multiorgan failure, and the other was a 92-year-old man with diabetes and cellulitis. No association was found between *emm* type and year of study, season, source of infection, or cellulitis location.

Six patients had recurrent bacteremia, ranging from 2 to 4 episodes per patient (Table 3). All six patients had a community-acquired recurrent cellulitis and were given treatment similar to that received by patients who did not have a recurrent infection. Five patients had chronic lymphatic abnormalities at the infection site compared to 11 of 42 patients with nonrecurrent cellulitis (odds ratio [OR] 14.1, 95% confidence interval [CI] 1.5–134.3, p = 0.012). Two patients had recurrent infection with the same *emm* type, *stG*840.0 (one patient had three episodes, 1 and 7 months apart; the other patient had 2 episodes, 6 months apart). Three patients had a different *emm* type each episode. The isolates of the patient with four recurrent episodes were not available for *emm* typing. PFGE results indicated that isolates recovered from the same patient that shared the same *emm* type were highly genetically related (Figure 2).

Discussion

GGS are widely distributed in nature and are recognized as both commensals and pathogens in animals as well as in humans. *S. dysgalactiae* subsp. *equisimilis* is part of the normal bacterial flora in humans. *S. dysgalactiae* subsp. e*quisimilis* is the most common species of large colony-forming serogroup G streptococci that is β-hemolytic on sheep blood agar. The animal-associated *S. canis* has rarely been implicated as a human pathogen, although accurate data are not available (11,24). Asymptomatic pharyngeal carriage of GGS has been described in up to 23% of humans (25), and vaginal carriage in 5% of asymptomatic puerperal women (1).

This 12-year retrospective survey identified 94 episodes of GGS bacteremia at the Hadassah-Hebrew University Medical Center. It is one of the two largest series of GGS bacteremia described in the literature to date (26). Some similarities were found in the epidemiologic characteristics of group G streptococcal bacteremia between our study and other studies. Patients were predominantly elderly men. Most patients (92%) had underlying diseases, similar to those (74%–92%) reported previously (2–6,9).

As shown by Auckenthaler et al. (3) and Woo et al. (6), malignancy was one of the most important underlying conditions associated with GGS bacteremia, affecting 35% of our patients. In our patients, the most common malignancy associated with cellulitis was carcinoma of the breast. The most common source of infection among patients with bacteremia with a known source was soft tissue infection, especially cellulitis (61%), 66% of these infections were confined to the lower limbs. Similarly, 48% of our patients with cellulitis had an underlying skin lesion; in 35% of the patients, it was related to lymphatic abnormalities. These abnormalities were mostly attributed to malignancy, surgery, or radiation. Two patients had a congenital lymphatic malformation.

Nineteen percent of our patients had primary bacteremia, which is within the range (11%–52%) described by others (2,3,6,7). The death rate in our study (5%) was lower than that previously reported (8%–30%) (3,6,9). One reason for this finding may be the relatively younger age of our patients: Only 22% of our patients were >75 years of age compared to those from Sylvetsky's group, where 63% were >75 years of age (9). The small number of patients who eventually died of bacteremia precluded analysis of risk factors associated with death.

In contrast to the previous study from a different hospital in Jerusalem, Israel, describing GGS bacteremia during

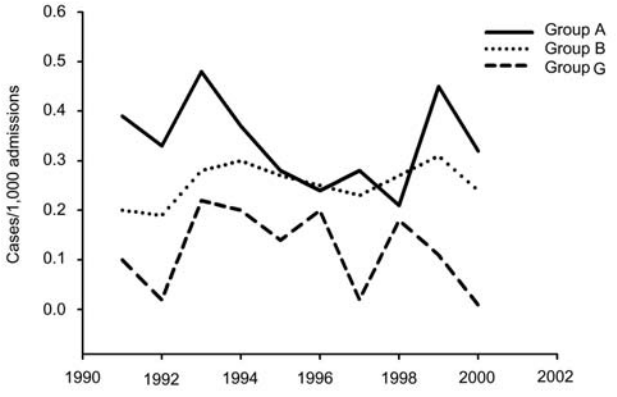

Figure 1. Trends in β-hemolytic streptococcal bacteremia at the Hadassah Medical Center.

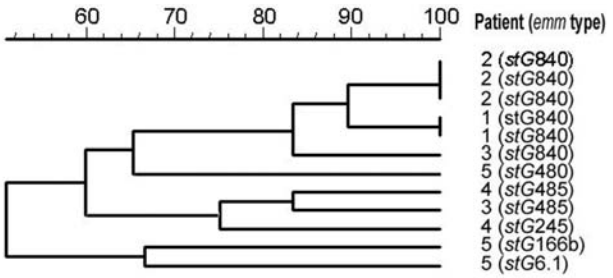

Figure 2. Dendrogram of pulsed-field gel electrophoresis analysis of isolates from patients with recurrent bacteremia. "Patient" refers to numbers from Table 3.

the same years as our study (9), the yearly incidence of GGS bacteremia did not increase. The dissimilarity in our two groups may be attributable to the differences in ages of the patients and the proportion of men. In addition, the other hospital is a community hospital with a large geriatric department, without neurosurgery or bone-marrow and solid organ transplants.

A noteworthy finding in our series was the high frequency of recurrent GGS bacteremia. We identified six patients with bacteremia, all of whom had recurrent cellulitis. Five had lymphatic drainage abnormalities. Two patients had recurrent bacteremia attributable to the same *emm* type (stG840, patients 1 and 2, Table 3). PFGE results indicated that within each patient the isolates recovered at different time points were clonal (Figure 2) but that the two pairs of *stG*840 isolates had differing PFGE patterns, which suggests that they were different clonal types.

Since two of the patients with recurrent bacteremia had an infection by the same clone, these infections may have been relapses. Recurrence of bacteremia suggests that the initial infection may not provide protective immunity. The question of protective immunity to this bacterium is addressed by Bisno et al. in a murine model of recurrent GGS cellulitis (27). Despite recurrent skin challenge with GGS, the lesion did not decrease in severity, size, or time to heal. Bisno et al. found that, despite the development of demonstrable humoral immune response to M protein, acquired protective immunity did not occur. Possibly GGS downregulates M protein in vivo, thus allowing it to evade these specific antibodies. Immediately initiating antibiotic therapy for the first GGS infection might contribute to the low level of immunity, as has been demonstrated in cases of recurrent GAS tonsillitis (28).

Cellulitis can recur in extremities or other sites where venous and lymphatic circulation has been compromised by processes such as malignancy, lymph node dissection, prior irradiation, trauma, or saphenous venectomy (10,29,30). Nongroup A β-hemolytic streptococci have been implicated as a major cause of cellulitis in the setting of circulatory compromise (31,32). Focusing on the 48 patients with cellulitis, we found that recurrent cellulitis with bacteremia was 14.1 times more likely to develop in patients with lymphatic drainage abnormalities when compared to patients without such abnormalities. Our report is the first to describe the phenomenon of recurrent GGS cellulitis associated with bacteremia in patients with lymphatic abnormalities. Recurrent GGS bacteremia seems to be more common than recurrent GAS bacteremia. In our study of 90 patients with GAS bacteremia (33) who were admitted to the Hadassah Medical Center during a 6-year period, none had a recurrence compared to 6 of 84 patients in this study ($p < 0.013$). Our patient with four recurrences of GGS bacteremia had in effect five additional episodes, which were not included in our report. Three episodes were cellulitis with GGS bacteremia at other hospitals, and two episodes were severe cellulitis without proven bacteremia. These cases are examples of recurrence of GGS infection in a manner not known to occur with GAS. A recent report suggested that allelic variation of human leukocyte antigen II contributes to the differences in severity of GAS infections (34). The relationship between bacterial factors and host mechanisms of defense in this patient and others with recurrent bacteremia needs further investigation.

To illuminate the unique characteristics of patients with GGS bacteremia, we compared our group of GGS bacteremia patients with two groups of GAS bacteremia patients (33,35). Sex and age of the patients with GGS bacteremia were similar to those with GAS bacteremia in a retrospective study. However, when we compared patients with GAS bacteremia from our hospital participating in a nationwide prospective, population-based study, GGS patients were older and more likely to be men than the GAS patients (35). Thus, the characteristics of the GGS patients may reflect institutional and selection bias attributable to different study methods and may not be a true tendency for older patients.

Serologic M typing was developed years ago for GAS typing, but it has also been used for GGS (36). M protein encoded by *emm* is a virulence factor of GGS similar to the GAS surface protein (20). *emm* typing for both GAS and GGS is based on the heterogeneity of the 5′ ends of the gene, which give rise to different sequence types. More than 120 *emm* types are recognized for GAS, and approximately 40 types of GGS and group C *Streptococcus* (GCS) have been identified (http://www.cdc.gov/ncidod/biotech/strep/emmtypes.htm). Despite the similarities between GAS and GGS, Geyer and Schmidt (37) found that in GCS and GGS, two types of arrangements in the *emm* region differ significantly from the known types of *mga* region in GAS. The conclusion was that Mgc is related to Mga proteins of various types of GAS but forms a distinct cluster.

Table 3. Characteristics of recurrent episodes of GGS bacteremia

Patient	*emm* type				Time to recurrence (mo.)
	Episode 1	Episode 2	Episode 3	Episode 4	
1	*stG*840.0	*stG*840.0			6
2	*stG*840.0	*stG*840.0	*stG*840.0		1, 7
3	*stG*840.0	*stG*485.0			12
4	*stG*485.0	*stG*245.0			21
5	*stG*480.0	*stG*6.1	*stG*166b.0		3, 1
6	Unknown	Unknown	Unknown	Unknown	34, 13, 12

In previous studies, Lawal et al. used serologic M typing for 103 isolates of GGS. Fifty-six isolates (54%) could be serologically typed into eight serotypes (38). Of 128 isolates, 40 (31%) could be serotyped with six antisera (39). The inability to type a large proportion of GGS by the older serologic method is similar to the situation that exists for GAS (12).

In our 56 GGS isolates available for sequence typing, we found 13 *emm* types. None of our isolates were *emm* nontypeable. Kalia et al. (40) *emm* typed 18 GGS isolates from human infections obtained from various countries. They found 13 *emm* types, the most common of which was *stG*480.0 (3 of 18 isolates compared to 3 of 56 in our study); our most common type was *stG*485.0 (10 of 56 isolates). Thus, *emm* typing provides a useful tool for identifying isolates when compared to traditional M typing.

Tyrrell found a correlation between clusters of GAS M types and patient age (41). We could not find a correlation between *emm* type and clinical features such as patient sex, age, and source of infection or cellulitis location, although our database may not be sufficiently large to draw these correlations.

We found among our GGS isolates *emm* types that were previously described for GCS (*stC*36.0) and for GAS (*stG*245.0). Although *stG*245.0 was originally associated with *S. dysgalactiae* subsp. *equisimilis* harboring group A antigen, it is usually associated with GGS (B. Beall, unpub. data). Kalia et al. described a few GCS *emm* types, which in our study were found in group G streptococci. Certain types are occasionally found in both GGS and GCS (Beall, unpub. data). The dynamics of interspecies transfer of virulence loci between GAS, GGS, and GCS (18,42–45), as well as potential genetic transfer or intragenomic events causing interconversion of group antigen types, remains to be resolved. Our findings that several isolates with *emm* type *stG*840 have different PFGE patterns may support the notion that isolates of GGS with the same *emm* type are not of the same clone. The relationship between GGS *emm* type and clone remains to be examined. In general, GAS *emm* typing, when restricted to the same geographic area and time period, is indicative of clonal type; however Beall et al, (46) demonstrated numerous examples of apparently unrelated GAS strains (as judged by independent T agglutination phenotype and opacity factor [*sof*] sequence) sharing the same *emm* sequence type, and, more recently, nonrelatedness between distinct GAS strains of identical *emm* types has been demonstrated through MLST (47).

In summary, we describe 94 cases of GGS bacteremia, observed mainly in older patients with underlying medical conditions. The most frequent portal of entry was the skin. The high rate of recurrence of GGS bacteremia was an unusual and unexpected finding. Clinicians should be alert to this phenomenon, which seems to be more common than recurrent GAS bacteremia. We found that lymphatic drainage disorders were a highly significant risk factor for recurrence, and that *emm* type *stG*840.0 may have a special role in recurring disease.

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Print Media Response to SARS in New Zealand

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To examine the media response to severe acute respiratory syndrome, we reviewed New Zealand's major newspaper (261 articles for 3 months). While important accurate health messages were frequently included, some were missed (e.g., hand washing in only 2% of articles). No incorrect information was identified, and health spokespersons were accurately quoted.

Severe acute respiratory syndrome (SARS) is a new viral disease in humans that emerged in southern China in November 2002 (1). The World Health Organization (WHO) issued a global alert about SARS on March 12, 2003, and an unprecedented public health response was subsequently mounted. An important part of that response was probably the intense global media coverage given to this disease. To derive lessons for addressing future threats to public health, we examined the media response in New Zealand's major daily newspaper.

The Study

We searched the Internet-based electronic archive of the New Zealand Herald for a 3-month period beginning with WHO's first global alert (March 13–June 11, 2003). We chose this paper because it has the largest circulation of a daily paper in the country (i.e., 530,000 readers out of a national population of 4 million), and its reporting is likely to represent that of other mainstream media. The advanced search capacity at the newspaper's Web site (2) was used with the key search term being "SARS" (combined with the other terms detailed in Tables 1 and 2; [3–5]). The search was confined to the news section of the archive because stories on SARS in the business and sports sections rarely provided information on health aspects.

We compared information in the articles on SARS with that in the Medline-indexed literature (to July 2003). Information attributable to health officials in New Zealand was compared to the information on the Ministry of Health's Web site and its media releases (n = 19) (6). For comparison purposes, we obtained from WHO the weekly numbers of new cases of SARS from four areas that had ongoing SARS transmission in the Western Pacific Region; China was excluded because of its irregular pattern of reporting.

SARS dominated the health-related news in this newspaper during the study period, with 261 news articles (i.e., 3.3 articles per issue). The rate of articles mentioning SARS (87 per month) was greater than that for smoking and tobacco (59 per month), cancer (43 articles), diabetes (12 articles), heart disease (10 articles), and asthma (6 articles). The number of articles mentioning SARS rose and fell, more or less in line with disease activity (Figure). Of the 261 articles, 48% had a headline with the word SARS.

In the 261 articles, no technically incorrect information about the clinical or epidemiologic features of SARS appeared in print. Also, the views or comments attributed to Ministry of Health spokespersons were consistent with messages promoted by the ministry in its media releases and on its SARS Web site (31 articles). The impression conveyed was that the spokespersons were credible, and their reported statements imparted information and reassurance, and sometimes put the risk for SARS into a broader risk perspective. Some statements by officials pro-

Table 1. Information on the clinical features of SARS in the New Zealand Herald [a]

Clinical feature	No. (%) of articles (N = 261)
Symptoms detailed on the Ministry of Health's SARS Web site	
Cough or fever	67 (26)
Cough	55 (21)
Fever[b]	54 (21)
"Shortness of breath"	15 (6)
"Trouble breathing" or "difficulty breathing"	5 (2)
"Body aches" or "muscle pain" (myalgia[b])	3 (1)
"Diarrhoea"[b] or "discomfort"	2 (1)
Additional symptoms of SARS from the literature (3–5)	
Chills[b]	12 (5)
Headache	5 (2)
Other[c]	3 (1)
Other words relating to clinical features	
"Pneumonia" or "flu"	103 (39)
Pneumonia	67 (26)
Flu	53 (20)
Flu-like	38 (15)
Influenza	17 (7)
"High fever"	34 (13)
Temperature	23 (9)
Temperature of 38°C	9 (3)
"High temperature"	6 (2)
"Respiratory symptoms"	3 (1)

[a]SARS, severe acute respiratory syndrome. Quotation marks refer to actual phrases in newspaper articles.

[b]These were the symptoms considered to predict SARS most strongly in the early stages of illness, according to Rainer et al. (4).

[c]Other signs and symptoms included loss of appetite, malaise, rigor, vomiting, sore throat, dizziness, sputum, night sweat, coryza, abdominal pain, neck pain, nausea, arthralgia ("joint pain"), chest pain, rhinorrhea ("runny nose").

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Table 2. Information on SARS transmission and control measures reported in the New Zealand Herald [a]

Information on SARS	No. (%) of articles (N = 261)
SARS transmission	
Transmission by "droplets" or "sneezing" or "coughing"	16 (6)
"Close contact," "dire ct contact," or "physical contact" with an infected person as a risk factor for transmission	16 (6)
"Close contact" or "contacts ," the definition used for outbreak control purposes	13 (5)
Possible transmission through a contaminated "surface" or "object" or lift "button" or door "handle"	13 (5)
"Person-to-person" transmission	7 (3)
Possible risk posed by bodily "secretions" (or "faecal" contamination, "faeces ," or "stool")	7 (3)
Possibility of "airborne" transmission	5 (2)
"Casual contact" not being a risk factor for transmission	2 (1)
No evidence for "airborne" transmission (or unlikely)	2 (1)
Touching one's "eyes", or "nose," or "mouth" with potentially contaminated hands as a risk factor	1 (0.4)
SARS control or personal protection	
"Quarantine"	85 (33)
"Isolation"	62 (24)
"Mask"	60 (23)
"Hand washing" for prevention	4 (2)
Advice to seek medical attention if relevant symptoms are present	4 (2)
Lack of health insurance cover age for travellers to affected areas	2 (1)
Groups at increased risk of infection and or death	
Health workers (including nurses and doctors)	24 (9)
"Elderly" (and other terms for older persons)	7 (3)
Persons with diabetes or other chronic conditions	3 (1)

[a]SARS, severe acute respiratory syndrome. Quotation marks refer to actual phrases used in newspaper articles.

moted the theme of civic responsibility by stating that persons who ignored official travel advisories were placing others at risk.

The symptoms of cough or fever were mentioned in 26% of articles, and the terms "pneumonia" or "flu" were also commonly used (39%) (Table 1). The word "flu" was used in the articles far more frequently than "influenza." More detailed information on symptoms was rare; 3% of articles mentioned the specific temperature of 38°C (the figure used in official information).

All the countries with in-country transmission of SARS were mentioned, with particular emphasis on China (65% of articles). Travel advice from official agencies was from WHO (12% of articles), the Ministry of Health (8%), and other government agencies (4%). However, few articles included the specific ministry advice that recent travelers from SARS-affected countries avoid nonessential medical visits but seek medical attention if relevant symptoms developed (Table 2).

Articles frequently described public health responses involving quarantine or isolation (Table 2). Masks were frequently mentioned (or shown in photographs), and information on disease transmission was sometimes provided. This information appeared to be accurate, although occasionally unconfirmed means of transmitting SARS (e.g., airborne spread, as opposed to spread by droplets from coughing, and spread through cockroaches and rodent feces) were mentioned. Eight articles (3%) provided a Web site address for SARS information. The Ministry's SARS Web site and three relevant 0800 telephone helplines were infrequently mentioned (n = 4 [2%] and n = 8 [3%], respectively).

In retrospect, some comments reported were overly pessimistic. For example, an economist was reported as saying that the disease "was on its way to New Zealand, and once here it was unlikely to go away quickly." International health officials were also quoted as saying that SARS "is probably here to stay" and "is now probably entrenched in the population [in China]."

Particular terms were used that could be considered alarming (e.g., "outbreak" in 38% of articles, "deadly" in 32%). Similarly, at least one of the following terms was used in 15% of headlines: kill, killer, deadly, panic, and death (n = 38). Some examples of headline phrasing included the following: "doctor dies of killer virus"; "nature's terrorism strangles Hong Kong"; "SARS deaths leap"; "panicking crowds flee"; "creeping panic over epidemic"; and "SARS virus… mutating rapidly."

Discussion

This analysis is limited by its focus on only one New Zealand newspaper (albeit the one with the largest daily circulation). It also lacks the broader context that could have been obtained from interviews with key personnel. Nevertheless, it provides some insight into the media response to an emerging public health threat.

SARS clearly dominated the health news during this study period, although total coverage was possibly limited by competition from another major event, the war in Iraq. This media interest might be due to a number of newsworthy features concerning SARS, namely, its new disease status, exotic aspects (e.g., possibly arising from wild animals), relative infectiousness, uncertain transmission modes, high case-fatality rate, and limited treatment options.

Information on disease symptoms was frequently provided but often with little accompanying detail. This finding highlights the importance of health authorities' keeping

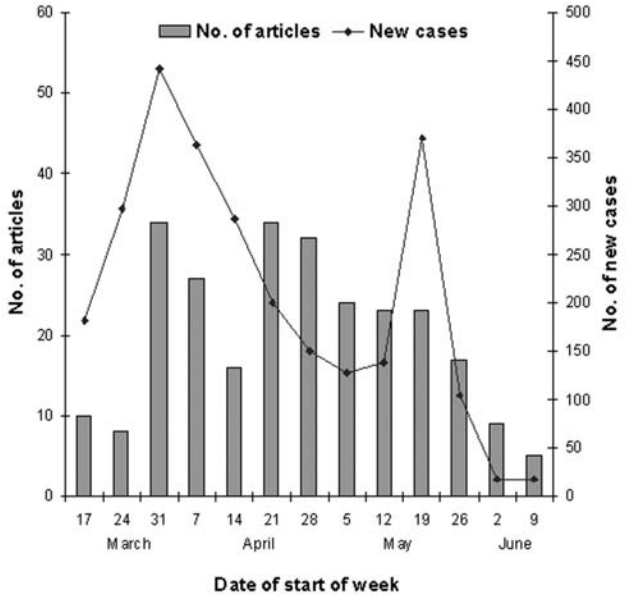

Figure. Articles on severe acute respiratory syndrome (SARS) in the New Zealand Herald and new cases of SARS (Singapore, Hong Kong, Vietnam, Taiwan).

key messages short and using well-published Web sites for providing more detail. The media appear to be much more likely to use some words than others (e.g., "flu" versus "influenza" and "outbreak" versus "pandemic"). This finding suggests the need for health authorities to use simple language and to use it consistently.

Although some prominence was given to describing disease control interventions (e.g., quarantine and isolation), relatively few articles provided information on basic personal preventive measures such as hand washing. Little coverage was given to how to access Web sites or telephone helplines, perhaps because newspapers only partly see themselves as a "public good information service" and may expect health authorities to pay to advertise such details. This finding suggests that if critical health messages are not picked up by the media in a crisis, then paid advertising could be a backup option (especially to list and explain access to key Web sites). Indeed, health budgets could ideally take into account such contingencies.

Conclusions

This analysis showed that official health spokespersons were accurately quoted and that no technically incorrect information on the clinical or epidemiologic features of SARS was published. Such a response is reassuring and highlights the potential value of the health sector's use of the media to inform the public.

This newspaper sometimes used headlines and particular words (e.g., "deadly") that could be considered alarming. Nevertheless, some articles quoting health officials and others did provide reassuring information and messages.

Media analyses could be extended in a number of ways to provide a broader and deeper understanding of the response to SARS. A range of newspapers could be studied, along with key interviews with health reporters and health sector spokespersons. Such work could be justified, given the importance of risk communication in dealing with the possible reemergence of SARS (7) and the importance of information for the public and the media on the control of this disease (8). These issues are also relevant in handling the threat of pandemic influenza (9) or diseases associated with bioweapons that could spread globally (10).

In summary, this particular major daily newspaper provided generally useful and accurate information to the public on SARS. This finding highlights the potential value of the media for communication about public health issues and pandemic threats.

We thank the New Zealand Ministry of Health for supporting the initial phase of this work, which was undertaken when the first author was working on SARS control for the Ministry. The findings are those of the authors and do not reflect the views of the Ministry of Health.

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Crimean-Congo Hemorrhagic Fever in Bulgaria

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We report the epidemiologic characteristics of Crimean-Congo hemorrhagic fever in Bulgaria, as well as the first genetic characterization of the virus strains circulating in the country in 2002 to 2003 that caused disease in humans.

Crimean-Congo hemorrhagic fever virus (CCHFV) (genus *Nairovirus*, family *Bunyaviridae*) causes severe disease with a fatality rate as high as 30%. CCHFV is endemic in the Balkan Peninsula; a number of cases occur every year, sometimes in an epidemic form. Cases have been reported in Albania (1), Kosovo (2,3), and Bulgaria (4). Mountains approximately 1,500 m to 2,500 m high separate these countries from Greece, where no case of the disease has yet been identified. However, a CCHFV strain was isolated in Greece from *Rhipicephalus bursa* ticks, collected in May 1975 from goats of a flock in Vergina village, 80 km west of Thessaloniki (5). Antibodies against the virus were detected in the Greek human population (6). CCHFV is also endemic in Russia and in parts of Asia and Africa.

The virus is transmitted to humans by the bite of ixodid ticks (primarily of the *Hyalomma* genus) or by contact with blood or tissues from infected persons or infected livestock. The risk for spread of the virus from person to person is high, which occasionally results in nosocomial outbreaks. After an incubation period of 3 to 7 days, the patient has sudden onset of fever, chills, myalgia, and headache, which rapidly progress to severe illness; a hemorrhagic state follows with bleeding from the mucous membranes and petechiae, associated with thrombocytopenia and leukopenia (7).

CCHFV, like all members of the genus, is a negative-stranded RNA virus with a tripartite genome consisting of a small, medium, and large segment encoding the nucleocapsid protein; the glycoprotein precursor, which results in the two envelope glycoproteins G1 and G2; and the putative RNA-dependent polymerase, respectively (8).

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We report (for the first time in English) the epidemiologic characteristics of the disease in Bulgaria. We also provide the first genetic characterization of the CCHFV strains circulating in the country from 2002 to 2003 that caused disease in humans.

Bulgaria is a country of 8 million inhabitants in the eastern part of the Balkans (Figure 1). CCHF was first recognized in the country in 1952 and became a reportable disease in 1953. In 1968, CCHFV was isolated from blood samples of two patients. Results from serologic investigations showed that approximately 20% of patients living in disease-endemic areas who reported a tick bite had antibodies to CCHFV (4). The seropositivity in animals in the disease-endemic areas can be as high as 50%. Most cases were reported from Plovdiv and Pazardgik (central Bulgaria), Haskovo and Kardgali (southeastern Bulgaria), Shumen (northeastern Bulgaria), and Burgass (eastern Bulgaria) (4). The most prevalent tick in Bulgaria is *Ixodes ricinus*; however CCHFV strains have been isolated from *Hyalomma plumbeum* (*H. marginatum*), *Rhipicephalus sanguineus*, and *Boophilus calcaratus* (9).

From 1953 to 1974, 1,105 CCHFV cases were reported to the Bulgarian Ministry of Health; the fatality rate was approximately 17%. Of them, 20 cases were nosocomial infections and 52% were fatal. In 1974, an immunization program was introduced for medical workers and military personnel in CCHF-endemic areas. The treatment regimen consisted of mouse brain preparation inactivated by chloroform, heated at 58°C, and adsorbed on $Al(OH)_3$. The first two doses were given at day 0 and day 30; a third dose was given 1 year later, and another dose was given 5 years after that (10). As a result, between 1975 and 1996, the number of reported CCHF cases was reduced to 279, with a fatality rate of 11.4%. No infection was reported from vaccinated military personnel (11).

Since 1997, a total of 124 cases occurred in Bulgaria, 27 of them fatal (Table 1). Most patients had been bitten by a tick; however, a few were infected through direct contact with CCHF patients. Only the eastern part of the country

Figure 1. Bulgaria and neighboring Balkan countries.

Table 1. Distribution of Crimean-Congo hemorrhagic fever cases and related deaths in Bulgaria, 1997–2003

Y	No. of cases	No. of deaths (%)
1997	20	4 (20)
1998	15	3 (20)
1999	5	2 (40)
2000	10	1 (10)
2001	18	5 (28)
2002	56	12 (21)
2003	14	2 (14)

has been affected; two main foci exist, one in the southeast and a second one in northeast. The mean age of patients is 52 years (range 11–79 years). Most patients are men (74%), probably because they are more frequently exposed to ticks bites during outdoor activities. The disease occurs mainly from March to July when ticks are more active. The main clinical symptoms are fever, malaise, nausea, epistaxis, petechiae, and bleeding from the gastrointestinal tract; the main laboratory findings are leukopenia, thrombocytopenia, and elevated transaminase levels.

To investigate the genetic relationships of the CCHFV strains circulating recently in Bulgaria, RNA was extracted from cell culture supernatant from six virus isolates. The virus had been isolated in a Vero E6 cell line from blood samples taken from CCHF patients who were infected in 2002 and 2003. The epidemiologic characteristics of the patients are shown at Table 2. A reverse-transcriptase–nested polymerase chain reaction (PCR) was applied to amplify a partial fragment of the S RNA genome segment by using two sets of primers, F2-R3 and F3-R2 (12). Purified PCR products were sequenced; the nucleotide sequences were submitted to the GenBank database and assigned the accession numbers AY550253–AY550258. After aligning the obtained Bulgarian CCHFV sequences with respective ones retrieved from GenBank, we constructed a phylogenetic tree with PHYLIP software (13) (Figure 2). All Bulgarian isolates were found to cluster together, with a genetic homology of 98.4% to 100% at the nucleotide level. Identical sequences were obtained from isolates originating from the same region in the same year.

The Bulgarian CCHFV strains were found to cluster with other Balkan strains from Kosovo and Albania, with a mean genetic difference of 2% and 1.2%, respectively. All Balkan strains clustered in the same branch with CCHFV strains from European Russia, such as STV/HU29223 strain, isolated in 2000 from human blood in Stavropol (mean genetic difference 2.5%), and ROS/TI28044, isolated in 2000 from *Hyalomma marginatum* ticks in Rostov (mean genetic difference 3.7%) (14). A "European CCHFV group," distinct from all others, is evident. An exception to the European group is the Greek strain AP92, isolated from *R. bursa* ticks (5), which forms an independent clade, which differs from the Bulgarian strains by 24%. This genetic difference is likely attributable to the different species of related ticks or to reassortment. Studies on the Greek strain are still in progress; they will help explain the genetic and pathogenic differences

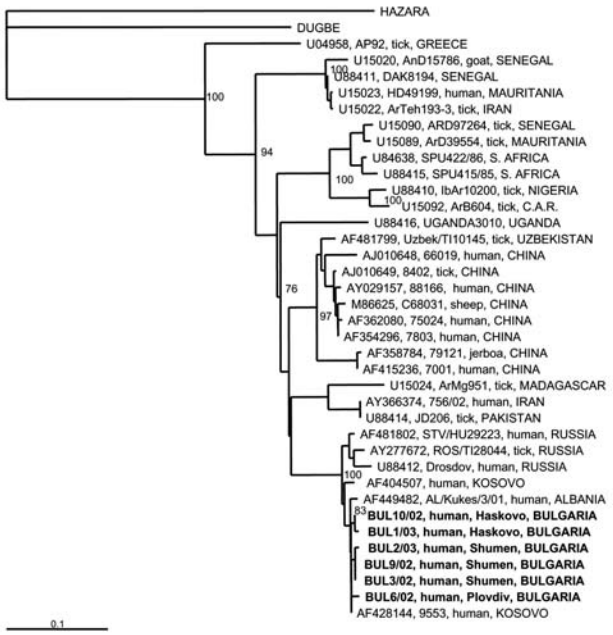

Figure 2. Phylogenetic tree based on 255-nt fragment from the S RNA segment, showing the clustering of the sequences obtained from this study and respective representative Crimean-Congo hemorrhagic fever virus strains from GenBank database. Sequences of two other nairoviruses, Dugbe and Hazara, were included; Hazara virus was used as outgroup. The numbers indicate percentage bootstrap replicates (of 100); values below 70% are not shown. Horizontal distances are proportional to the nucleotide differences. The scale bar indicates 10% nucleotide sequence divergence. Vertical distances are for clarity only. Sequences used in the analysis are indicated at the tree as: GenBank accession no., strain, host, country. C.A.R., Central African Republic.

Table 2. Epidemiologic characteristics of patients whose blood samples yielded Crimean-Congo hemorrhagic fever virus strains

No. of isolate	Date of disease onset	Sex	Age (y)	Area
BUL1/03	June 2003	Male	60	Haskovo
BUL2/03	June 2003	Male	45	Shumen
BUL3/02	April 2002	Female	62	Shumen
BUL6/02	June 2002	Male	73	Plovdiv
BUL9/02	June 2002	Male	23	Shumen
BUL10/02	August 2002	Male	39	Haskovo

among this strain and respective strains from neighboring countries.

Although the genetic divergence among European strains is low, a great divergence is seen among European CCHFV strains and strains from other continents (Asia and Africa). As the number of CCHFV sequences derived from the S genome segment is growing, eight distinct clades can be seen: 1) strain AP92 from Greece; 2) strains from Senegal, Mauritania, and Iran; 3) strains from Senegal, Mauritania, and South Africa; 4) strains from Nigeria and Central African Republic; 5) strain from Uganda; 6) strains from Central Asia and China; 7) strains from Madagascar, Iran, and Pakistan; and 8) European strains (Russia, Albania, Kosovo, and Bulgaria) (Figure 2).

In conclusion, this report shows that the CCHV is endemic in Bulgaria and causes severe disease in the whole Balkan Peninsula (except Greece) and that the Bulgarian CCHFV strains are genetically similar to other Balkan virus strains (except AP92). CCHFV evolves relatively slowly, which suggests that the great genetic divergence among the strains is not time-dependent. Whether this divergence is because of the different tick species, the different geographic location, or any other reason, remains to be elucidated.

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Recombinant Human Enterovirus 71 in Hand, Foot and Mouth Disease Patients

Yoke-Fun Chan* and Sazaly AbuBakar*

Two human enterovirus 71 (HEV71) isolates were identified from hand, foot and mouth disease patients with genome sequences that had high similarity to HEV71 (≥93%) at 5'UTR, P1, and P2 and coxsackievirus A16 (CV-A16, ≥85%) at P3 and 3'UTR. Intertypic recombination is likely to have occurred between HEV71 and CV-A16 or an as-yet to be described CV-A16-like virus.

Hand, foot and mouth disease (HFMD) is a common illness of infants and young children <10 years of age. It is characterized by fever, ulcers in the oral cavity, and rashes with blisters that appear on the palm and sole. The most common causal agents of HFMD are coxsackievirus A16 (CV-A16) and human enterovirus 71 (HEV71), but other enteroviruses, including CV-A5 and CV-A10, can also cause it. When caused by CV-A16 infection, it is usually a mild disease, and patients normally recover without requiring any special medical attention.

In rare instances, the disease leads to aseptic meningitis and more serious diseases, such as encephalitis or poliomyelitis-like paralysis. HFMD caused by HEV71 has been associated with the more severe forms of the disease, including a high number of cases of fatal encephalitis during the outbreaks in Malaysia in 1997 and Taiwan in 1998. During these outbreaks, several HEV71 subgenotypes were isolated; two subgenotypes, B4 and C2, were identified as the main causal agents associated with the fatal infections (1–3). During the Malaysia 1997 outbreak, HEV71 subgenotype B3 was the most prevalent subgenotype isolated from patients with the milder form of HFMD (4). The B3 virus was also found in neighboring countries in 1997 and western Australia in 1999; it has since disappeared (4–6).

To elucidate the mechanisms underlying the emergence of the different HEV71 subgenotypes with potentially different pathogenic potentials, we examined the whole genome sequence of representative HEV71 isolates of HFMD patients from the Malaysia 1997 outbreak. Virus was isolated and identified from patients' samples as described earlier (1). Initial characterization and construction of phylogenetic trees was performed by using the virus genome 5′ nontranslating region (NTR) sequences. From this initial tree, six isolates (UH1 [GenBank accession no. AJ238455], SHA89 [AJ586873], SHA63 [AJ238456], SHA66 [AJ238457], SHA52 [AJ238531], and SHA71 [AJ238535]) were randomly selected to represent the different HEV71 genotypes and were sequenced in their entirety. Sequence analysis, construction of phylogenetic trees, and potential recombination of the isolates were determined as previously described (1,7). Similarity plot and bootscan analyses for the recombination studies were performed with SimPlot version 3.2 (8,9). For the analysis, a sliding window of 400 nt was moved in increments of 20 nt at a time. Sequences were not corrected for multiple substitutions, all gaps were stripped, transition-to-transversion ratio of two was used, and 50% consensus files were used to exclude the poorly conserved sites. Recombination was identified when conflicting genome sequence profiles appeared, which suggested acquisition of sequences from a different parental genotype. Phylogenetic trees were then constructed for each of the putative recombinant sequences by using the maximum likelihood method, and support for the tree topology was determined by bootscanning analyses that used the bootstrapping procedures (9) with 100 resamplings. The crossover breakpoints were identified when χ^2 values were maximum (10). In addition, recombinant sequences were confirmed in patients' samples by using the reverse transcription–polymerase chain reaction (RT-PCR) with primers RECF (5′-CTCAACAGAGCTGTGCTAGTCATGCAATCC-3′; nucleotide positions 5229–5258) and RECR (5′-TCCACTGAGGTTGAGAAAACCATATTGCAC-3′; nucleotide positions 5748–5777), designed on the basis of isolates SHA63 and SHA66 genome sequences and DNA sequencing.

Initially, a phylogenetic tree depicting the genetic relationships of the isolates was constructed by using the whole genome sequence of the six isolates and those available in the GenBank (Figure 1A). As expected, the six isolates were placed into three different lineages (genotypes), and as no reports exist on the typing of the different HEV71 isolates with the whole genome sequences, the genotypes established with the virus capsid (VP1) gene sequence were adopted (5). The three HEV71 subgenotypes identified, B3, B4, and C2, represent the HEV71 genotypes found cocirculating in Malaysia during the 1997 outbreak. Sequence analyses performed by using the isolates whole genome sequences initially reaffirmed the genotyping of the isolates (Figure 1A). The isolates remained within the respective genotypes when the P1 and P2 genome regions were used to construct the phylogenetic trees (online Appendix 1 Figure, available at

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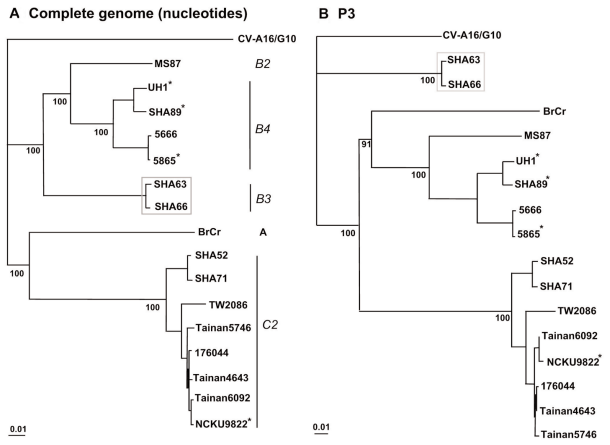

Figure 1. Phylogenetic trees showing genetic relationships among Human Enterovirus 71 (HEV71) isolates. The neighbor-joining trees were constructed from alignment of the whole genome sequences (panel A) and nucleotide sequences of P3 (nucleotides 5067-7325), (panel B). The bootstrap values are shown as percentage derived from 1,000 samplings, and the scale reflects the number of nucleotide substitutions per site along the branches. Isolates from fatal cases are denoted with asterisks.

http://www.cdc.gov/ncidod/eid/vol10no8/04-0059_app1.htm). A significant shift in the tree topology, which involved the positions of the two subgenotype B3 isolates, SHA63 and SHA66, was noted when the tree was constructed by using the P3 genome region, a region consisting of the nonstructural protein genes located towards the 3′ end of the genome (Figure 1B). At this genome region, these isolates clustered with the other known causal agent of HFMD, CV-A16. This conflicting tree topology raised the possibility that these isolates contained chimeric genome sequences, perhaps as a result of a previous recombination event involving HEV71 and a human enterovirus A (HEV-A). We performed similarity plot analysis using the consensus genome sequence of HEV71 subgenotype B3 isolates against several potential parental genomes, including all available HEV-A, and confirmed conflicting genome profiles resembling a pattern of recombination for the two subgenotype B3 isolates (Figure 2). The isolates showed high sequence similarity (≥93%) to subgenotype B4 at the 5′ terminus of the genome spanning the 5′ NTR region, the whole structural gene sequences, 2A and part of 2B gene (nt 1–3908). A conflict in the genome sequence showing high similarity (≥85%) to CV-A16/G10 was noted at the 3′ end of the genome involving the 3C protease, 3D polymerase, and 3′ NTR region gene sequences. Two estimated crossover points that resulted in a switch from HEV71 genotype B to CV-A16–like sequences ($p < 0.001$, Fisher exact test) were located within nt 3908–5603. However, the B3 genome sequence dissimilarity relative to subgenotype B2 and B4 viruses began as early as at nt 3617 (part of 2A gene), which does not rule out the possibility that this may be the first crossover point, even though the χ^2 values supporting the crossover point was most statistically significant at position 3908.

Locating a definite breakpoint was not possible within the B3 virus genome region, as the genome region consisting of part of 2B, 2C, 3A, 3B, and part of 3C genes had a more complex genome sequence with weak association (low bootstrap values) to all other HEV71 and CV-A16 isolate sequences. Hence, the genome region served as a distinct signature sequence for the subgenotype B3 isolates.

Specific RT-PCR for detecting CV-A16 VP1 (CV-A16-VP1), HEV71 VP1 (HEV71-VP1), and HEV71/CV-A16-like sequence (REC) performed on the patients' specimens amplified only enterovirus sequences from all the samples by using the generic enterovirus primer sets that amplified the 5′ NTR (11). This finding suggests that all the samples, except for the controls, contained enterovirus sequences. In contrast, only HEV71-VP1 and HEV71/CV–A16-like

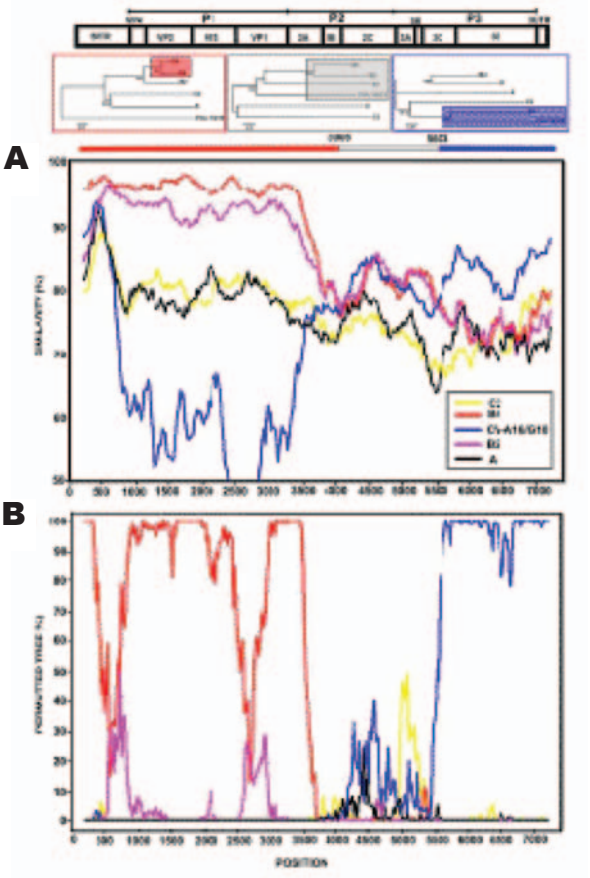

Figure 2. Identification of recombinant sequences in the genome of Human Enterovirus 71 (HEV71) subgenotype B3. A) shows the conflicting tree topology at the P3 genome region. B) shows results from similarity plot and bootscan analyses indicating the recombination sites. The window size of 400-nt slides in increments of 20 nt at a time. Positions containing gaps were excluded from the comparison.

sequences were amplified from SHA63 and SHA66 samples by using the respective PCR amplification primers (data not shown). No CV-A16-VP1 sequence was detected in either SHA63 or SHA66 patients' samples. These results suggest that the CV-A16–like sequences were not likely to have arisen from a serendipitous sequence amplification artifact involving samples with dual infections with HEV-71 and CV-A16. Furthermore, as both of the B3 isolates were obtained from two different HFMD patients (1), the existence of subgenotype B3 HEV71 with CV-A16–like sequences in nature is highly supported.

Further examination of all available 3D polymerase gene sequences (390 nt, subgenotype B3 nucleotide position 6696–7085) of all human enterovirus A (HEV-A) associated with HFMD (12), CV-A5, CV-A10, CV-A16, and HEV71, showed that subgenotype B3 isolates SHA63 and SHA66 had consistently higher nucleotide sequences similarity to CVA-16 (86%) than to all other HEV71 (75%–81%) and HEV-A (76%–78%) (online Appendix 2 Table available from http://www.cdc.gov/ncidod/eid/vol10no8/04-0059_app2.htm). The presence of this CV-A16–like sequence within the B3 virus genome suggested that a possible recombination event had previously occurred between HEV71 and CV-A16. The finding that the two viruses tend to cocirculate within the same population during most HFMD outbreaks (13) supported the likelihood that this recombination could have happened. Nonetheless, the parent virus may be a yet-to-be described HEV-A with high sequence similarity to CV-A16.

The CV-A16-like protease and polymerase gene within the B3 isolates genomes could influence the pathogenic potentials of the virus in humans. The B3 virus was likely less pathogenic as the B3 isolates, SHA63, and SHA66, were obtained from uncomplicated HFMD cases, and the B3 virus was not the main virus isolated from children with severe diseases during the outbreaks in Sarawak, Singapore, Malaysia, and Perth (4–6). However, whether all other B3 viruses shared similar characteristics is not known.

Recombination among nonsegmented RNA viruses was once thought to be uncommon. However, findings involving HIV, dengue virus, and poliovirus have established that intratypic recombination does occur among nonsegmented RNA viruses. Similar to enteroviruses (14) and dengue viruses (7), recombination could result in the emergence of viruses with altered pathogenic potentials. In our study, the discovery that two B3 isolates could have emerged as a result of a previous recombination event raises the possibility that recombination events among enteroviruses associated with HFMD occur more frequently in nature. These recombination events could be the mechanism driving the emergence of a number of newly described HEV71 lineages in Asia, some with differing pathogenic potentials.

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Syphilis in the HIV Era

Sigall Kassutto* and John P. Doweiko*

The incidence of syphilis has consistently increased from 2000 to 2002. We report a case of acquired syphilis with symptoms of Tullio phenomenon in a patient concurrently diagnosed with HIV infection. The resurgence of syphilis in HIV-positive groups at high risk has public health implications for prevention of both diseases.

The Case

A 35-year-old man with an unremarkable medical history sought treatment for headaches, hearing loss, and night sweats. The headaches were occipital and bilateral and had started 3 months earlier. They came on as the day progressed and were neither positional nor associated with nausea, vomiting, or visual changes. Three weeks before arriving at the hospital, he noted a sense of "fullness" in his ears; he said that spoken voices sounded muffled, and he had difficulty hearing telephone conversations. When someone at the restaurant where he worked dropped a dish, he heard that sound clearly and reported that it was almost painful, causing him to become dizzy. He otherwise denied ear pain or recent trauma. His appetite was good, and he had not lost weight. Although he reported no fever, he did report night sweats for several weeks. He had no rash, diarrhea, abdominal pain, chest pain, shortness of breath, joint complaints, or dysuria. The patient attributed his headaches to stress. He also said that he was exposed to dust at his workplace because of remodeling.

The patient was taking no regular medications and had no drug allergies. He had quit smoking 7 months earlier and drank 10–12 beers per week. He reported no history of intravenous drug use. He lived in New Hampshire, had no pets, and worked as part owner of a restaurant. Exposures included multiple male and female sexual partners with inconsistent condom use, and he had acquired several tattoos 8 months earlier while traveling in Spain and Italy. He had no known tuberculosis exposure. An HIV antibody test had been negative 18 months earlier.

On physical examination, he appeared healthy but anxious. Temperature was 36.7°C, blood pressure 128/84 mm Hg, pulse 80, respirations 16/minute. Sclerae were anicteric, and his pupils reacted to direct and consensual testing and responded normally to accommodation. Funduscopic examination showed no retinal abnormalities. The left tympanic membrane was retracted; both sides demonstrated a small effusion. No vesicles were seen. Bedside testing showed that his hearing was diminished to low volume sounds, but he was able to hear loud sounds, which he found painful. When the examiner clapped his hands loudly a few feet from the patient's ear, the patient exhibited nystagmus. His sinuses were not tender, and the oral mucosa had no lesions. The patient's neck was supple; a 1.5-cm, nontender lymph node was palpated in the left upper anterior cervical chain. No other lymphadenopathy was noted. The remainder of the examination was unremarkable, with negative Romberg test; normal gait; and normal motor, sensory, and reflex performance.

Laboratory evaluation (Table) showed low hematocrit and normal renal and liver function. HIV enzyme-linked immunosorbent assay and confirmatory test were positive. Rapid plasma reagin (RPR) was positive at a titer of 1:128 and was confirmed by a fluorescent treponemal antibody test. Subsequent studies showed a CD4 receptor–positive T-cell (CD4) count of 899/μL and an HIV viral load of 878 copies/mL. A lumbar puncture showed protein 33 mg/dL, glucose 78 mg/dL, 9 erythrocytes/μL, and 8 leukocytes/μL (100% lymphocytes). Cerebrospinal fluid venereal disease research laboratory test (CSF-VDRL) results were negative.

The patient was started on 24 mU of IV penicillin per day for 14 days. At the end of this period, he reported notable improvement in his headaches, hearing loss, and vertiginous symptoms. One month after completing treat-

Table. Selected laboratory test results 1 month after therapy for neurosyphilis in HIV-positive patient with neurosyphilis[a]

Laboratory test	Result
Complete blood count	
Leukocytes	5,700/μL
Hematocrit	36%
Platelets	295,000/μL
Electrolytes and renal fu nction tests	Within normal limits
Liver function tests	Within normal limits
Cerebrospinal fluid evaluation	
Leukocytes	8 cells/μL
Differential	100% lymphocytes
Erythrocytes	9 cells/μL
Protein	33 mg/dL
Glucose	78 mg/dL
CSF-VDRL	Negative
HIV-1 testing	
ELISA + WB	Positive
CD4 count	899 cells/μL
Viral load (RT -PCR)	878 copies/μL
Rapid plasma reagin testing	
Baseline	1:128
1 month posttreatment	1:32
3 months posttreatment	Nonreactive

[a]CSF-VDRL, cerebrospinal fluid venereal disease research labo ratory test; ELISA, enzyme -linked immunosorbent assay; WB, Western blot; RT -PCR, reverse transcription –polymerase chain reaction.

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ment, his RPR titer was 1:32; 3 months after he completed treatment, it was nonreactive.

Conclusions

The course of syphilis in an HIV-positive patient may be altered from the natural history of the disease in HIV-negative patients. An increased frequency of ocular disease, multiple and slower resolving primary chancres, and a higher titer RPR have been reported (1–6). In addition, delay or failure of titer decline after treatment, predilection for developing the Jarisch-Herxheimer reaction, and clinical relapse have also been described in HIV infection (1,4,7,8).

Treponema pallidum is thought to invade the central nervous system in 25% of patients with syphilis, irrespective of HIV status (9). Most of these persons successfully clear the infection (10), but other patients harbor the spirochete and remain at risk for sequelae of neurosyphilis. Syphilitic meningitis, meningovascular disease (often occurring with stroke), labyrinthitis, or cranial nerve palsies, as seen in this case, can be early findings.

The Tullio phenomenon is vestibular hypersensitivity to sound. In this condition, loud sounds or even routine acoustic stimuli can result in vertigo, nystagmus, or nausea and vomiting. The physiologic underpinnings of the Tullio phenomenon were first described in 1929, when Tullio noted that experimentally induced fenestrations in the bony capsule of the lateral semicircular canals of pigeons caused the canals to be sound-responsive, inducing vestibular activation (11,12). Shortly thereafter, Benjamins described a Tullio reaction in a human patient with fistulizing cholesteatoma (13). Today, the term has been generalized to include vestibular activation in response to stimulation by sound of any part of the vestibular apparatus (12).

The Tullio phenomenon is seen in a range of clinical contexts, including congenital deafness, Meniere disease, suppurative middle ear disease, and spirochetal infections, such as syphilis or Lyme disease. Nields et al. describe a woman who had nystagmus and vertigo with routine sounds; running tap water caused her to fall to the floor or retch in pain (14). Watson et al. describe a series of patients with oscillopsia induced by pencil tapping, telephone ringing, or the sound of cutlery falling on the floor. Patients often report a vague sense of ear blockage and an unpleasant awareness of their own voice vibrating in their ear (12).

Symptomatic cranial nerve VIII involvement in this patient prompted treatment for neurosyphilis, despite equivocal (but typical) CSF findings. Interpreting CSF findings in HIV-positive patients with syphilis is challenging because commonly encountered mild lymphocytic pleocytosis may be attributable to HIV. CSF-VDRL is often negative, with a 20%–70% false-negative rate (15).

Optimally managing patients with HIV and syphilis has been debated; to date routine recommendations are the same as for HIV-negative persons (16). Although any patient in whom syphilis is diagnosed and who has neurologic symptoms should undergo lumbar puncture, CSF evaluation should also be considered in asymptomatic patients in whom syphilis is diagnosed and who have an RPR titer $\geq$1:32 or a CD4+ count $\leq$350, as these markers have been associated with increased risk for neurosyphilis (10). Because of the high rate of relapse that has been reported in some series (4,7,8), close clinical and serologic follow-up is essential. Whether *T. pallidum* eradication is impaired in HIV coinfection remains controversial. A study of 59 patients with neurosyphilis showed that HIV-positive study participants were 2.5 times less likely to normalize CSF-VDRL reactivity than HIV-negative patients. This effect was even more pronounced in patients with CD4+ counts $\leq$200 (17). The findings suggest that more intensive therapy for neurosyphilis in immunocompromised patients merits further study.

After an all-time low case-rate of syphilis in 2000, reports of rising trends, specifically in groups at risk, such as men who have sex with men, were reported in 2001 (18). The outbreaks marked a 9.1% overall increased rate of primary and secondary syphilis (19) and were characterized by a high rate of HIV infection. Parallel increases in HIV infection rates were a concern because of the common mechanism of transmission and the increased efficiency of HIV transmission with coexistent genital ulcers (20). In 2001, increases in HIV rates were reported in several states with large urban populations; men who have sex with men accounted for the largest known subgroup at risk in incident adult or adolescent HIV cases (32%) and AIDS cases (44%) (21). New HIV diagnoses increased in 29 states with mandatory reporting from 1999 to 2002, notably by 17% among gay and bisexual men (22). Syphilis incidence also continued to rise in the United States in 2002, with a 12.4% increase since 2001 (19), and the highest trends were estimated to be among men who have sex with men. In San Francisco, primary and secondary syphilis rates increased by >1,000% from 1998 to 2002 among men who have sex with men (23). High-risk behaviors have been documented in this group (24) and factor prominently in syphilis reemergence in the early 21st century.

This case report describes a patient who had Tullio phenomenon as the index symptom of neurosyphilis with previously undiagnosed HIV infection. His RPR titer was 1:128, his CD4 count was preserved, and he responded well clinically and serologically to standard therapy. To our knowledge, this case is the first report of syphilis occurring as Tullio phenomenon in an HIV-positive patient. We suggest that syphilis be considered in patients who have cranial nerve VIII symptoms and an appropriate risk-factor profile. Eradicating treponemes may be limited

from sanctuary sites such as the CNS. Because control of syphilis likely depends not only on antimicrobial effect but also on host immune response, routine surveillance is a mainstay of therapy, particularly in patients with HIV infection. Awareness of unusual symptoms of a relatively common disease will benefit not only the patient but also public health efforts in managing both syphilis and HIV infection. Preventive and educational efforts focused on men who have sex with men may prove particularly important in modifying behaviors that foster the growth of both epidemics.

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Swimming and *Campylobacter* Infections[1]

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A matched case-control study was conducted to study risk factors for domestically acquired sporadic *Campylobacter* infections in Finland. Swimming in natural sources of water was a novel risk factor. Eating undercooked meat and drinking dug-well water were also independent risk factors for *Campylobacter* infection.

Campylobacter jejuni and *C. coli* are leading causes of human bacterial gastroenteritis in industrialized countries (1,2). In 1998, in Finland, the number of reported *Campylobacter* cases exceeded that of salmonella for the first time (2). A similar increase in *Campylobacter* incidence is evident in other industrialized countries (1,3), but the reason for this finding remains unknown (1).

Most human C*ampylobacter* infections are sporadic, and a seasonal peak in the distribution of the infections occurs during the summer months in several countries, including Finland (1–4). A variety of risk factors for *Campylobacter* infections have been identified, including handling and eating poultry (1,3,5–7) and drinking unpasteurized milk (1,3,5,7) or untreated water (1,8,9). In Finland, several waterborne outbreaks have been reported (10), but risks associated with sporadic *Campylobacter* infections are largely unknown (Table 1).

In our case-control study, we identified risk factors for and possible sources of infection for domestically acquired sporadic *Campylobacter* infections in Finnish patients from three geographic areas during the seasonal peak from July 1 to September 30, 2002.

The Study

Three clinical microbiology laboratories that served patients in the southern, central, and eastern parts of Finland participated in this multicenter, matched case–control study. A case-patient was defined as a person with stool culture, collected during the study period and tested at one of the three laboratories, that was positive for *C. jejuni* or *C. coli*. Patients from both outpatient clinics and hospitals were included. When a *Campylobacter*-positive patient was identified, personnel from the microbiology laboratory contacted the clinic or hospital for more information on the patient's recent travel history. If the patient had not traveled abroad within 2 weeks before illness, that patient's physician was contacted by phone and was asked to send to the patient information about our study and a questionnaire and a prepaid envelope to be returned to the researchers.

Two age-, sex-, and municipality-matched controls were chosen for each case-patient. Controls were selected from the Population Register Center, Espoo, Finland, an official register of all Finnish residents. Potential controls were contacted by mail and asked to fill in a questionnaire and mail it back in a prepaid envelope. Exclusion criteria for the controls were *Campylobacte*r infection, at least three loose stools per day, abdominal pain, or fever 30 days before filling out the questionnaire. If the questionnaire was not returned within 2 weeks, a new pair of controls was chosen, leading to a maximum of four controls per case.

The questionnaire sent to patients included questions on the disease, travel in and outside of Finland, dietary intake of food items (meat, fish, vegetables, fruit, and dairy products), quality of drinking water, contact with pets and other domestic animals, and swimming in water from natural sources. The controls answered similar questions except for those concerning illness. Case-patients and controls were excluded if they had traveled abroad within 2 weeks before illness (case-patients) or filling in the questionnaire (controls). The study was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa.

For sample-size calculation, the case-control ratio was 1:2. The exposure level among patients and controls was assumed to be 30% and 15%, respectively. The study was based on the estimate that 97 patients would be needed for the 5% significance level with 80% power. Only patients with at least one matched control were accepted for the final study set. Data entry was performed by EpiData 2.1b (EpiData Association, Odense, Denmark), and statistical analyses were made with EpiInfo 2002 (Centers for Disease Control and Prevention, Atlanta, GA). For risk factors with 95% confidence interval (CI) above one, conditional logistic regression examined these independently related to *Campylobacter* infection.

Of the 316 patients with stool culture–verified *Campylobacter* during the study period, 208 had no known foreign travel; the 634 controls also had not traveled out-

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[1]This study was in part presented at the 12th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms, Aarhus, Denmark, September 6–10, 2003.

Table 1. Matched multivariate analysis of significant risk factors for domestically acquired sporadic *Campylobacter* infection, July – September 2002, Finland [a]

Risk factor	Adjusted OR	95% CI	2-tailed p
Tasting or eating raw or undercooked meat	10.79	1.31–89.09	0.0272
Drinking water from a dug well	3.36	1.37–8.24	0.0082
Swimming in water from natural sources	2.80	1.23–6.39	0.0145

[a]OR, odds ratio; CI, confidence interval.

side of Finland. A total of 151 (73%) patients and 309 (49%) controls returned the questionnaire. Of the patients, 11 were excluded because of traveling abroad (according to the questionnaire), 3 for misunderstanding or missing information, 5 for having too long a delay (>37 days) between onset of symptoms and answering the questionnaire, and 11 because the delay between symptoms and answering the questionnaire could not be defined. In addition, a matched control was unavailable for 21 patients. Of the controls, 172 were excluded for the following reasons: traveling abroad (17 controls), gastrointestinal symptoms (56 controls), missing information (21 controls), and previous *Campylobacter* infection (2 controls); 76 were omitted because of the lack of a matching case. The final analysis was made up of 100 patients and 137 controls.

A total of 99 patients were infected with *C. jejuni* and 1 with *C. coli*. All cases were sporadic and not associated with any known outbreaks. Regional distribution and demographic characteristics of patients and controls are presented in Table 2. Patients and controls were matched in doubles (66 patients), triples (31 patients), and quadruples (3 patients). Patients filled in the questionnaires within 3 to 37 days from onset of illness, with a median delay of 16 days. The median interval between onset of illness of the patients and their controls responding to the questionnaire was 32 days. The median delay between patients and controls filling in the questionnaire was 15 days. The total number of exposures analyzed was 82. Factors significantly associated with an increased or a reduced risk for *Campylobacter* infection are shown in Table 3.

Of the 14 patients who ate undercooked or raw meat, 57% had eaten poultry and 36% minced meat, supporting previous studies that have identified eating undercooked poultry as a risk factor (7,8,11). Except for tasting or eating undercooked chicken meat, preparing or eating chicken was not associated with an increased risk for *Campylobacter* infection in our study.

Of the four significant risk factors in the initial univariate analysis, three were independently associated with *Campylobacter* infection in multivariate analysis: tasting or eating undercooked or raw meat, drinking untreated dug well water, and swimming in natural sources of water (Table 1). At least one of these three epidemiologically associated risk factors was found in 67% of the patients.

Conclusions

We identified, to our knowledge for the first time, swimming in natural sources of water to be an independently associated risk factor for sporadic *Campylobacter* infection. As the infective dose for *Campylobacter* infection is likely low, contaminated surface water may cause infection through swimming; campylobacters are commonly found in natural waters, such as rivers, streams, and lakes (12). However, in contrast to our study, in a recent Norwegian study (9), swimming in the sea, lakes, and swimming pools was associated with a reduced risk for *Campylobacter* infection.

Our study showed that private water supplies present a significant risk factor for sporadic *Campylobacter* infection. Kapperud et al. (9) also found that exposure to surface water or drinking nondisinfected water caused an increased risk. In Finland, in addition to the 310,000 households that use private wells, approximately 300,000 summer cottages have private water supplies (13). Dug wells are susceptible to surface water contamination. Furthermore, the summer of 2002 was exceptionally dry in Finland, resulting in poor water quality in these wells because of low groundwater levels. In our study, drinking

Table 2. Patient characteristics

Characteristic	Patients, N = 100 (%)	Controls, N = 137 (%)
Sex		
Male	42 (42)	56 (41)
Female	58 (58)	81 (59)
Age (y)		
1–4	4 (4)	6 (4)
5–9	1 (1)	2 (2)
10–19	3 (3)	5 (4)
20–29	13 (13)	18 (13)
30–39	9 (9)	13 (10)
40–49	15 (15)	18 (13)
50–59	26 (26)	32 (23)
≥60	29 (29)	43 (31)
Median age (y)	51	51
Municipality		
Helsinki	35 (35)	47 (34)
Kuopio	44 (44)	62 (45)
Joensuu	21 (21)	28 (20)

Table 3. Matched univariate analysis of exposure factors for domestically acquired sporadic *Campylobacter* infection, July–September 2002, Finland [a]

Risk factor	Patients, n = 100	Controls, n = 137	Adjusted OR	95% CI	2-tailed p
Increased risk					
Tasting or eating undercooked or raw meat [b]	14/88	3/124	12.00	1.54–93.77	0.0052 [c]
Drinking water from a dug well	31/96	22/137	3.19	1.58–6.45	0.0017
Swimming in water from natural sources	48/100	40/134	2.27	1.24–4.16	0.0089
Eating strawberries	70/89	79/124	2.90	1.21–6.95	0.0287
Reduced risk					
Eating					
Black and red currants	17/73	73/126	0.17	0.07–0.41	< 0.0001
Blueberries	20/73	56/118	0.43	0.21–0.89	0.0115
Carrots	43/83	89/126	0.44	0.24–0.82	0.0039
Yogurt	51/90	83/121	0.35	0.15–0.85	0.0332
Pasteurized milk	55/93	99/131	0.44	0.22–0.85	0.0075
Cooked or fried fish	50/93	98/128	0.35	0.18–0.67	0.0004
Liver (beef)	4/69	15/105	0.18	0.04–0.87	0.0083
Drinking water produced by a large water plant	52/97	88/137	0.52	0.26–1.02	0.0371
Eating at a friend's house	24/49	44/71	0.35	0.13–0.96	0.0195
Others					
Eating					
Minced meat (pork)	45/83	70/116	0.54	0.28–1.06	0.0438
Minced meat (beef)	64/90	97/128	0.78	0.42–1.46	0.3459
Drinking					
Water produced by a small water plant	23/97	34/137	0.80	0.37–1.72	0.4528
Water from bedrock well	20/97	17/137	1.96	0.89–4.34	0.1210
Bottled water	15/97	27/137	0.75	0.37–1.51	0.3062
Contact with cat	27/88	40/118	0.87	0.43–1.76	0.5768
Contact with dog	53/93	76/128	1.02	0.55–1.89	0.9385
Contact with farm animals	4/83	9/118	0.36	0.06–2.14	0.1426
Eating outside the home	69/98	100/137	0.78	0.41–1.48	0.3686
Eating chicken prepared from					
Nonmarinated pieces	9/71	20/111	0.32	0.10–1.06	0.0274
Marinated pieces	34/81	47/111	0.76	0.38–1.58	0.3613
Nonmarinated strips	11/70	13/114	1.06	0.33–3.46	0.8357
Marinated strips	19/77	40/118	0.61	0.29–1.28	0.1324
Drinking unpasteurized milk	7/80	9/111	1.40	0.45–4.37	0.7768

[a]OR, odds ratio; CI, confidence interval.
[b]Of 14 exposed patients, 13 specified meat type: 8 (57 %) had tasted u ndercooked poultry, and 5 (36%) had tasted minced meat.
[c]Fisher exact test.

water from a large water plant protected against sporadic *Campylobacter* infection. Large water plants usually have surface water as their source and use multistage purification and disinfection procedures before drinking water is distributed to consumers, which substantially reduces risk for waterborne infections.

Eating strawberries, although a significant risk factor in univariate analysis, was not an independent risk factor in the multivariate analysis. During the same time period but outside the study region, a small cluster of cases was reported for which the suspected source was eating strawberries directly from the field (14).

Reduced risk for the disease was associated with eating other berries, such as red and black currants and blueberries, and carrots. These findings are consistent with the literature (7–9), although no one fully understands the role of these protective factors.

In Finland, because most sporadic *Campylobacter* infections occur during July to September, our study could not identify risk factors that may have varied seasonally. The median age of our patients and controls was considerably high (51 years of age), which may have influenced the results. This age group, however, may be typical for Finland, since in our previous study sporadic, domestically acquired *Campylobacter* infections were frequent in certain parts of the country in elderly men (15).

In addition to the known risk factor of eating raw or undercooked meat, this study clearly identified water as an important risk for domestically acquired *Campylobacter* infections in the summertime in Finland. The novel finding that swimming in water from natural sources was associated with increased risk for infection further emphasizes the importance of other water-related exposure factors.

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Spotted-Fever Group *Rickettsia* in *Dermacentor variabilis*, Maryland

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Three-hundred ninety-two adult *Dermacentor variabilis* were collected from six Maryland counties during the spring, summer, and fall of 2002. Infection prevalence for spotted fever group *Rickettsia* was 3.8%, as determined by polymerase chain reaction. Single strand conformational polymorphism (SSCP) analysis followed by sequencing indicated that all infections represented a single rickettsial taxon, *Rickettsia montanensis*.

The Study

Several species of spotted fever group (SFG) rickettsiae have been isolated from ticks in the United States; however, the only species considered to cause human disease in Maryland is *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF). The potential pathogenicity of rickettsial organisms is most often predicted by the ability of the species to cause disease in guinea pigs. The reliability of this method has been debated, and researchers have suggested that "every rickettsial species may have pathogenic potential, provided that its reservoir arthropod is capable of biting humans" (1,2).

The prevalence of SFG *Rickettsia* infection in *Dermacentor variabilis*, the primary vector of *R. rickettsii* in the eastern United States, has been estimated in several studies. Prevalences from 0.2% in Ohio (3) to 8.6% in Maryland (4) have been reported. Many studies have implied that these infections were *R. rickettsii*, but few have confirmed these identities (5). Numerous SFG-rickettsial species have been isolated or partially characterized from molecular evidence in the eastern United States; these species include *R. rickettsii*, *R. rhipicephali*, *R. montanensis* (=*R. montana*), *R. parkeri*, and "*R. amblyommi*" (3,6–8). These species have been identified, either together or separately, in areas where RMSF is endemic. As the distributions of different SFG-species in disease-endemic areas become better understood, determining the relationship between the rickettsiae involved in human disease and those isolated from vector ticks and mammal and tick reservoirs may be necessary.

Differentiating the tick-borne SFG *Rickettsia* before the 1990s depended largely on culture and epitope recognition techniques, such as immunoflourescence and agglutination tests and mouse serotyping with monoclonal antibodies. Genotypic studies of rickettsiae conducted during the 1990s led to two rickettsial genes that can be used to identify rickettsial infections: citrate synthase (*gltA*) and *rOmpA* (9). Citrate synthase encodes the first enzyme of the tricarboxylic acid cycle and is highly conserved among all *Rickettsia* species, serving as a polymerase chain reaction (PCR) target to identify any rickettsial infection. *rOmpA* encodes a surface-expressed protein of SFG-rickettsiae that is important for adhesion to host cells (10). Only SFG *Rickettsia* contain the *rOmpA* gene (11), making it an ideal PCR target to identify SFG *Rickettsia* infections.

Approximately 35 cases of RMSF are reported annually in Maryland. From 1994 through 1998, Maryland ranked 8th nationally, reporting 112 cases. These cases, confirmed by the Maryland Department of Health and Mental Hygiene, meet the Centers for Disease Control and Prevention (CDC) case definition, yet not much information exists to characterize the infection rate of SFG rickettsiae in *D. variabilis* in the state. This cross-sectional study examined the prevalence and composition of SFG *Rickettsia* in *D. variabilis* in Maryland.

In 2002, genomic DNA was extracted from 392 adult *D. variabilis* collected by flagging in Anne Arundel, Baltimore, Calvert, Charles, Prince George's, and St. Mary's Counties, Maryland. Quality of the modified hexadecyltrimethylammonium bromide (CTAB) DNA extractions was verified by amplifying a tick 16S mtDNA fragment (12). Modifying the existing extraction procedure involved an additional phenol:chloroform:isoamyl alcohol (25:24:1) extraction step to further stabilize the extracted DNA. Tick extractions were screened by PCR for evidence of infection with *Rickettsia* by using primers specific to the *Rickettsia* citrate synthase gene (9). The *Rickettsia* infection rate was 6.1% (24/392, 95% confidence interval [CI] 4.0%–9.0%). All *Rickettsia*-positive tick extractions were subsequently screened by PCR for SFG *Rickettsia* by using primers for the *rOmpA* gene of SFG-*Rickettsia* (9). The prevalence of SFG *Rickettsia* infection was 3.8% (15/392, 95% CI 2.2%–6.2%). Single strand conformational polymorphism (SSCP) banding patterns were identical for all tick-derived *rOmpA* PCR amplicons. Similarly, SSCP banding patterns of the tick-derived citrate synthase amplicons for the SFG-*Rickettsia*–positive samples were monomorphic. These results suggest that these tick infections represent a single

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Table 1. Characteristics of *Dermacentor variabilis* collected in Maryland, 2002 [a]

Characteristic	N	% infection with any *Rickettsia* organisms (95% CI)	% infection with SFG *Rickettsia* (%) (95% CI)
All ticks	392	6.1 (4.0–9.0)	3.8 (2.2–6.3)
Sex		p[b] = 1.000	p = 1.000
Male	185	5.9 (3.0–10.4)	3.8 (1.5–7.6)
Female	207	6.3 (3.3–10.5)	3.9 (1.7–7.4)
County of collection		p = 0.052	p = 0.024
Anne Arundel	1	0 (0–97.5)	0 (0–97.5)
Baltimore	342	6.1 (3.8–9.2)	3.5 (1.8–6.0)
Calvert	17	0 (0–19.5)	0 (0–19.5)
Charles	18	0 (0–18.5)	0 (0–18.5)
Prince George's	1	100 (2.5–100)	100 (2.5–100)
Saint Mary's	13	15.4 (1.9–45.4)	15.4 (1.9–45.4)
Month collected		p = 0.007	p = 0.101
April	146	4.8 (1.9–9.6)	4.8 (1.9–9.6)
May	108	4.6 (1.5–10.5)	1.9 (0.2–6.5)
June	78	2.6 (0.3–9.0)	1.3 (0.03–6.9)
July/August	58	17.2 (8.6–29.4)	8.6 (2.9–19.0)
Unknown[c]	2	0	0

[a]CI, confidence interval; SFG, spotted fever group.
[b]Fisher exact p values.
[c]Ticks with unknown month of collection were excluded from the statistical analyses for this characteristic.

SFG *Rickettsia* taxon (13). Citrate synthase and *rOmpA* PCR products from three ticks were sequenced with the citrate synthase and shortened *rOmpA* PCR primers, respectively. Sequences of each respective gene fragment derived from these ticks were identical and confirm the SSCP findings (GenBank accession no.: *gltA*, AY548828–AY548830, *rOmpA*, AY543681–AY543683). The derived sequences were also compared to rickettsiae sequences in the public domain and were identical to those derived from *R. montanensis* from *D. andersoni* (GenBank accession no. RMU55823 *rOmpA* and RMU74756 *gltA*).

Prevalence estimates were reported as percentages with exact 95% CI based on the binomial distribution. Fisher exact test was used to compare infection prevalence across the strata of selected characteristics. The association between each characteristic and the prevalence of infection was quantified as odds ratios (OR), calculated with logistic regression or exact methods for categorical data when the data were highly unbalanced. All statistical analyses were performed with STATA (version 7.0; Stata Corporation, College Station, TX) or StatXact (version 5.0.3; Cytel Software Corporation, Cambridge, MA).

The variation in prevalence of *Rickettsia*-positive ticks across all counties was marginally significant (p = 0.052), with a higher prevalence in St. Mary's County compared to all other counties (OR 5.1, 95% CI 0.5–27.2, p value = 0.08). However, only 13 ticks were collected from St. Mary's County, so this estimate was based on limited data. In contrast to the equivocal results for the geographic distribution of *Rickettsia*-positive ticks, temporal heterogeneity was evident, as the prevalence of *Rickettsia*-positive ticks varied significantly with month of collection (p = 0.007). Risk for infection was significantly elevated for any *Rickettsia* organism in ticks collected in July or August (OR 4.1, 95% CI 1.5–11.5) compared to those collected in April. Further analyses combining the data from the spring and early summer months showed that the risk for

Table 2. Univariate odds ratio (OR) associated with any *Rickettsia* organism and with *R. montanensis*

	Rickettsia genus–positive			*R. montanensis*–positive		
Variable	OR	95% CI[a]	p value	OR	95% CI	p value
Sex						
Female	1.0	Reference		1.0	Reference	
Male	0.94	0.41–2.16	0.890	0.98	0.35–2.75	0.967
County of collection						
Baltimore	1.02	0.29–3.57	0.969	0.57	0.16–2.09	0.397
All other counties	1.0	Reference		1.0	Reference	
Month collected						
April	1.0	Reference		1.0	Reference	
May	0.96	0.30–3.12	0.951	0.37	0.08–1.84	0.227
June	0.52	0.11–2.58	0.425	0.26	0.03–2.13	0.209
July/August	4.14	1.49–11.47	0.006	1.87	0.57–6.16	0.301

[a]CI, confidence interval.

infection with any *Rickettsia* organism in July or August was even higher (OR 4.7, 95% CI 2.0–11.3). The risk for infection with *R. montanensis* with the late summer months, compared to the spring and early summer months, was somewhat less but still approached statistical significance (OR 3.0, 95% CI 0.8–10.2, p value = 0.06). This observation may be an artifact of diminishing tick abundance later in the summer months.

Conclusions

The prevalence of SFG *Rickettsia* in *D. variabilis* estimated from this study (3.8%) was lower than that in previous reports from Maryland. However, in regions where RMSF is observed annually, prevalence estimates range widely, from 2% in Connecticut to 10% in Alabama, with intermediate prevalences in New York, Kentucky, Tennessee, and Arkansas (5). In addition, *R. montanensis* had not been previously recognized in Maryland. Most earlier studies of SFG *Rickettsia* infection prevalence did not identify the *Rickettsia* to the species level, although the SFG-positive samples were sometimes assumed to represent *R. rickettsii*. One study in Maryland in which 26 *Rickettsia* isolates were obtained from *D. variabilis* determined the species composition of the rickettsiae. Two isolates were *R. rickettsii*, 1 isolate was *R. bellii* (non-SFG), and 23 (88%) were identified as WB-8-2, a then-unnamed SFG-*Rickettsia* (5). Weller et al. performed a phylogenetic analysis and found WB-8-2 ("*R. amblyommii*") to be closely related to *R. montanensis* (14), although they can be differentiated by serotyping.

R. montanensis has been isolated from ticks in other eastern states. During the 1980s, Feng et al. reported that *R. montanensis* represented 41 (91%) of 45 of the SFG isolates from *D. variabilis* collected in Cape Cod, Massachusetts (7). Anderson et al. reported isolation of *R. montanensis* from *D. variabilis* in Connecticut (6), and in 1990, Pretzman et al. reported that most SFG *Rickettsia* isolated from *Dermacentor* ticks throughout Ohio was *R. montanensis* (3). Further, these researchers noted that *R. rickettsii* were not isolated from ticks collected in several Ohio counties where RMSF was considered endemic. These studies illustrate that the rickettsial composition and dynamics within the RMSF-endemic areas are complex and need to be addressed with greater scrutiny.

The role of SFG *Rickettsia* in human health is largely unknown, and many are considered to be nonpathogenic either because the bacteria have not been isolated from humans or they do not demonstrate pathogenicity in animal models. For example, *R. montanensis* is avirulent in guinea pigs but virulent in voles (15). These findings have led to caution when labeling rickettsiae as nonpathogenic (2). *R. montanensis* and other "nonpathogenic" SFG *Rickettsia*–infected ticks may also benefit human health by decreasing *R. rickettsii* in tick populations as a result of the "interference" phenomenon (15).

The findings of this study and others raise important questions. In 2000, a total of 495 cases of RMSF were reported to CDC and 4 deaths were attributed to spotted fever caused by *Rickettsia rickettsii*. The extent to which *R. rickettsii* is the agent responsible for reported cases of RMSF should be reevaluated, considering the number of studies completed in RMSF-endemic regions, including this one, that have found non–*R. rickettsii* as the predominant or only detectable SFG *Rickettsia*.

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Human *Escherichia coli* O157:H7 Genetic Marker in Isolates of Bovine Origin

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The antiterminator *Q* gene of bacteriophage 933W (Q_{933}) was identified upstream of the *stx2* gene in 90% of human disease–origin *Escherichia coli* O157:H7 isolates and in 44.5% of bovine isolates. Shiga toxin production was higher in Q_{933}-positive isolates than Q_{933}-negative isolates. This genetic marker may provide a useful molecular tool for epidemiologic studies.

Escherichia coli O157 is recognized worldwide as an important cause of diarrheal disease, which in some patients is followed by hemolytic uremic syndrome and death (1). A primary virulence factor of this pathogen is the prophage-encoded Shiga toxin (2). Greater Shiga toxin production per bacterium is associated with increasing severity of human disease (3,4). Because of its location in the phage genome, the *stx*-gene variant dubbed stx_2 is under similar regulatory control as other phage late-genes, as it is governed by the interaction of the transcription antiterminator Q with the late promoter P_R' (5).

Although cattle and other ruminants appear to be the natural reservoir for *E. coli* O157 and other Shiga toxin–producing *E. coli* (STEC), only a small fraction of STEC serotypes routinely present in cattle are frequently isolated from human patients. Mounting evidence suggests that considerable genetic, phenotypic, and pathogenic diversity exists among these pathogens (6–8). Furthermore, genetic subtypes or lineages of *E. coli* O157 do not appear to be equally distributed among isolates of bovine and human origin (7). The purpose of this study was to examine the distribution of specific sequences upstream of the stx_2 gene among *E. coli* O157:H7 of human and bovine origin, along with corresponding magnitudes of Shiga toxin production.

The Study

A total of 158 stx_2-encoding *E. coli* O157:H7 isolates were assayed, 91 isolates of bovine origin and 67 originally isolated from ill persons (see online Appendixes 1 and 2; http://www.cdc.gov/ncidod/EID/vol10no8/03-0784_app1.htm and http://www.cdc.gov/ncidod/EID/vol10no8/03-0784_app2.htm). All isolates demonstrated unique banding patterns on pulsed-field gel electrophoresis (PFGE). For polymerase chain reaction (PCR) analysis, 5 µL of DNA obtained from boiled stationary-phase bacteria was added to a 50-µL PCR master mix containing a final concentration of 1.5 (Q_{933}) or 2.5 (Q_{21}) mmol $MgCl_2$, 200 µmol/L each deoxynucleoside triphosphate, 1 U *Taq* polymerase, 0.6 pg/µL of primer 595 (5′-CCGAAGAAAAACCCAGTAACAG-3′) (9), and 0.6 pg/µL of either primer Q_{933} (5′-CGGAGGGGATTGTTGAAGGC-3′;Q_{Stxf}) (9) or primer Q_{21} (5′-GAAATCCTCAATGCCTCGTTG-3′; this study). PCR consisted of an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 s, 52°C (Q_{933}) or 55°C (Q_{21}) for 1 min, and 72°C for 1 min; and a final 10-min extension step at 72°C. *E. coli* strain 933 or FAHRP88 was used as a positive control and master mix alone as a negative control. All PCR products were separated by gel electrophoresis (100 V) in 1% agarose gels, stained with ethidium bromide, and visualized by using UV illumination.

Shiga toxin production was determined by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Premiere EHEC, Meridian Diagnostics, Cincinnati, OH). Briefly, log-phase cells from Luria-Bertani broth enrichments were diluted to 0.6 optical density (OD) at 600 nm, subsequently pelleted, resuspended in phosphate-buffered saline, and induced by exposure to UV light (240 nm) for 3 s (10). A 1:9 volume of a 10x concentrate of brain heart infusion broth was added to each culture and shaken at 37°C for 2.5 h. Replicate cultures that were not exposed to UV light (noninduced controls) were maintained at 4°C. Two hundred microliters of each induced and noninduced enrichment was subsequently used as the specimen in the EHEC ELISA, as described (11). OD results were recorded for each isolate both with and without UV induction. The relative change in Shiga toxin production after induction was calculated for each isolate; $(OD_{induced})/OD_{noninduced})$. *E. coli* O157 (EDL933) and a toxin-negative control isolate were assayed as positive and negative controls each time the assay was repeated.

E. coli O157 isolates were classified on the basis of the presence or absence of bands of the predicted size on the Q_{933}-595 and Q_{21}-595 PCR reactions (Figure). A chi-square test was used to determine whether different PCR genotypes were equally distributed among isolates of bovine and human origin. Likewise, a chi-square test was used to assess the equality of distribution of PCR genotypes among bovine isolates from different countries. One-way analysis of variance for nonparametric data (Kruskal-Wallis test) was used to identify differences in

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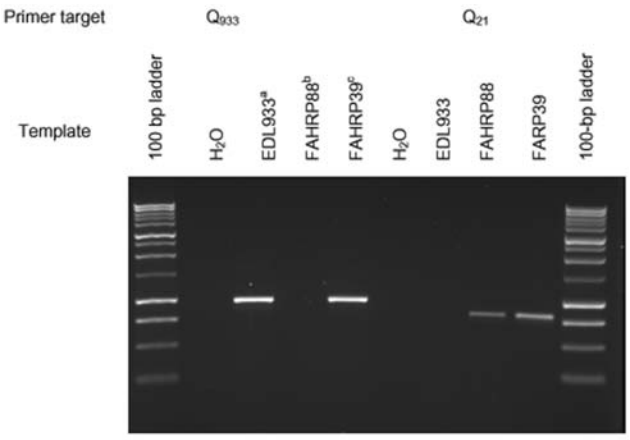

Figure. Ethidium bromide–stained gel of the amplification products obtained from Q933-595 and Q21-595 polymerase chain reactions. [a]EDL933, human isolate (ATCC43895). Obtained from the STEC Center, Michigan State University. [b]FAHRP88, isolated from Ohio dairy cow. [c]FAHRP39, human isolate (E29962) (12).

ranked-transformed toxin production among noninduced and induced *E. coli* O157 isolates as well as to determine significant differences in the percent increase in toxin following induction.

Previously, Kim et al. described a nonrandom distribution of *E. coli* O157 subtypes among cattle and humans by using an octamer-based genome-scanning method (7). We tested several of the isolates that had been previously characterized. Nine had been previously identified as belonging to the lineage I genotype and seven isolates as belonging to the lineage II genotype. We found that all nine lineage I isolates consistently amplified the Q_{933} target, regardless of species of origin. All four bovine isolates classified as lineage II by Kim et al. amplified the Q_{21} target. One lineage II human isolate (NE015) amplified the Q_{933} target, and another lineage II isolate (NE037) produced no amplicons in either PCR reaction. One human isolate classified as lineage II (ATCC 43889) amplified both target sequences, presumably because of polylysogeny.

The distribution of the specific *Q*-gene alleles found upstream of the prophage *stx* region among bovine isolates may have a geographic component. The distribution of *E. coli* O157 phage genotypes collected from healthy cattle from diverse geographic areas is consistent with the variable incidences of human disease in different countries (Table 1). For example, six (75%) of eight Scottish bovine isolates examined amplified the Q_{933} target, the same target that is frequently present in human isolates of human disease origin. Scotland reports some of the highest incidence rates of human *E. coli* O157–related diseases and hemolytic uremic syndrome (13). In contrast, none of the seven Australian *E. coli* O157 bovine isolates amplified the 1750-bp fragment. Contrary to the situation in Scotland and the United States, *E. coli* O157 infection of humans is rarely reported in Australia (14).

Conclusions

The Q_{933} gene target was more commonly identified in human disease–associated strains of *E. coli* O157 than from strains of bovine origin. Amplification of the Q_{933} target, either alone or in combination with amplification of the Q_{21} target from the same isolate, was identified in 60 (9%) of 66 (55/66 alone and 5/66 in combination with Q_{21}; 1 isolate amplified neither target) compared to 40 (44%) of 91 (32/91 alone, and 8/91 in combination with Q_{21}) of bovine isolates ($p < 0.001$). Furthermore, these genetic subtypes were nonrandomly distributed among the *E. coli* O157 isolates of bovine origin obtained from different countries ($p < 0.05$) (Table 1).

These limited data suggest that the distribution of *E. coli* O157 strains in cattle may differ between countries or regions, thereby providing an explanation for geographic differences in the incidence of human *E. coli* O157 infection. More isolates from cattle need to be analyzed with these methods to better characterize the *E. coli* O157 in the bovine reservoir of each country.

A positive reaction with the Q_{933} target was significantly associated with higher OD results on the Shiga toxin ELISA (both noninduced and induced) and higher-fold increases in toxin production following induction than isolates amplifying the Q_{21} target alone ($p < 0.0001$) (Table 2). Despite these differences, we did not identify any clinical associations between the magnitude of Shiga toxin production and severity of human disease could be identified in this study. Other, non–Shiga toxin–related virulence factors and host susceptibility are also believed to play

Table 1. Distribution of polymerase chain reaction results from bovine *Escherichia coli* O157 isolates based on geographic origin[a]

		Q allele		
Country of origin	No. tested	933	21	Both
		N (%)	N (%)	N (%)
USA	46	20 (44)	25 (54)	1 (2)
Scotland	8	– (0)	2 (25)	6 (75)
Australia	7	– (0)	7 (100)	– (0)
Japan	17	3 (18)	14 (82)	– (0)
Total	78	23 (29)	48 (62)	7 (9)

[a]–, not detected. Percentages are read across rows, not down columns. Significant difference in proportion of *Q* alleles isolated from different countries ($p < 0.05$, chi-square test for homogeneity).

Table 2. Shiga toxin production by *Escherichia coli* O157:H7 by *Q* allele

Assay	*Q* allele	Response		
		Median	Minimum	Maximum
OD_{600nm} noninduced	Q_{933}	0.442	0.153	2.814
	Q_{21}	0.170	0.120	0.413
OD_{600nm} induced	Q_{933}	1.228	0.172	2.896
	Q_{21}	0.165	0.084	1.210
Fold increase in OD_{600nm} after induction [a]	Q_{933}	2.2	0.3	7.7
	Q_{21}	0.9	0.4	5.1

[a] $(OD_{induced})/(OD_{noninduced})$. The maximum and minimum optical density readings at 600 nm listed in each row are not necessarily from the same isolate; therefore, the maximum -and minimum-fold increase cannot be calculated directly from the table.

essential roles in the outcome of clinical STEC infections. The Q_{933}-negative isolates obtained from human disease might have lost this Q_{933}-containing prophage by the time of isolation, or these isolates might have been recovered from patients also infected with STEC containing Q_{933}-type prophage (15). Whether specific *Q*-gene alleles directly correlate with the magnitude of Shiga-toxin production or whether other (unstudied) factors within the phage lytic cascade genetically linked to specific *Q* alleles instead are responsible for the magnitude of toxin production is not known.

The antiterminator Q, the protein product of the *Q* gene, and $P_{R'}$, the late promoter, are reputed to be involved in regulating phage late-genes and, because of the location of $P_{R'}$ in prophage genome, of Shiga toxin production as well (5). In *E. coli* O157 phage 933W (GenBank no. 9632466) and *E. coli* O157 stx_{2vhd} (GenBank no. 15718404), the 359-bp sequence immediately upstream of the stx_2 gene is nearly identical (>95% nucleotide identity). However, further upstream of this area of identity, DNA sequences differ significantly. In *E. coli* O157 933W, this gene is identified as the antiterminator *Q* gene. In contrast, in *E. coli* O157 stx_{2vhd} this area is occupied by a gene with >95% sequence identity with the antiterminator *Q* gene of bacteriophage 21 (gi 4539472). The *Q* gene of bacteriophage 21 does not share DNA sequence homology with the *Q* gene of bacteriophage 933W, and only 36% predicted amino acid homology. Since the *Q* gene is reputed to play an important role in regulating toxin production, our results provide a plausible explanation (differential regulation of Shiga toxin production) of why certain *E. coli* O157 genotypes are more commonly isolated from human patients (7).

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SARS Risk Perception, Knowledge, Precautions, and Information Sources, the Netherlands

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Severe acute respiratory syndrome (SARS)–related risk perceptions, knowledge, precautionary actions, and information sources were studied in the Netherlands during the 2003 SARS outbreak. Although respondents were highly aware of the SARS outbreak, the outbreak did not result in unnecessary precautionary actions or fears.

Severe acute respiratory syndrome (SARS) is one of the latest examples of an emerging infectious disease confronting the world (1). Outbreaks of diseases like SARS are expected to recur, and they may rapidly spread across the globe. Measures to control outbreaks include not only identifying new organisms, developing vaccines, and initiating appropriate therapies, but also adequately informing the public about risks and precautions. In an unaffected country like the Netherlands, true risk may have been low, but SARS still received broad media attention, which may have increased perception of risk. Perceived risk, not actual risk, determines the population's reaction (2,3), even though these perceptions are often biased (3). The public may be optimistic when familiar risks are perceived to be largely under volitional control; pessimism, sometimes leading to mass panic, is more likely a result of perceiving risks to be uncontrollable (2–5). Persons who perceive themselves to be at risk for SARS may engage in precautionary behavior, but they may also stigmatize those who are perceived as possible sources for infection (6). To promote realistic risk perceptions and effective precautions, communication through various information sources is essential (7,8).

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The Study

We explored SARS-related risk perceptions, knowledge, actions, and use of information sources in an area where no cases occurred during the 2003 SARS outbreak. Respondents were drawn from a random sample of 500 members of an Internet research panel who completed an electronic questionnaire on a Web site June 19–26, 2003. Respondents were 373 persons ages 19–78 years; 48% were male. Of the respondents, 37.2% had a low level of vocational or secondary education; 39.6% had an intermediate level of vocational or secondary education; 21.5% had professional or university training; and the remainder were missing values.

Data were collected with an electronic questionnaire developed by the SARS Psychosocial Research Consortium (G.D. Bishop et al., unpub. data; full questionnaire is available from http://www.eur.nl/fgg/mgz/papers.html). Risk perceptions were obtained by asking respondents how they estimated their risk of acquiring and dying from SARS. To compare the SARS-related risk perceptions to other potential threats, respondents were asked to indicate how likely they thought it was for them to get other diseases or have accidents (Table 1). Respondents were also asked how worried they were about contracting SARS, a family member getting SARS, SARS occurring in their region, SARS emerging as a health problem, and the likelihood of other persons acquiring SARS.

Knowledge about SARS was assessed with four questions on whether respondents had ever heard of SARS, knew what SARS is, knew its causes, and knew the death rate for people with the condition. A total SARS-related knowledge score was computed by adding the correct answers to the questions (range 0–4).

Respondents were asked whether they felt able to avoid contracting SARS and which actions they had taken to avoid getting it (Table 2). The total number of actions taken was regarded as an overall SARS precautionary behavior score (range 0–19, $\alpha = 0.72$). Diagnostic actions that could be indicated included taking one's temperature; going to a physician; paying attention to coughing, sneezing, feelings of fatigue, and headaches; and calling a SARS

Table 1. Perceived risk of being affected by SARS and other diseases or accidents [a]

	Mean (SD)	% likely or very likely
SARS	1.5 (0.8)	1.0
Flu or common cold	4.0 (1.0)	72.9
Accident at home	3.5(1.0)	52.0
Cancer	3.0 (1.0)	18.5
Heart attack	2.9 (0.9)	21.7
Traffic accident	2.8 (0.9)	16.1
Food poisoning	2.8 (1.0)	21.4
HIV/AIDS	1.5 (1.9)	1.9

[a]SARS, severe acute respiratory syndrome; for the scores, 1 = very unlikely and 5 = very likely.

Table 2. Proportion of respondents (N = 373) who reported specific actions to prevent severe acute respiratory syndrome (SARS)

Precautionary action	Percentage
Avoided travel to SARS-infected areas	39.9
Made sure to get sufficient sleep	8.3
Wore a mask	3.8
Avoided eating in "food centers"	2.9
Took an herbal supplement	2.4
Avoided large gatherings of people	2.1
Washed hands more often	2.1
Used disinfectants	2.1
Were more attentive to cleanliness	1.9
Avoided particular types of people	1.6
Ate a balanced diet	1.6
Avoided travel by airplane	1.1
Did not go to school or work	1.1
Avoided shaking hands	1.1
Avoided travel by taxis	0.5
Avoided travel on subways or buses	0.3
Avoided eating in restaurants	0.3
Exercised regularly	0.3

hotline. The total number of actions was regarded as a diagnostic behavior score (range 0–8, $\alpha = 0.77$). Respondents were asked to indicate how likely they were to avoid different persons to prevent SARS. Finally, respondents were asked to indicate how much information about SARS they obtained from different sources and how much confidence they had in these sources (Table 3).

Results

All but two of the respondents had heard of SARS. Most respondents knew that it is a severe type of pneumonia (91.2%) and caused by a virus (88.7%). The correct estimate of 15% for the death rate for SARS-infected patients was reported by 9%, while 34.1% made estimates close to that number (10%–20%). Equal proportions of the respondents underestimated (44.5%) and overestimated (46.4%) the death rate. A mean knowledge score of 2.9 (standard deviation [SD] = 0.5) was observed; 83.9% of the respondents answered three or more knowledge questions correctly.

While 38.9% were worried about SARS as a health problem, few respondents were worried about getting SARS themselves (4.9%), about family members acquiring it (8.3%), or about SARS in the Netherlands (4.9%). Only 2.6% rated their risk of getting SARS as high or very high; 1.6% thought it likely or very likely that they might die from SARS. The perceived likelihood for getting SARS was lower than for getting a heart attack and cancer but comparable to that for HIV/AIDS (Table 1). Thirty-three percent of respondents thought that their risk for SARS was lower than that for other persons of the same sex and age; 7.7% perceived their risk to be higher than that of others.

Perceived capability to avoid SARS was rated as good or very good by 40.5%; 12.3% rated their capability as poor or very poor. All respondents reported taking at least one precautionary action; 41.3% reported one or more specific actions, especially avoiding travel to a SARS-endemic area; the other respondents indicated they had done "something else" to avoid getting SARS (Table 2). A mean score of 2.9 (SD = 0.5) was obtained for precautionary actions.

Substantial proportions of respondents reported that they would avoid persons from a SARS-endemic area (50.0%), a person who has a family member with SARS (46.1%), persons possibly from a SARS-endemic area (27.8%), and strangers wearing a protective mask (31.9%). A few respondents (<7%) reported they would avoid healthcare workers or persons who had a cough, looked unwell, had a fever, or sneezed.

SARS diagnostic behavior was rare, with "paying close attention to coughing" (3.5%) reported most often. Only 2.7% had visited a doctor because of SARS-related worries, and 1.1% had called a SARS information telephone service. The mean score for diagnostic action was 0.1 (SD = 0.6).

Pearson correlations indicated that perceived risk of acquiring SARS was positively associated with worries and self-reported precautionary actions to avoid SARS, while negative associations were found with perceived ability to avoid SARS. Precautionary action to avoid SARS was further associated with worries related to the syndrome, and knowledge about SARS was associated with worries about the condition as a health problem (Table 4).

Multiple linear regression analyses with SARS-related risk perceptions and worries as dependent variables and

Table 3. Sources of information about severe acute respiratory syndrome (SARS) and confidence in those sources[a]

Information source	Amount of information, mean (95% CI)	Confidence in the information, mean (95% CI)
Television	3.9 (3.8–4.0)	3.6 (3.5–3.7)
Newspapers	3.5 (3.3–3.6)	3.4 (3.3–3.5)
Internet	2.3 (2.2–2.5)	3.0 (2.9–3.1)
Magazines	2.1 (2.0–2.3)	2.7 (2.6–2.8)
Health officials	1.7 (1.6–1.8)	3.3 (3.2–3.5)
Friends	1.6 (1.5–1.7)	2.5 (2.3–3.6)
Physicians	1.3 (1.2–1.4)	3.2 (3.1–3.4)

[a]Scale ranged from 1 = very little to 5 = very much. CI, confidence interval.

Table 4. Pearson correlations between severe acute respiratory syndrome (SARS)-related risk perceptions, knowledge, and actions

	1	2	3	4	5	6	7
1. Perceived risk of acquiring SARS							
2. Perceived risk of acquiring SARS compared to others	0.43[a]						
3. Worry about getting SARS	0.64[a]	0.31[a]					
4. Worry about SARS as a health problem	0.40[a]	0.34[a]	0.45[a]				
5. Knowledge about SARS	–0.10	0.02	–0.05	–0.02[b]			
6. Self-reported precautionary actions to avoid SARS	0.16[c]	0.05	0.23[a]	0.10	0.00		
7. Perceive d ability to avoid SARS	–0.33[a]	–0.27[c]	–0.30[a]	–0.22[a]	–0.03	0.04	
8. Perceived ability to avoid SARS compared to others	–0.27[a]	–0.49[c]	–0.23[a]	–0.21[a]	–0.09	–0.03	0.30[a]

[a]p < 0.001.
[b]p < 0.05.
[c]p < 0.01.

sex, age, and education as independent variables showed a significant association between sex and risk perceptions (standardized regression coefficient [β] = 0.23, p = 0.005) and between years of education and worries (β = –0.18, p = 0.007). Women perceived their risk as higher than men, and less educated persons were more worried about SARS than those with more years of education. No significant associations were found in regression analyses with precautionary actions or SARS-related knowledge as dependent variables.

Conclusions

This study is the first to report on public perceptions of SARS outside the affected area. The results indicate that the Dutch population was well aware of the SARS outbreak, knew what SARS was, was not overly concerned about their risk, and obtained their information primarily from television and newspapers, which were also rated as trustworthy sources of information. Many respondents reported that they took precautionary actions to reduce their risk for SARS, but very few took possible diagnostic actions.

The present study builds upon earlier work from the SARS Psychosocial Research Consortium (G.D. Bishop et al., unpub. data). In that study, more respondents underestimated the death rate of SARS patients than in the present study (71% vs. 45%), with no significant difference between affected and unaffected countries. Our study was conducted later, which may have meant that more knowledge about SARS was available. Earlier studies (9,10) have reported on SARS-related risk perceptions during the outbreak in Hong Kong, and these studies reported quite different perceptions of high personal risk, ranging from 9%–30%. In our study, the perceived likelihood of getting SARS was rated high by few persons. Women reported higher perceptions of risk than men, and people with less education expressed more worries about the disease. Earlier studies on different topics reported mixed findings on differences in risk perceptions according to level of education (11–13). Higher perceptions of risk were associated with more worry and more self-reported precautionary actions, which is in line with predictions from risk perception theory and previous research (2,9). Avoiding air travel was the only precautionary action that was mentioned relatively often.

We conclude that the 2003 SARS outbreak did not lead to unwarranted precautionary actions or fears. Even though no SARS cases were discovered in the Netherlands, the Dutch population was well aware of the outbreak and was well informed about SARS, primarily through television and newspapers. The methods and results of the present study can be used for risk perception research during new outbreaks of SARS or other emerging infectious diseases.

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Flies and *Campylobacter* Infection of Broiler Flocks

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A total of 8.2% of flies caught outside a broiler house in Denmark had the potential to transmit *Campylobacter jejuni* to chickens, and hundreds of flies per day passed through the ventilation system into the broiler house. Our study suggests that flies may be an important source of *Campylobacter* infection of broiler flocks in summer.

Campylobacteriosis, caused by *Campylobacter jejuni*, is the most common foodborne infection in industrialized countries, where it causes millions of cases of illness every year (1). Chicken products are the food items most often reported to be the source of human campylobacteriosis (1). Thus, eliminating *Campylobacter* from broilers is important for the safety of the food supply for humans (2,3) and is a priority in animal industrial production and health programs. In spring 2003, a Danish program against foodborne *Campylobacter* infection was launched. The strategy addresses multiple steps in the food supply chain "from stable to table" but focuses on reducing the prevalence of *Campylobacter* in chicken production by having comprehensive hygiene barriers between broiler houses and the environment (4,5).

For unexplained reasons, *Campylobacter* infection cannot be controlled during summer. Even strict compliance with all biosecurity regulations has failed to control the infection. In August 2003 in Denmark, for example, 72.1% of broiler flocks were infected (Danish Zoonosis Centre, www.dfvf.dk), a situation that left selling of *Campylobacter*-contaminated chicken meat to consumers inevitable. Although similarity between *Campylobacter* isolates from broiler flocks and animals in the surrounding areas has been shown (6), the transmission routes are not understood, as no contact between broiler flocks and animals outside the broiler house takes place in closed production systems. However, indirect contact may be established by flies that take up *Campylobacter* as they forage on fresh animal feces. We show that *Campylobacter*-infected flies entered a broiler house in large numbers through the ventilation systems, which suggests that flies may be an important vector in summer.

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The Study

The number of flies transported by means of ventilation air into a broiler house in Denmark was counted from July 22, 2003, to July 28, 2003, and the *Campylobacter* carriage rate of flies captured in the environment of the broiler house was estimated. The study period was chosen because flies generally peak in activity and abundance in July to August in Denmark. In addition, chickens in the broiler house and the animals in the area around the broiler house (5 sheep, 4 horses, and 1 dog with 10 puppies) were tested for carriage of *Campylobacter*. DNA from all *C. jejuni* isolates was analyzed by pulsed-field gel electrophoresis (PFGE) to determine if strains from different animals were similar.

The broiler house (80 m x 15 m) was located at Universal Transverse Mercator Grid zone 32, East 564,137 m, North 6,294,759 m (http://mac.usgs.gov/mac/isb/pubs/factsheets/fs07701.html). The facility was negative-pressure ventilated through a total of 84 wall valves for air intake (16.5 cm x 52.5 cm) and 12 round chimneys (diameter 62 cm) for active air outlet through the roof. The house was emptied, and the chickens (n = 28,235) were slaughtered on July 29, 2003. Reports of local weather data from the Danish Meteorological Institute (www.dmi.dk) for that week were a maximum day temperature of 25.4°C, a minimum night temperature of 11.9°C, and days with bright sunshine with no wind or rain.

Flies were collected in polyester nets equipped at two wall inlets (one net at each end of the house) in the dynamic air flow measured (7) at a pressure of 21 Pa of influx ventilation air (speed 3.6 m/s, volume 1,213 m^3/h per inlet valve). After the nets were harvested, flies were visually sorted from other insects and counted. The flies, identified primarily to the order *Diptera* and the families *Muscidae* and *Calliphoridae*, were counted; the count showed that 917 ± standard deviation (SD) (843.5–990.5) flies (a + b)/2 x 84, with a and b representing the number of flies in the two nets) had entered the broiler house per day through the ventilation system, or approximately 1 fly per 2,700 m^3 of ventilation air (1,213 m^3/h x 84 x 24 h/917). For this specific broiler house, this amount equals approximately 30,000 flies per broiler cycle in the summer season. To estimate the possibility of roof inlets as entrance route for flies into houses with an air inlet in the roof, the fan of one chimney was switched off. This step generated inlet air in this chimney. The result showed 167 flies per day entered through this one chimney, or an average of 4.5 flies per 2,700 m^3 air. The flies captured in the ventilation system

were used for counting only, since they were dead and dried out at the time of harvest.

To determine the prevalence of *Campylobacter* in flies around the broiler house, a total of 96 flies were captured singly within a distance of 50 m from the house by rackets equipped with small disposable plastic bags. Captured flies were narcotized with CO_2. After species or genus was determined, each fly was transferred with a pair of tweezers to live storage in a sterile plastic tube with 1 mL of saline. The tubes were kept in an insulated container, which was transported to the laboratory within 24 hours after capture.

For *Campylobacter* detection, each fly was macerated in a sterile mortar, suspended in 2 mL of 0.9% saline. The mixture was centrifuged at 15,870 x *g* for 7 min. The pellet was resuspended in 2 mL of Bolton broth (CM0983 with SR0183 and SR0048) (Oxoid, Basingstoke, UK) and vortexed before incubation for enrichment at 37°C for 24 h. After enrichment, the tube was again centrifuged, and 100 µL of the sample from each of 49 tubes was streaked onto modified cefoperazone charcoal deoxycholate agar (mCCDA) (blood-free agar base supplemented with CM739 + SR155) (Oxoid). These plates were incubated at 42°C for 48 h in a microaerobic atmosphere (6% O_2, 6% CO_2, 4% H_2 in N_2). The tubes from the remaining 47 samples were subjected to DNA analysis by a nested polymerase chain reaction (PCR) (8).

The results of conventional culture showed that, of 49 flies tested, *C. jejuni* was isolated from 4 (8.2%) (Figure 1A); when a nested PCR was used, 33 (70.2%) of the 47 flies were *Campylobacter*-positive (Figure 1B). The species distribution according to the nested PCR when species-specific primers were used showed that, of 47 samples, 56.4% were positive with *C. jejuni* primers, 18.0% were positive with *C. coli* primers, and 25.6% were positive with *Campylobacter* species primers. Coinfection with *C. jejuni* and *C. coli* or *Campylobacter* spp. was found in six flies. However, PCR also detects nonculturable and dead *Campylobacter*. The reason for dividing the flies into two equal portions, one for conventional culture and the other for PCR, was to avoid reducing the assumed low number of *Campylobacter* on each fly.

Cloacal or rectal swab samples from 20 broilers, 5 sheep, and 4 horses were cultured on mCCDA agar, as described above. Rectal swabs from 11 dogs were streaked onto cefoperazone amphoricin teicoplanin (CAT) agar plates (blood-free agar base with CM739 and SR174) (Oxoid), and incubated at 42°C for 96 h. Subsequently, the swabs were tested for *Campylobacter* by our laboratory's routine PCR for feces (9). *C. jejuni* was isolated from 20 broilers and 4 sheep and *C. upsaliensis* from 11 dogs. One sheep and the four horses were culture-negative. However, all fecal swab samples were *Campylobacter*-positive by the routine PCR.

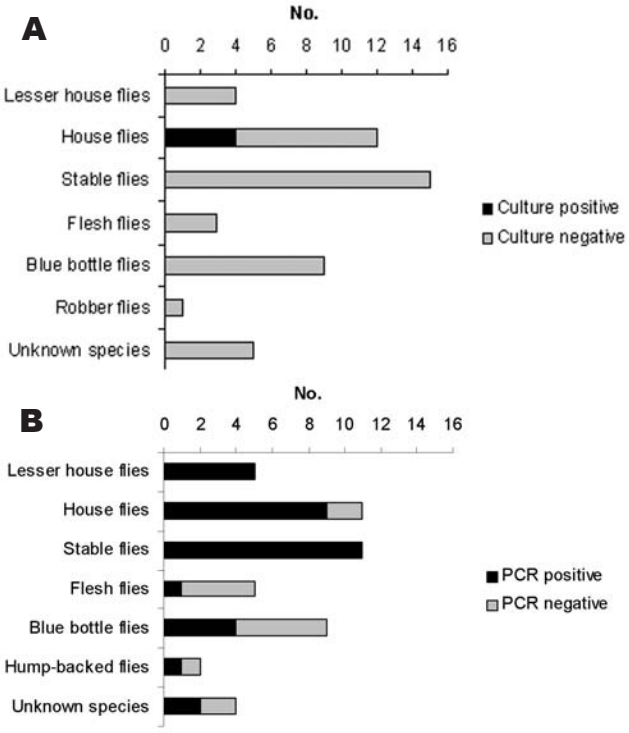

Figure 1. *Campylobacter*-infected flies captured around a broiler house in Denmark. Flies are grouped according to species. *Campylobacter*-positive and -negative by A), *Campylobacter* bacteriologic culture, and B), nested polymerase chain reaction.

A total of 28 *C. jejuni* isolates from 20 broilers, 4 flies, and 4 sheep were fingerprinted with PFGE with two different restriction enzymes, *Sma*I and *Kpn*I. With both enzymes, 27 of the isolates had an identical PFGE pattern

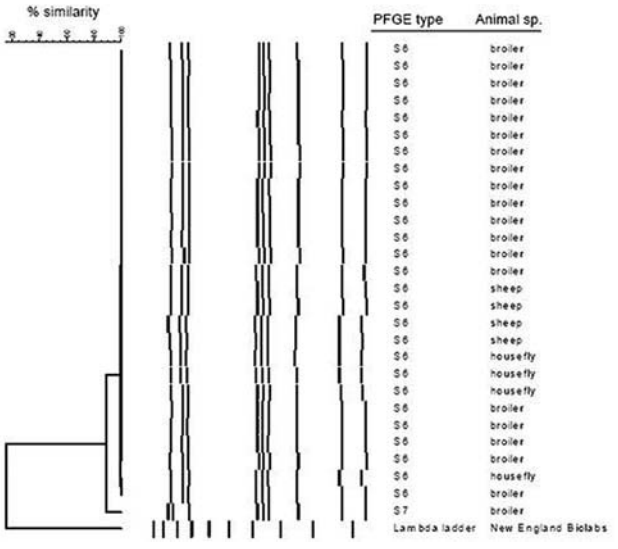

Figure 2. Dendrogram of pulsed-field gel electrophoresis types with *Sma*I of *Campylobacter jejuni* isolates from chickens in a Danish broiler house and from animals outside (flies and sheep).

(S6), whereas a single broiler isolate had a slightly different, but closely related pattern (S7), which probably was derived from the more prevalent pattern. The *Sma*I patterns are shown in Figure 2. Twenty-seven of 28 isolates from three animal sources, broilers, sheep, and flies, and from both inside and outside the broiler house, belonged to the same clone.

Under experimental conditions (10), flies are able to transmit *Campylobacter* among chickens. Moreover, a high prevalence of *Campylobacter*-infected flies captured in a broiler house has been found (11). However, no study has yet been able to demonstrate a significant role of flies captured in the houses for transmitting infection from flock to flock (5). Our results suggest that the potential of flies to transmit infection depends upon a current supply to the broiler house of *Campylobacter*-infected flies from the outside. Furthermore, the number of flies entering the broiler house must increase as the need for ventilation air increases as a consequence of the growth of chickens. Thus, the risk of introducing *Campylobacter* to the house increases with the age of the chickens.

Conclusions

This study has demonstrated that flies pose a threat of *Campylobacter* infection, from which chickens currently are unprotected from April to October, when insects are in season in the Northern Hemisphere. We found that in July hundreds of flies per day passed through the ventilation system into a broiler house and that 8.2% of flies captured in the environment had the potential to transmit *C. jejuni* from outside animals to chickens in the broiler house. These results warrant further research on how to combat the summer peak of *Campylobacter* in broilers to improve the safety of the human food supply.

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Rickettsia parkeri in *Amblyomma triste* from Uruguay

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Our goal was to detect whether spotted fever group *Rickettsia* are found in the suspected vector of rickettsioses, *Amblyomma triste*, in Uruguay. *Rickettsia parkeri* was detected in *A. triste*, which suggests that this species could be considered a pathogenic agent responsible for human rickettsioses in Uruguay.

In South America, cases of rickettsioses produced by the genus *Rickettsia* have been described in several countries in the last 20 years. The first three native cases of rickettsioses in Uruguay were reported in 1990. Patients had an initial small necrotic lesion (eschar) on the tick-bite point of attachment, fever and regional lymphadenopathies, an erythematous maculopapular rash, or any combination of these symptoms. Ticks involved in these cases were classified as *Amblyomma triste* (1), formerly thought to be *A. maculatum* (2).

A. triste is a neotropical tick species with a variety of hosts (3,4). It is the main tick species feeding on humans in Uruguay, and it is the primary candidate vector for transmitting rickettsioses in this country (5). According to the literature (2), *Rickettsia conorii* has been the causative agent of rickettsial diseases in Uruguay, but the evidence has been only serologic (by antirickettsial microimmunofluorescence testing) in all patients with suspected rickettsioses (6,7). Neither rickettsial isolation nor polymerase chain reaction (PCR) amplification from human blood samples from patients from Uruguay have been performed. However, as has been suggested (8), other tick-transmitted rickettsiae could be present in Uruguay.

The Study

The aim of this study was to identify the spotted fever group (SFG) rickettsial species present in the suspected vector of SFG rickettsioses in Uruguay (*A. triste*). From 1999 to 2004, in Uruguay, ticks were collected from humans (with and without rickettsial syndrome), other mammals, and vegetation and preserved in ethanol 70% at room temperature. Species, sex, and stage of development were determined by members of the Facultad de Veterinaria, Universidad de la República (Uruguay). Classified adult ticks (N = 91) were sent to the Hospital de La Rioja (Spain) for analysis with molecular biologic techniques. Thirty-six ticks recovered from 14 humans were attached but nonengorged. Only one tick removed from a human, the one corresponding to human 3, was attached and engorged. A total of 16 *A. triste* were captured walking on three different humans (nonattached). The remaining ticks were attached to two goats (n = 3), a rodent of the species *Scapteromys tumidus* (n = 4), and three dogs (n = 30; 19 of them were engorged). One tick was recovered from vegetation. Details are shown in the Table.

DNA from the ticks was extracted by using the Tissue DNA Spin Kit (Genomed, Granada, Spain) according to the manufacturer's instructions. PCR testing for *ompA*, *gltA*, and 16S rRNA genes was performed as previously described (9–11). Two negative controls (one of them with template DNA but without primers and the other with primers and containing water instead of template DNA) as well as a positive control (*R. conorii* Malish #7 grown in Vero cells) were included in all PCR assays. Restriction analysis of *ompA* amplicons was also carried out under conditions reported by Roux et al. (12). Each PCR-amplified fragment of *ompA* gene was sequenced twice for all positive samples (Universidad de Alcalá de Henares, Spain) to confirm the identification of rickettsiae. Data were aligned with homologous sequences of reference strains of the SFG rickettsiae retrieved from the GenBank database.

Six ticks (three females and three males) collected on three humans and three dogs yielded positive PCR products of the expected sizes for *ompA*, *gltA*, and 16S rRNA, respectively (Table). One of these ticks infected with SFG *Rickettsia* (the only one that was engorged) was removed from a woman (human 3) diagnosed with rickettsial syndrome in the Instituto de Higiene, Facultad de Medicina, Universidad de la República (Uruguay). This patient showed a small initial maculopapulous lesion on her scalp at the tick-bite point, followed by regional lymphadenopathies and fever. Diagnosis was made on the basis of the clinical picture and indirect immunoglobulin (Ig) G immunofluorescent technique with *R. conorii* antigen (Biomerieux Laboratories, Marcy l'Etiole, France). Serum specimens were collected during the acute phase (day 0) and convalescent phase (1 month later). The patient showed seroconversion for *R. conorii* with IgG, and she had a benign disease course after treatment with oral tetracyclines. No clinical signs of infection were confirmed for the remaining two humans bitten by ticks infected with SFG *Rickettsia* (humans 6 and 7), but ticks were removed

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Table. *Amblyomma triste* ticks collected from different origins in Uruguay[a]

Host	No. of ticks (N = 91)		Location	Date of isolation	PCR amplification			SFG *Rickettsia* species found in ticks
	Males	Females			*ompA*	*gltA*	16S rRNA	
Human 1	0	1	Maldonado	Nov 1999	–	–	–	
Human 2	2	0	Canelones	Oct 2000	–	–	–	
Human 3[b]	1	0	Montevideo	Oct 2000	+	+	+	*R. parkeri*
Human 4	2	0	Maldonado	Dec 2000	–	–	–	
Human 5	1	0	San José	Oct 2001	–	–	–	
Human 6	0	1	Canelones	Sep 2002	+	+	+	*R. parkeri*
Human 7	1	0	Montevideo	Dec 2002	+	+	+	*R. parkeri*
Human 8	1	2	Montevideo	Oct 2002	–	–	–	
Human 9	2	7	Montevideo	Oct 2002	–	–	–	
Human 10	1	1	Canelones	Aug 2003	–	–	–	
Human 11	1	1	Canelones	Aug 2003	–	–	–	
Human 12	2	1	Montevideo	Oct 2003	–	–	–	
Human 13	0	1	Montevideo	Sep 2003	–	–	–	
Human 14	3	4	Canelones	Sep 2003	–	–	–	
Human 15	4	8	Montevideo	Oct 2003	–	–	–	
Human 16	2	0	Canelones	Nov 2003	–	–	–	
Human 17	1	0	Canelones	Nov 2003	–	–	–	
Human 18	0	2	Montevideo	Jan 2004	–	–	–	
Goat 1	1	0	Maldonado	Nov 1999	–	–	–	
Goat 2	0	2	Canelones	Oct 2000	–	–	–	
Rodent	2	2	Montevideo	Oct 2000	–	–	–	
Dog 1	3	21	Maldonado	Dec 2000	+ (3)	+ (3)	+ (3)	*R. parkeri*
Dog 2	0	1	San José	Oct 2001	–	–	–	
Dog 3	1	4	Canelones	Sep 2002	–	–	–	
Vegetation	0	1	Montevideo	Dec 2002	–	–	–	

[a]PCR, polymera se chain reaction; SFG, spotted fever group.
[b]Human 3 had rickettsioses.

immediately after attachment in these cases. For all six positive samples, sequence analysis for *ompA* amplicons showed 100% similarity with the homologous sequence of *R. parkeri* (GenBank accession no. U43802). Profiles obtained with *Rsa*I for *ompA* PCR fragments were also in accordance with these data.

Conclusions

SFG *Rickettsia* isolated from arthropods and initially classified as nonpathogenic to humans are increasingly recognized as causing emerging rickettsial diseases (13). In the last 10 years, different *Rickettsia* species and subspecies, such as *R. aeschlimannii* (14), *R. sibirica* strain *mongolotimonae* (15), and *R. slovaca* (16), among others, have been implicated as human pathogens. Very recently, a new tickborne *Rickettsia*, *R. parkeri*, has been identified as a cause of human disease in the southern United States (17). According to Paddock et al., *R. parkeri* rickettsioses may also occur in other regions of the Western Hemisphere, e.g., in Uruguay.

We report *R. parkeri* infection in *A. triste* ticks collected in Uruguay. Several cases of rickettsioses have been described in this country but, to date, no *Rickettsia* has been isolated, cultivated, and characterized as the causative agent. A few years ago, *R. conorii* was presumptively considered the etiologic agent, but diagnosis was established with serologic assays (indirect microimmunofluorescence testing) as reference technique (6). Cross-reactions are noted within SFG *Rickettsia* antigens, and available serologic tests cannot be used to implicate a specific pathogen. In Uruguay, *A. triste* frequently bites humans, and rickettsioses frequently develop in them (5). Our finding of *R. parkeri* infection in one *A. triste* tick collected from a patient with rickettsiosis suggests that *R. parkeri* could be a pathogenic SFG *Rickettsia* involved in rickettsial diseases in Uruguay. Traditionally, this agent was reported as nonpathogenic to humans, but the first report of a human infection with *R. parkeri* was recently published (17). It has also recently shown to be mildly pathogenic to guinea pigs (18). In our study, *R. parkeri* was the only detected SFG *Rickettsia* in *A. triste* ticks from Uruguay. Our data suggest that *A. triste* is a host of SFG *Rickettsia* in Uruguay, and *R. parkeri* could be the causative agent of human cases of rickettsioses in Uruguay.

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Hantavirus Infection in Anajatuba, Maranhão, Brazil

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In 2000, the first outbreak of hantavirus pulmonary syndrome was recognized in the Brazilian Amazon (Maranhão State). An epidemiologic study identified a 13.3% prevalence of hantavirus-specific immunoglobulin G. The analysis of risk factors suggests that persons are occupationally exposed to infected rodents in the crop fields.

Hantavirus pulmonary syndrome (HPS), caused by a hantavirus later identified as Sin Nombre virus, was identified for the first time in May 1993 in the southwestern United States (1). The natural reservoirs of members of *Hantavirus*, a genus belonging to the *Bunyaviridae* family, are wild rodents of the Rodentia order, Muridae family, and Sigmodontina subfamily. The human disease is a zoonosis and is acquired by inhaling aerosols containing urine, feces, or saliva particles from infected wild rodents (2–4). The disease has been described in North, Central, and South America (4).

In 2000, the first outbreak of HPS occurred in the Brazilian Amazon region (5), specifically in Quebra and São Jerônimo, in a rural area of Anajatuba, state of Maranhão, Brazil (Figure). These two villages combined had a population of 535 inhabitants. The climate is semihumid tropical, and the main economic activities are raising cassava, rice, and corn on large plantations and fishing.

The Study

All of the inhabitants (or their legal guardians) in both towns who provided blood samples and signed the written and informed consent were included in the study. Those who did not provide blood samples were excluded (n = 137, 25.6%). No statistically significant differences were found with respect to sex and age between those studied and those excluded.

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The study was conducted in two stages. First, we performed a cross-sectional analysis to determine the prevalence of hantavirus-specific immunoglobulin (Ig) G and to identify risk factors for human infection by a hantavirus. The portion of the population whose blood samples showed hantavirus antibodies were considered seropositive. In the second stage, 6 to 24 months after the first collection, we retested the portion of the population whose blood samples did not show hantavirus antibodies (seronegative cohort).

The measure of association used was the prevalence rate ratio (PRR) at the 95% confidence interval (CI). The Wald test was also used, and statistical significance was set at the 0.05 level. Those variables with $p < 0.20$ in the unadjusted analysis were included in the adjusted analysis. The variables with $p < 0.10$ were maintained in the final model after stepwise backward elimination was performed. Because prevalence of infection was >10%, the results were adjusted for confounding factors by using the Poisson regression model. Standard errors were adjusted according to the robust method, and the cluster effect was taken into account.

We used a hierarchical modeling strategy, in which the variables were divided into three blocks: block 1, socioeconomic variables (education, marital status, occupation [farm worker or housewife]); block 2, behavioral variables (storing grains inside the home, fishing, using dead rats for fishing bait, bathing in rivers, drinking water from streams or rivers, sweeping the home,

Figure. Map showing Anajatuba municipality, Maranhão State, Brazil.

seeing rats at home or in the wild, seeing rat feces inside the house, having the ability to recognize wild rats, killing a rat either at home or in the field, being bitten by a rat); and block 3, demographic variables (sex and age). The adjusted analysis was performed in three steps. In the first step, the PRR of the socioeconomic variables (block 1) was adjusted; in the second step, the PRR of the behavioral variables (block 2) was adjusted for the statistically significant variables in the first step. Finally, in the third step, the PRR of the demographic variables (block 3) was adjusted for the statistically significant variables in the second step.

Antibodies of the IgG class were detected by enzyme-linked immunosorbent assay (ELISA), by using antigen of Sin Nombre virus (Centers for Disease Control and Prevention, Atlanta, GA). The serologic tests were performed in the Department of Viruses Transmitted by Arthropods at the Instituto Adolfo Lutz, São Paulo. The samples of human serum underwent a series of dilutions and were tested for recombinant nucleocapsid protein antigen of Sin Nombre virus and for the control recombinant antigen. One conjugate of antihuman IgG, prepared in mice and marked with peroxidase and the chromogen ABTS (2,2-azino-di [3-ethybenthiazoline sulfonate]), was used to show the reaction. Samples were considered positive when they showed an optical density higher than the value of the reactivity limit at a dilution of ≥1:400.

Of the 535 residents of Quebra and São Jerônimo, 398 (74.4%) participated in the study. The overall seroprevalence was 13.3% (95% CI 10.1%–17.1%).

In the unadjusted analysis, age >17 years, being illiterate, living in consensual union, working as an agricultural laborer, fishing, using dead rats as bait for fishing, house sweeping, and killing rats in the field or inside the home were all significantly associated with infection by hantavirus. Those who had seen rats in the fields, had been bitten by a rat, or could recognize wild rats also were more likely to become infected (Table 1).

The Poisson regression analysis was done in three steps. In the first step (testing the significance of socioeconomic factors), illiteracy, consensual union, and agricultural work were associated with hantavirus infection. In the second step (studying the effect of behavioral variables), seeing rats in the field conferred a higher risk of infection. In the third step (assessing effects of demographic variables), age >17 years was associated with hantavirus infection (Table 2).

In the second stage of the study, a cohort of 292 seronegative persons was tested initially by hantavirus-specific IgG 6 months after the initial collection, with one seroconversion. Of the 291 persons who remained seronegative, 234 were retested for antibodies 24 months after the initial collection; 4 seroconverted. The survival table estimated a probability of seroconversion in 24 months of 1.7% (95% CI 0.5%–4.3%). Among those who seroconverted, two reported fever during the follow-up period.

Table 1. Unadjusted analysis of risk factors for hantavirus infection in Anajatuba, Maranhão State, Brazil, 2000

Variable	PRR (95% CI)[a]
Male vs. female	1.29 (0.77–2.17)
Age (y)	
18–40 vs. ≤ 17	4.90 (1.99–12.11)
41–64 vs. ≤17	13.4 (5.80–30.9)
>65 vs. ≤17	17.2 (6.62–44.5)
Living with a companion versus living alone	3.62 (2.22–5.93)
Being illiterate	3.33 (1.97–5.62)
Being a farm worker	3.65 (1.90–7.00)
Being a housewife	1.83 (1.10–3.03)
Seeing rats in the wild	5.94 (2.11–16.7)
Being bitten by a rat	3.19 (1.82–5.59)
Being able to recognize wild rats	3.18 (1.69–6.01)
Using dead rats for fishing bait	2.87 (1.20–6.85)
Fishing	2.61 (1.22–5.57)
Sweeping the home	2.36 (1.04–5.32)
Killing a rat in the field	2.02 (1.22–3.35)
Killing a rat at home	1.99 (1.14–3.47)
Seeing rats at home	1.55 (0.76–3.17)
Bathing in streams	1.55 (0.98–2.46)
Seeing rat feces inside the home	1.28 (0.78–2.10)
Storing grains inside the home	1.08 (0.53–2.20)

[a]PRR, prevalence rate ratio; CI, confidence interval.

Conclusions

The seroprevalence of hantavirus antibodies varies considerably according to the species of hantavirus and the rodents involved. A low prevalence of 1.7% for Sin Nombre virus antibodies was described in 1993 in the southwestern United States (6). In Central and South Argentina, where the genotypes Lechiguanas, Hu39694, and Andes are the most important, seroprevalence was also low, varying from 0.1% to 1.5% (7). A high prevalence, such as that observed in the area of Anajatuba, has also been described in other regions of the Americas. In the northern region of Argentina, where Orán is the most important genotype, seroprevalence is >20%. In Chile, where Andes virus predominates, a seroprevalence as high as 7.5% has been observed (8). In Paraguay, where Laguna Negra virus is the most important, the analysis of a nonrandom sample found a seroprevalence of 12.8%, while in indigenous communities a prevalence of up to 57% has been found (9). In Brazil, a serologic study in three cities in São Paulo, where Juquitiba virus was associated with HPS, detected a seroprevalence of 0.4% to 4.5% (10).

A case-control study in the southwestern United States, to examine risk factors associated with HPS, showed no association between sex, age, and HPS (11). HPS patients were more likely to have observed rodents near the home,

Table 2. Adjusted analysis of risk factors for hantavirus infection in Anajatuba, Maranhão State, Brazil, 2000

Variables	PRR (95% CI) [a]	p
First step [b]		
Illiterate		0.001
No	1	
Yes	2.49 (1.45–4.26)	
Farm worker		0.025
No	1	
Yes	2.44 (1.12–5.32)	
Living with a companion		0.022
Yes	1	
No	2.05 (1.10–3.80)	
Second step [c]		
Seeing rats in the field		0.013
No	1	
Yes	4.22 (1.36–13.11)	
Third step [d]		
Age group (y)		< 0.001
≤17	1	
18–40	3.65 (1.34–9.94)	
41–64	9.56 (3.65–25.04)	
≥65	13.43 (4.86–37.10)	

[a]PRR, prevalence rate ratio; CI, confidence interval.
[b]Adjusted PRR of socioeconomic variables (block 1).
[e]PRR of behavioral variables (block 2), adjusted for statistically significant variables in the first step.
[d]PRR of demographic variables (block 3), adjusted for statistically significant variables in the second step.

to have stored food in the home, and to have cleaned food storage areas. In our study, age >17 years as well as being illiterate and living in a consensual union were associated with infection by hantavirus in the adjusted analysis. However, storing food in the home was not associated with a greater seroprevalence.

The risk for exposure at home versus risk for occupational exposure must be clarified. In Anajatuba, the only behavioral variable that was independently associated with hantavirus infection was seeing rats in the crop fields, adding evidence to the theory that this disease could be linked to occupational exposure.

Hantavirus transmission to humans through wild rodent bites has been reported in cases of hemorrhagic fever with renal syndrome (12). Among those who reported rat bites, seroprevalence was 38.1%, $p < 0.001$ in the unadjusted analysis. However, in the adjusted analysis, this variable had a borderline association with seroprevalence.

Follow-up results from a seronegative cohort demonstrated that none of the persons that seroconverted met the criteria that would define a case of HPS, indicating that mild or asymptomatic clinical forms of the disease developed with greater frequency in those who became infected than did the classic form of HPS.

The results we observed must interpreted with caution because of the small population studied and the possibility of colinearity, since many of the variables correspond to activities with a similar potential for rodent exposure. Risk factors may vary according to the virus involved. The possibility of having a mixed group of case-patients exists because the antigen detects different hantaviruses.

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West Nile Virus in British Columbia

Michael Aquino,* Murray Fyfe,† Laura MacDougall,† and Valencia Remple†

We investigated personal protective behaviors against West Nile virus infection. Barriers to adopting these behaviors were identified, including the perception that DEET (N,N-diethyl-m-toluamide and related compounds) is a health and environmental hazard. Televised public health messages and knowing that family or friends practiced protective behaviors were important cues to action.

Personal protective behaviors are the primary means of preventing human illness from West Nile virus (WNV) infection (1). To plan effective WNV prevention and control programs, we must know the factors that influence adopting protective behaviors (2). The health belief model is a theoretical framework that has investigated health behaviors related to infectious diseases, including tuberculosis, HIV, influenza, and measles (3–8). The major tenet of this model is that persons will take action to ward off an illness if they believe that they are susceptible, the illness has serious consequences, the course of action is beneficial, or the anticipated benefits of action outweigh the costs (9). To investigate the determinants of engaging in WNV protective behaviors in British Columbia (B.C.), we developed a questionnaire using the health belief model as a framework.

The Study

Participants were randomly selected from a systematic random sample of B.C. residential telephone records. Telephone interviews were conducted from July 2 through August 18, 2003. The study concluded after 309 interviews were completed, a predetermined endpoint based on allowable resources.

A questionnaire was designed specifically for this study. We measured the frequency (1 = never to 5 = always) with which participants said they practiced protective behaviors (applied mosquito repellent, eliminated standing water, and avoided mosquitoes). The following predictor items measured concepts of the health belief model: knowledge (mode of transmission, risk groups), susceptibility to illness, severity of illness, barriers to action (safety concerns, cost), benefits of action, and cues to action (behavior of relatives, sources of information). Response options for predictors were measured on a 5-point Likert scale and signified the respondent's agreement with a statement (1 = strongly disagree to 5 = strongly agree) or belief that an event would occur (1 = not at all likely to 5 = very likely). Response options for predictors measured with multiple-choice questions were scored as correct or incorrect. The subscore for a concept was calculated by averaging the scores obtained from questions specific to that concept. The subscore for the knowledge concept was calculated by counting the number of correct responses. We ascertained the participant's sex, age, ethnicity, education, and income. Participants were also asked to report the level of mosquito activity near their residence (low, medium, high).

A response rate of 64.6% (307 of 477) was calculated by dividing the number of completed surveys by the number of persons contacted. Respondents were more likely to be women, older, more educated, and in a higher income bracket than the general population.

Most respondents said they obtained information about WNV by watching television (n = 180, 62.9%). While almost all (n = 285, 98.6%) were aware that WNV was transmitted by mosquitoes, 57.9% (n = 159) were aware that adults ≥50 years of age are at greatest risk for serious illness. Of those unaware of this fact, 52.0% (n = 63) were ≥50 years of age.

At least occasional practice of the following specific protective behaviors was reported: 197 (68.2%) removed standing water, 168 (58.1%) practiced mosquito avoidance behavior, and 162 (56.0%) used DEET-based mosquito repellents (Figure). When asked if information about WNV had influenced them to remove standing water, agreement or strong agreement was reported by 213 (73.7%) respondents, 147 (50.9%) for using DEET (N,N-diethyl-m-toluamide and related compounds)-based mosquito repellent, and 110 (38.1%) for avoiding mosquitos.

The most prominent barriers to practicing protective behaviors were perception that DEET is a health and environmental hazard, the time required to remove standing water, and participating in outdoor leisure activities during peak mosquito hours. Approximately half (n = 113, 45.9%) of respondents claiming to have spent time outdoors during peak mosquito hours did so to participate in leisure activities (walking, playing with kids, gardening). More than one third (n = 101, 35.1%) agreed or strongly agreed that removal of standing water was time consuming. More than one third (n = 101, 35.1%) believed DEET is hazardous to the environment, and more than one quarter (n = 78, 27.1%) disagreed or strongly disagreed that it is safe for human use.

The proportional odds ordinal regression model was fit to model the frequency at which persons reported avoiding mosquitoes, applying DEET, and removing standing water (Table). Predictors that were investigated included the

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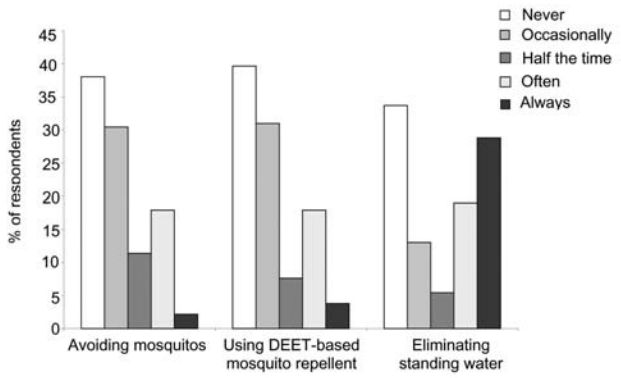

Figure. Reported frequency of personal protective behavior practice. DEET, N,N-diethyl-m-toluamide and related compounds.

composite scores for each of the health belief model concepts, demographic variables (sex, age, area code, education, income, ethnicity), and residential mosquito activity level.

Barriers to action and cues to action were important predictors in each of the three models. In addition, perceived susceptibility was significant in modeling the frequency of practicing avoidance of mosquitoes ($p < 0.01$) and had an associated odds ratio of 1.61 (confidence interval 1.22–2.12). All other investigated predictors were not significant in multivariate models at the $\alpha = 0.05$ level.

Conclusions

Before the study, the British Columbia Centre for Disease Control and Prevention issued three formal press releases and provided 110 interviews with local or provincial media outlets about WNV and associated protective behaviors. Most respondents in our study cited television as their main source of information, which demonstrates the ability of public health messaging to reach audiences through broadcast media. This finding is consistent with findings from other studies (10) and underscores the important role that this medium plays in educating the public (11).

The proportion of respondents who said they used DEET-based mosquito repellent or practiced mosquito avoidance behavior was comparable to the proportion found in similar studies conducted in Connecticut (2,10). Eliminating standing water was not specifically investigated in these studies. A national U.S. study reported smaller proportions of respondents who said that they avoided the outdoors during dawn or dusk (24%), used DEET-based mosquito repellent (31%), and eliminated standing water (31%) (12). Differences may be the result of the varying levels of WNV activity throughout the United States.

Regular systematic evaluations of the knowledge, attitudes, and behaviors of the public are needed to ensure the effectiveness of public health messages (2). By using the health belief model as a theoretical framework, we were able to identify barriers to the practice of protective behaviors. The fear that DEET-based mosquito repellents are hazardous to human health and the environment is a barrier of particular concern. These repellents are a mainstay for the personal prevention of WNV (11) and were demonstrated to be an important protective behavior option, given the participation of many respondents in outdoor activities during peak mosquito hours. Instructions for the safe use of DEET are outlined in the literature (13) and should be conveyed to address public fears. Literature on the effects of DEET on the environment is limited. DEET does not readily degrade by hydrolysis at environmental pHs (14) and has been identified as a ubiquitous pollutant in aquatic ecosystems, but the effect of this is unknown (15).

A deficiency was also observed in the proportion of respondents who were aware that persons ≥50 years of age were at greatest risk for serious illness from WNV. More than half of those unaware were ≥50 years of age. Making perceptions of susceptibility and severity in this population more consistent with the actual susceptibility and severity could help to influence the adoption of WNV protective behaviors (9).

Our study supports the ability of public health education campaigns to influence the practice of WNV protective behaviors. Specifically, we found that most respondents reported that information about WNV influenced them to engage in protective behaviors, and cues to action significantly increased the odds that respondents practiced protective behaviors more frequently. Together, these findings suggest the potential for public health messages that endorse WNV protective behaviors to have a

Table. Results of modeling reported frequency of personal protective behavior with proportional odds ordinal regression[a]

Outcome modeled	Significant predictor variables	β	OR (95% CI)	p value
Practicing mosquito avoidance behavior	Perceived barriers to ac tion	–0.77	0.46 (0.35–0.62)	< 0.01
	Cues to action	1.08	2.96 (2.32–3.77)	< 0.01
	Perceived susceptibility	0.48	1.61 (1.22–2.12)	< 0.01
Reported frequency of using DEET-based mosquito repellent	Perceived barriers to action	–0.70	0.50 (0.31–0.79)	< 0.01
	Cues to action	1.19	3.30 (2.49–4.38)	< 0.01
Reported frequency of eliminating standing water	Perceived barriers to action	–1.25	0.29 (0.20–0.42)	< 0.01
	Cues to action	1.27	3.56 (2.49–5.09)	< 0.01

[a]OR, odds ratio; CI, confidence interval; DEET, N,N-diethyl-m-toluamide and re lated compounds.

"snowball effect"; public health education can influence persons to practice protective behaviors, and these persons can influence friends and family to do the same.

A number of limitations were associated with our study. First, we depended on self-reporting to measure frequency of practicing WNV protective behaviors, and no effort was made to validate the participants' responses. If participants attempted to please interviewers, the frequency of protective behavior practice may have been overestimated. Second, administering the interview by telephone excluded persons who did not have telephones or only had cellular phones, which may have contributed to the observed demographic differences between our study population and the general population. Consequently, findings may not be generalizable outside the study population. Third, information on nonrespondents was not obtained, and differences between them and the study population could not be ascertained. Thus, the effects of this bias could not be determined. Despite these limitations, this study will help public health officials achieve the goal of promoting WNV protective behaviors and reducing the risk for infection.

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SARS Transmission and Commercial Aircraft

To the Editor: Severe acute respiratory syndrome (SARS) is an emerging transmissible disease first reported in Asia in February 2003. The disease is characterized by acute onset of fever with nonproductive cough, myalgia, shortness of breath, or difficulty breathing (1). Approximately 14% of case-patients require mechanical ventilation (1,2). The syndrome is caused by the previously unrecognized SARS-associated coronavirus (SARS-CoV) (3). The primary mode of SARS transmission is through close person-to-person contact. In March 2003, the World Health Organization (WHO) issued two travel advisories to SARS-affected countries. Despite these advisories, probable case-patients traveled by air internationally, thereby spreading the disease globally. The extent of risk posed by probable cases for in-flight transmission of SARS is unclear.

A study was conducted by the Robert Koch Institute in Berlin, Germany, to document SARS transmission during international flights. On April 11, 2003, the Institute was notified that a probable SARS-infected person had flown from Hong Kong to Frankfurt, Germany, on March 30 to 31, 2003, and then traveled extensively in Europe after onset of symptoms. In 5 days, the traveler, a 48-year-old Hong Kong businessman, had flown on seven flights throughout Europe (Table). On March 31, symptoms of SARS, including fever and general malaise developed; whether he had a cough at this time is unclear. He was admitted to a hospital in Hong Kong on April 8, and mechanical ventilation was initiated. He was reported to WHO as a suspected SARS patient on April 9 and diagnosed with SARS on April 10. Polymerase chain reaction analysis conducted on the patient's nasopharyngeal aspirate showed positive results for SARS-CoV on April 14.

Passenger manifests from the seven flights on which the patient had flown were requested by the local health departments and the Institute. In a previous study, Kenyon et al. indicated that airline passengers seated within two rows of an infectious tuberculosis patient were at greatest risk for infection (4). To determine an association between seating proximity to the SARS patient and transmission of SARS, a study that included all airline passengers seated within four rows (i.e., front, back, and same row) of the index patient (4) was conducted. Passengers ≥18 years of age who lived in Germany were contacted by the Institute and asked to participate in the study; all participants gave informed consent for inclusion in the study. Passengers in other countries were not included in the study because contact information was not available. Passengers <18 years of age were not included in the study; ethical approval from an Institutional Review Board, which would have delayed the study, would have been necessary. Contact information for study participants was forwarded to local health departments so that public health officials could provide follow-up care. Study participants were interviewed approximately 3 months after their flights because contact information was not available earlier. A standardized questionnaire was developed to collect information on demographics, flight details, countries visited before the flights, use of mask, and symptoms. Furthermore, 5–10 mL of whole blood was drawn and tested for SARS-CoV antibodies by using immunofluorescence assay.

A total of 250 passengers were identified and selected for the study. Contact information was available for 109 passengers; 69 of the 109 were living in Germany. Sixty-two of those 69 passengers were contacted, and 41 passengers agreed to participate in the study. Thirty-six participants completed questionnaires and had blood samples taken. The male-to-female ratio was 3:5, and the median age was 41 years (25–59 years). Contact information was not available for five passengers, which made their inclusion in the study impossible. All serologic samples (N = 36) tested were negative for SARS-CoV immunoglobulin G antibodies, and none of the 36 passengers reported symptoms characteristic of SARS. Ten passengers complained of cough, headache, and muscle aches. One passenger reported a cough, muscle aches, and fever, but symptoms started 10 days after the flight. An analysis of the seating arrangement showed that the study participants were randomly distributed around the index patient.

Table. Flight itinerary of SARS patient [a]

Departure city	Arrival city	Date/time departure	Date/time arrival	Duration (h:min)
Hong Kong	Frankfurt	March 30/23:10	March 31/05:35	12:25
Frankfurt	Barcelona	March 31/09:05	March 31/11:10	2:05
Onset of symptoms after arrival in Barcelona on March 31, 2003				
Barcelona	Frankfurt	April 2/07:05	April 2/09:15	2:10
Frankfurt	London	April 2/10:15	April 2/11:30	2:15
London	Munich	April 3/15:25	April 3/18:10	1:45
Munich	Frankfurt	April 4/14:50	April 4/16:00	1:10
Frankfurt	Hong Kong	April 4/17:40	April 5/10:35	9:55

[a]SARS, severe acute respiratory syndrome.

No SARS transmission was shown among contacted passengers seated in close proximity to the index patient; these results suggest that in-flight transmission of SARS is not common. These results are consistent with other studies that assessed the risk for in-flight transmission of SARS (5,6). The results also suggest that SARS-CoV is not efficiently transmitted, as reflected in its basic reproduction number R_0 (range 2–4) (7). The SARS-infected patient on the indicated flights was in his first week of illness; infectivity is greatest in the second week (8). Therefore, the likelihood of SARS transmission on the indicated flights was not high. These results are further supported by the fact that all contacts were asymptomatic 13 days after their last contact with the SARS patient. No information was available on health-care contacts. Although we did not observe any SARS transmission, we cannot rule out the possibility that it may have occurred. We had no contact information on 56% of the passengers on the indicated flights and, therefore, had to exclude them from the investigation. Obtaining complete contact information from the remaining passengers was difficult, which severely impeded the investigation. Similarly, we were unable to contact crew members and had to exclude them. Recent studies have documented SARS transmission to passengers seated more than four rows away from an index patient (5,9); thus, studying the passenger proximity to the patient may not be sufficient. Because of these limitations, our final sample size was small and probably biased. Since we did not observe any evidence to indicate in-flight transmission of SARS, we were unable to assess the importance of seat assignment proximity as a risk factor.

The study shows that the roles of public health authorities and the aviation industry should be to "harmonise the protection of public health without the need to avoid unnecessary disruption of trade and travel" in public health emergencies such as global SARS transmission (10). We recommend strengthening the collaboration between national health authorities and the airline industry. Furthermore, the International Air Transport Association should establish procedures to ensure that complete contact information is available for all passengers and that rapid notification can be accomplished in case of potential exposure to infectious diseases.

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Estimating SARS Incubation Period

To the Editor: In a recent article, Meltzer described a simulation method to estimate the incubation period for patients infected with SARS with multiple contact dates (1). In brief, he assumed a uniform distribution of all possible incubation periods derived from these contact dates for each patient and randomly selected an incubation period from all contact dates for each patient to obtain a distribution of the incubation period for all 19 patients. The process is

repeated 10,000 times to obtain an overall frequency distribution of the incubation period.

Instead of using this cumbersome iterative approach, the same results can be obtained by a simple method. When a uniform distribution is assumed for all possible incubation periods, the expected frequency for a day *x* as the incubation period is either 0 or 1/(total number of possible days). Taking the first patient (Canada 1) in (1) as an example, the expected frequency for 1, 2, 3, …, 18 days is 0, 1/11, 1/11, 1/11, 1/11, 1/11, 1/11, 1/11, 1/11, 1/11, 1/11, 1/11, 0, 0, …, 0. The expected frequencies for the other patients are available online from: http://www.cdc.gov/ncidod/EID/vol10no8/04-0284.htm#table.

The total expected frequency for each day is the sum of the expected frequencies for all patients for that day. Therefore, the frequency distribution of the incubation period is given by dividing each total expected frequency by the sum of the total expected frequencies (x 100%) and is 7.6, 22.1, 14.2, 9.0, 6.5, 11.5, 4.6, 3.7, 3.7, 6.4, 3.7, 1.7, 1.1, 1.1, 0.7, 0.7, 0.7, 0.7. This is identical to the frequency distribution shown in Figure 1 of the paper by Meltzer (1).

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In Reply: Drs. Wong and Tam (1) are correct in stating that their method of calculating mean frequencies of possible incubation periods for patients with severe acute respiratory syndrome (SARS) is simpler than the method that I presented (2). However, their method cannot replicate the confidence intervals shown in Figure 1 in my article. Their suggested methodology can only replicate Figure 2 in my article, which shows the cumulative distribution of the mean frequencies of individual incubation periods.

The comparative complexity of my method provides data that are essential for making public health decisions. For example, public health officials need to know incubation periods to determine appropriate periods of quarantine and isolation and how long to conduct intensive (and expensive) surveillance after the last clinical case has been reported. To reduce costs and to enhance public support, public health officials may keep quarantine and isolation periods to a minimum. They also need to know the risk for failure of such interventions attributable to patients with relatively long incubation periods. Both Figure 2 in my article and Drs. Wong and Tam's data show that approximately 95% of the mean incubation period will be ≤12 days (i.e., 5% will incubate for 13 to 18 days). By summing the 95th percentiles for days 13 through 18 from my Figure 1, it can be seen that there is a probabiltiy that ≤30% of patients will have incubation periods >12 days (the actual probability of any given percentage incubating for >12 days can be easily calculated by using the spreadsheet which is an appendix to my article). Public health officials need to understand the degree of variability associated with any data used to make public health policies. Sole reliance on the mean incubation periods (or mean frequencies) will hide more than is shown, which increases the probability of failed public health interventions.

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Detecting Bioterror Attack

To the Editor: In a recent article (1), Kaplan et al. addressed the problems in detecting a bioterror attack from blood-donor screening. The main point of this comment is the "early approximation" used by Kaplan et al. to derive the probability of detecting an attack. The simplification used by Kaplan et al. leads to a probability that does not account for the size of the exposed population and can lead to incorrect results and misinterpretations.

Consider a single bioterror attack that infects a proportion p of an exposed population of size N at time $\tau = 0$, such that the initial number of infected is $I_0 = Np$. The quantity of interest is the probability $D(\tau)$ of finding at least one positive blood donation and detecting the attack within time τ. For attacks conducted with contagious agents that could lead to an epidemic, Kaplan et al. used the early approximation solution of the classic epidemic models (2) to describe the progression of the number of infected persons.

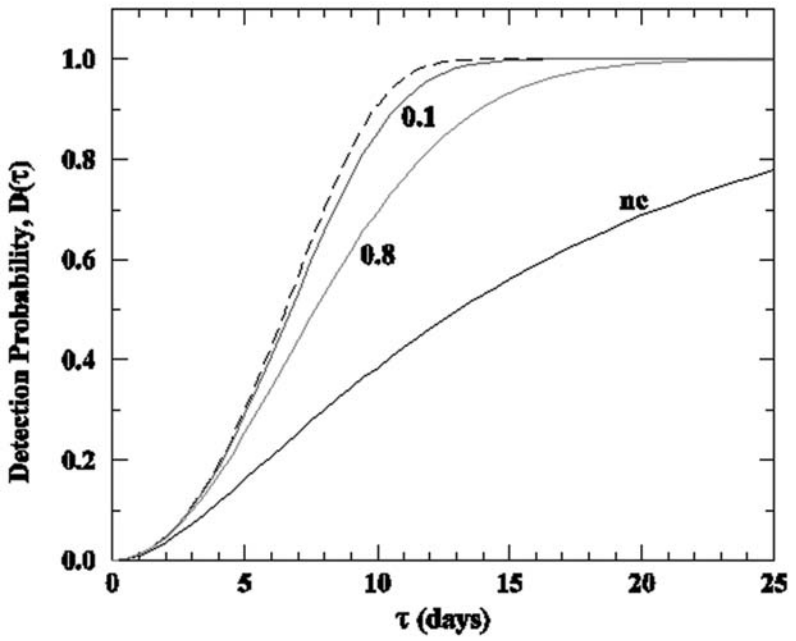

Figure. Probability of attack detection delay for a contagious agent. Dashed line represents the early approximation, solid lines the full solution (where the numbers represent the fraction p of the population initially infected)*, and the symbol "nc" stands for noncontagious agent ($R_0 = 0$). The parameters are as follows: blood donation rate k = 0.05 per person per year, screening mean window period ω = 3 days, mean duration of infectiousness 1/r = 14 days, basic reproductive number $R_0 = 5$, and the initial attack size Np = 500. Note that the exposed populations are therefore 5,000 and 625 for p = 0.1 and p = $(R_0 - 1)/R_0 = 0.8$, respectively.

Consequently, the resulting probability of attack detection [noted $D_{es}(\tau)$] is dependent only upon the initial size of the release I_0, the basic reproductive number R_0 (the mean number of secondary cases per initial index case), and other variables (the blood screening window ω, the mean number k of blood donations per person and per unit of time, and the mean duration of infectiousness 1/r) (see online Appendix at: http://www.cdc.gov/ncidod/EID/vol10no8/03-1044.htm). Early approximation can lead to unreliable results because it is valid only at earlier stages of the epidemics and in the limit where the proportion p of initially infected is much smaller than the intrinsic steady proportion $(R_0-1)/R_0$ of the epidemics (online Appendix). Relaxing this approximation and using the full solution for the progression of the number of infected persons leads to the probability D(τ) that takes into account the size of the exposed population (online Appendix). The latter is important because, in contrast to $D_{es}(\tau)$ that leads to the same conclusion, $D(\tau)$ indicates that the probabilities of detecting an attack within two exposed populations of different sizes, but with the same numbers of initially infected, are not identical. As illustrated in the Figure, when the other variables are fixed, d$D(\tau)$ ecreases as the proportion p of initially infected increases because the epidemic size decreases as p approaches the threshold $(R_0-1)/R_0$. These subtleties of a simple epidemic model are even less reliable when using the blood screening to detect a bioterror attack with agents that cause diseases of very short incubation period.

Nonetheless, detecting a bioterror attack is very similar to detecting the response of pathogen-specific immunoglobulin M antibodies (as an indicator of recent contact of hosts with pathogens) within a population of hosts by using serologic surveys. Therefore, the reasoning developed for a bioterror attack can be extended and applied to detect and time the invasion or early circulation of certain pathogens within a population. In that perspective, it might be useful to develop an analysis that includes more details of the epidemic progression within this framework.

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In Reply: As stated and argued throughout our article (1), we conducted a best-case analysis under assumptions that favored blood-donor screening to detect bioterror attacks; if such an analysis fails to justify donor screening, no analysis will. Bicout (2) is concerned about our assumption of exponential infection growth after attack; however, this assumption was one of several we made deliberately as part of our best-case scenario (1).

Bicout's calculations actually reinforce rather than refute our analysis. By relaxing our assumption of exponential infection growth and using the well-known logistic solution to the basic epidemic model (equation 1 in

Bicout's letter), Bicout shows that more time is required to detect a bioterror attack than when exponential infection growth is assumed (Figure accompanying Bicout's letter). The number of persons infected over time under the logistic model will be fewer than the number of persons infected if exponential growth is assumed; therefore, screening blood donors to detect a bioterror attack is even less attractive than using our best-case assumptions. The take-home message from our article was and is: It makes little sense to screen blood donors to detect a bioterror attack.

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Aeromonas spp. and Infectious Diarrhea, Hong Kong

To the Editor: Vila et al. reported the prevalence of *Aeromonas* spp. associated with traveler's diarrhea in Spain (1). Some of the patients described in this study had traveled to countries in Asia, such as Thailand and India. This report details the prevalence of this pathogen in patients with acute infectious diarrhea who were treated in emergency department settings in Hong Kong.

Over a 12-month period, we retrospectively studied all adult patients who showed clinical features of acute infectious diarrhea, were treated as outpatients with or without observation in the emergency department, and had a positive stool culture (2–4). Our data were collected at an urban university-affiliated hospital with 1,400 beds and an emergency department with an annual census of 190,000 patient visits. *Aeromonas* spp. were isolated from stool samples by standard culture procedures, which included introduction onto xylose lysine desoxycholate agar plate and thiosulphate citrate bile sucrose plate, and subsequent screening by triple iron sugar slant (acid butt with no H_2S), positive oxidase, negative urease, fermentation of mannitol but not dulcitol and inositol, resistance to vibriostatic agent 0/129, and ability to grow at 0% NaCl. The main species of *Aeromonas* were identified by the differential biochemical reactions of gas production from D-glucose, arginine dihydrolase, ornithine and lysine decarboxylase; esculin hydrolysis; Voges Proskauer reaction; fermentation from arabinose, sucrose, mannitol, salacin, and D-sorbitol; and citrate and glycerol utilization (5).

Of 130 patients with positive stool cultures, *Aeromonas* spp. were isolated in 9 patients (6.9%), including *A. caviae* in 4 patients, *A. hydrophila* in 2 patients, and *A veronii* in 3 patients. The cases were not epidemiologically linked. In one of these isolates (*A. caviae*), another enteropathogen (*Vibrio parahemolyticus*) was also isolated. None of the patients reported recent travel abroad or to mainland China before treatment.

Our review of the clinical features of these nine patients found that the mean highest body temperature at the time of treatment or during the patient's stay in the emergency department was 37.4°C (95% confidence interval [CI] 36.9–38.0). Two patients (both with *A. caviae* isolated) had temperatures >37.5°C. Bloody diarrhea was present in two patients (one with *A. veronii* and one with *A. caviae*). The mean number of unformed stools per day was 8.6 (95% CI 4.0–13.2). Abdominal pain in eight patients and vomiting in four patients was reported. Five patients required admission to the emergency department's observation unit before discharge. Of these, four patients needed intravenous fluid therapy. Empiric ciprofloxacin was given to one patient with a temperature of 38.3°C. Stool culture results were available within 3 days for positive isolation of *Aeromonas*. All A*eromonas* strains were susceptible to ciprofloxacin, cefotaxime, cotrimoxazole, and chloramphenicol, while two of nine isolates (one *A. caviae* strain and one *A. hydrophila* strain) were susceptible to ampicillin. All patients had recovered satisfactorily by the time stool culture results were available, and antimicrobial therapy was not necessary, except for the patient who was given ciprofloxacin empirically.

In conclusion, *Aeromonas* spp. are responsible for a small proportion of cases of bacterial gastroenteritis encountered in an urban emergency department setting in Hong Kong. Patients affected do not necessarily have a history of travel to a nonindustrialized region. In a substantial proportion of cases, the symptoms are severe enough to require intravenous fluid therapy and observation. However, symptoms generally would have resolved by the time the pathogen was isolated from stool culture. In contrast to the report of Vila et al., persistent diarrhea is uncommon, and antimicrobial therapy is usually unnecessary in our particular setting. *Aeromonas* spp. are susceptible to a wide range of antimicrobial drugs, except ampicillin. Whether empiric antimicrobial drugs given at the time of treatment would

have significantly shortened the duration of the symptoms is not known.

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Trichinella papuae in Saltwater Crocodiles (*Crocodylus porosus*) of Papua New Guinea

To the Editor: Until 1995, reptiles were not known to be hosts of *Trichinella*; however, in that year *Trichinella* was detected in 40% of farm-raised crocodiles (*Crocodylus niloticus*) in Zimbabwe. These crocodiles were infected with a new species, *T. zimbabwensis*, which was experimentally infective in mammals, including primates (1).

The infection of reptiles with *Trichinella* species that are potentially infective for humans has become more important since demand for the meat of crocodiles, caimans, and alligators has increased in many areas of the world. This trend has resulted in the development of national breeding programs in more than 30 countries in North, Central, and South America; Africa; Asia; and Australia (2), which generated an income of approximately $60 million in 1998 (3).

In 1999 in Papua New Guinea, wild and domestic pigs infected with a new species, *T. papuae*, were found (4,5); this new species was capable of completing its life cycle in reptiles that were infected experimentally (6). *Trichinella* infection has also been found in farm-raised saltwater crocodiles (*C. porosus*) in Papua New Guinea, where a national program for crocodile meat and skin products exists.

Papua New Guinea has one crocodile breeding farm that processes approximately 6,000 animals per year. Following the discovery of *Trichinella*-infected crocodiles in Zimbabwe, the Australian government requested that Papua New Guinea conduct *Trichinella* testing on the crocodile meat exported to Australia. Muscle samples from crocodiles were digested by pepsin and HCl solution according to the standard technique (7). When available, approximately 100 larvae from each infected crocodile were given by mouth to laboratory rats, and 10–20 larvae were stored in 90% ethyl alcohol for molecular identification. Multiplex polymerase chain reaction (PCR) was used to characterize the larvae, according to a published protocol (8). The primer set oTsr1 and oTsr4 was used to amplify the expansion segment V of the large subunit ribosomal RNA (9). The larvae of all *Trichinella* reference strains were used as controls. PCR products were gel-purified and directly sequenced by using the same primers as those used for PCR amplification. All sequences were aligned by using the Clustal W program from OMIGA 2.0 (Accelrys, San Diego, CA). Final alignment of the expansion segment V sequences was performed manually so microsatellites could be compared.

Muscle samples from 118 saltwater crocodiles (46 farm-born, 71 wild-born and farm-raised, and 1 killed in the wild near the Bensbach River) were tested. All samples from the farm-born crocodiles were negative for *Trichinella*. Of the samples from the 72 wild-born crocodiles (including the 1 killed in the wild), 16 (22.2%) were positive for *Trichinella* larvae, with an average of 7 larvae/g in the biceps. All of the infected crocodiles originated in the Kikori area (Figure). The prevalence of *Trichinella* infection in crocodiles from this area was 32.0% (16/50). Samples from the remaining 21 wild-born and farm-raised crocodiles, and the 1 killed in the wild, were negative for *Trichinella*. These crocodiles originated in nine different locations (Figure).

PCR analysis showed that the parasites belonged to *T. papuae*. However, the crocodile isolates differed from the reference strain of this species by the deletion of a TG dinucleotide and by a single base mutation (G vs. A) in the expansion segment V sequence. Testing for *Trichinella* in crocodile meat has been conducted in Zimbabwe and Papua New Guinea only, and infected crocodiles have been found in both countries. Crocodiles in other parts of the world are also likely to be infected. Since

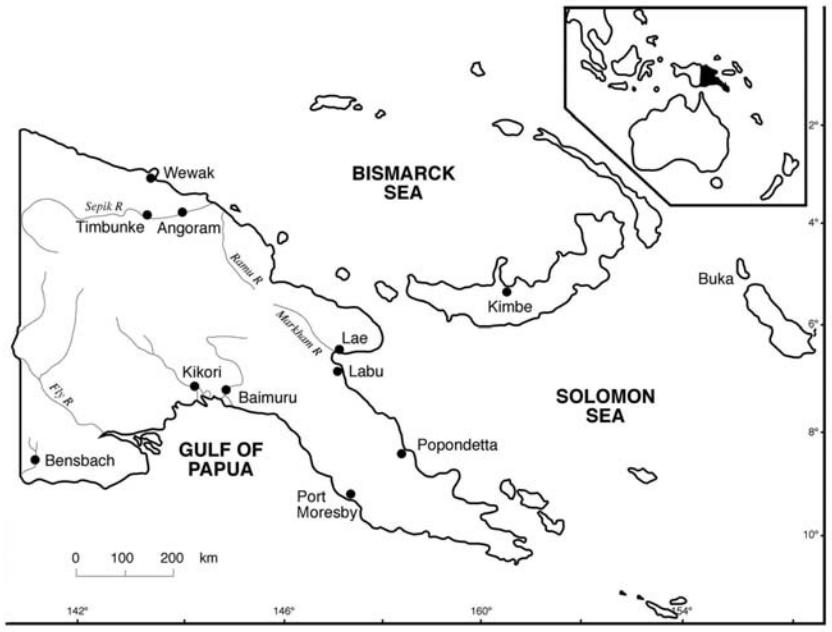

Figure. Papua New Guinea showing the areas of origin of the 72 wild-born saltwater crocodiles (*Crocodylus porosus*): 1 each from Baimuru, Angoram, Timbunke, Kimbe, Bensbach River, and Buka; 2 from Labu; 7 from Wewak; 7 from Popondetta; and 50 from Kikori.

both *T. zimbabwensis* and *T. papuae* infection can develop in reptiles and mammals, eating crocodile meat is a risk. In one region of Papua New Guinea, a high percentage of the local human population had anti-*Trichinella* antibodies (10). Moreover, the risk for human infection may be rising, given the increased marketing of meat from crocodiles, caimans, and alligators in many parts of the world (2). The meat of other carnivorous reptiles, although consumed in very few areas, may also represent a source of infection, as suggested by the large number of larvae of both *T. papuae* and *T. zimbabwensis* in the muscles of experimentally infected monitor lizards (6).

The presence of a TG dinucleotide in the expansion segment V sequence could be a useful marker for tracing the region of origin of infected meat. The infected crocodiles, all of which were born in the wild, likely acquired infection before they arrived on the farm, since none of the farm-born crocodiles was infected. In Zimbabwe, the source of infection was the *Trichinella*-infected crocodile meat that had been fed to the other crocodiles; the farm in Papua New Guinea does not engage in this practice, which would explain why none of its farm-born animals was infected.

This study shows the importance of implementing measures to prevent the spread of *Trichinella* infection. For instance, since both *T. papuae* and *T. zimbabwensis* can be easily transmitted from crocodiles to mammals, the discarded parts of crocodiles should be properly destroyed to avoid transmission to synanthropic animals, and the waste products should not be fed to domestic animals, unless the products are frozen or cooked before use. Crocodile-breeding farms should adopt the artificial digestion method used in many countries to screen pigs for *Trichinella* infection (7). Freezing crocodile meat, as practiced in Papua New Guinea, can also prevent infection because freezing destroys *T. papuae* and *T. zimbabwensis* larvae in muscles (1,4). By contrast, salting, drying, smoking, or preserving crocodile meat in brine will not destroy trichinellae; these curing methods are not standardized, and the survival of *Trichinella* larvae can depend on factors such as salt concentration, moisture, and temperature (7). Similarly, crocodile meat is frequently vacuum sealed, and the *Trichinella* larvae can retain their infectivity for several months in this environment (7).

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Panton-Valentine Leukocidin–positive *Staphylococcus aureus*, Singapore

To the Editor: Necrotizing community-acquired pneumonia attributable to Panton-Valentine leukocidin–producing strains of *Staphylococcus aureus* has been described as a distinct clinical syndrome with a high death rate in young, immunocompetent patients (1,2). This letter details the first reported case of necrotizing pneumonia caused by Panton-Valentine leukocidin-positive *S. aureus* in a southeastern Asian country, Singapore.

An 18-year-old girl of Chinese ethnicity with a 4-day history of fever, cough, hemoptysis, and dyspnea sought treatment at Singapore General Hospital in October 2003. This episode had immediately followed an influenza-like prodromal illness for which a general practitioner had prescribed oral erythromycin ethinylsuccinate and medications for symptomatic relief. Her medical history showed an intrauterine *Toxoplasma gondii* infection that had resulted in developmental delay and slight mental retardation. She had never traveled outside Singapore.

On admission, the patient's temperature was 38.4°C, blood pressure was 130/70 mm Hg, and her pulse rate was 108 per min. Bibasal crackles were heard on auscultation of her lung fields, and her respiratory rate was 30 per min despite the use of supplemental oxygen. The results of physical examination were otherwise unremarkable. Initial chest x-ray showed air-space shadowing of the right upper and middle lobes of the lung, as well as blunting of the right costophrenic angle. Blood tests gave the following results: leukocyte count 7.42 x 10^9/L, neutrophil count 6.53 x 10^9/L, platelet count 287 x 10^9/L, hemoglobin level 8.6 g/dL, prothrombin time 15.3 s, and activated partial thromboplastin time 28.7 s. She was experiencing acute renal failure with a serum creatinine level of 783 μmol/L. Liver biochemistry was abnormal with the following values: alkaline phosphatase 513 U/L, alanine aminotransferase 38 U/L, and aspartate aminotransferase 65 U/L. Serum bilirubin level was within the normal range.

The patient was prescribed intravenous ceftriaxone and azithromycin, and hemodialysis was initiated. Within 6 hours of hospitalization, the patient became hypotensive and hypoxemic and required inotropic support and mechanical ventilation. Intravenous ceftazidime and high-dose cloxacillin were substituted for ceftriaxone at that time. Blood cultures obtained on admission were sterile, but penicillin-resistant *S. aureus* grew from cultures of aspirated endotracheal tube secretions. Results of immunofluorescent tests conducted on bronchial washings for viral antigens of influenza virus A and B, parainfluenza virus, respiratory syncytial virus, and adenovirus were negative. Computed tomographic scan of the thorax on day 3 of hospitalization showed widespread confluent consolidation of the right lung with right pleural effusion and patchy consolidation of the lingular lobe of the left lung. The total leukocyte count increased to 26.3 x 10^9/L, and disseminated intravascular coagulopathy developed. Results of repeated blood and endotracheal cultures were positive for *S. aureus*, and intravenous gentamicin and rifampicin were added to her antimicrobial cocktail. A transthoracic echocardiogram showed a normal heart with no evidence of endocarditis.

Despite aggressive support, the patient's condition continued to deteriorate. A hemopyopneumothorax developed on the right side on day 4 of hospitalization, which required chest tube insertion. Hemoptysis persisted, and inotropic and ventilatory requirements progressively increased. The patient died on day 20 of hospitalization.

The severity of the patient's infection and the clinical symptoms suggested the presence of Panton-Valentine leukocidin genes in the causative *S. aureus*; tests confirmed the suspicion. *S. aureus* was identified on the basis of colony morphologic characteristics, the coagulation of citrated rabbit plasma (bioMérieux, Marcy l'Etoile, France), and production of a clumping factor (Staphyslide

test; bioMérieux). DNA was extracted from cultures grown on agar plates and amplified following a previously described protocol (1). The following oligonucleotide primer sequences were used: *luk-PV1*, 5′-ATCATTAGGTAAAATGTCTGGACATGATCCA-3′; *luk-PV2*, 5′-GCATCAAGTGTATTGGATAGCAAAAGC-3′. Polymerase chain reaction products were sequenced commercially and submitted to GenBank (accession no. AY508231).

This case is the first in Singapore of community-acquired pneumonia caused by *S. aureus* in which an attempt was made to detect Panton-Valentine leukocidin genes. Given that the patient had not traveled, she likely acquired the lethal strain of Panton-Valentine leukocidin–positive *S. aureus* locally. This idea is further supported by a recent study which reported that the Panton-Valentine leukocidin gene is found worldwide, albeit in community-acquired strains of methicillin-resistant *S. aureus* (3).

The incidence of severe community-acquired pneumonia attributable to Panton-Valentine leukocidin–positive *S. aureus* is unknown in many parts of the world. With one exception (4), cases of Panton-Valentine leukocidin–positive *S. aureus* causing community-acquired pneumonia have been reported sporadically only from European countries and the United States (1,2,5–8). These results may be attributable to the lack of recognition rather than to the rarity of the condition. A previous report showed that 7.6% of cases of severe community-acquired pneumonia in patients requiring ventilatory support in Singapore were caused by *S. aureus* (9), and a large proportion of these would fit the clinical syndrome described by Gillet et al. (2). Given the ease of transmitting the infection to close contacts (7,10), with the real possibility of a consequent outbreak (10), Panton-Valentine leukocidin testing should be conducted on *S. aureus* strains isolated from all patients with community-acquired necrotizing pneumonia and furunculosis for infection control purposes. Implementing standard hospital methicillin-resistant *S. aureus* measures resulted in control of the outbreak described by Boubaker et al. (10). This measure seems especially relevant given the dismal prognosis offered by conventional therapy in which the death rate of patients with necrotizing pneumonia may reach 75% (2). Further research on the epidemiology, optimal therapy, and prevention of this infection is needed.

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Balamuthia Amebic Encephalitis Risk, Hispanic Americans

To the Editor: *Balamuthia mandrillaris*, a free-living soil ameba, can cause granulomatous amebic encephalitis as well as nasopharyngeal, cutaneous, and disseminated infections in humans, nonhuman primates, and other animals. Approximately 100 published and unpublished cases of *Balamuthia* amebic encephalitis (BAE) have been reported; most were fatal. Diagnosis of BAE is usually made at autopsy, and rarely by biopsy, in part because the amebas can be overlooked in

histopathologic preparations. In recognizing BAE as a type of encephalitis that might otherwise be undiagnosed, the California Encephalitis Project (1) has been screening selected serum samples from patients with encephalitis for evidence of antibodies to *Balamuthia*.

We describe cases of BAE in California and compare data with national data collected on *Balamuthia* infections since the discovery of the organism in 1990. Since 1998**,** serum and other samples (cerebrospinal fluid [CSF], throat and rectal swabs, brain tissue) from patients with encephalitis have been submitted to the California Encephalitis Project by participating physicians throughout California. The goal of the California Encephalitis Project is to provide enhanced diagnostic testing for etiologic agents of encephalitis through an intensive testing algorithm. The case definition of encephalitis is encephalopathy, plus one or more of the following: fever, seizures, focal neurologic findings, CSF pleocytosis, or electroencephalographic or neuroimaging findings consistent with encephalitis (1). Persons with HIV/AIDS, severely immunocompromised patients, and patients ≤6 months of age are excluded from the project.

Serum samples were selected for screening for *Balamuthia* antibodies if the patient had clinical or laboratory features suggestive of *Balamuthia* encephalitis (elevated CSF protein and leukocyte counts or compatible findings on neuroimaging) and a history of outdoor occupational (agriculture or construction work) or recreational (camping or swimming) activities during which they may have been exposed to pathogenic or opportunistic free-living amebas. During the study, 215 (approximately 25%) of the >850 serum samples collected in California were tested for *Balamuthia* infection by indirect immunofluorescence assay (2). Testing was conducted on acute-phase serum and a follow-up sample, when available. Serum samples were tested at dilutions from 1:2 to 1:4,096. Positive and negative control samples were run in parallel, with titers from 1:128 to 1:256 for the former and negative to 1:32 for the latter. Serum samples from patients with *Balamuthia* encephalitis did not cross-react with *Acanthamoeba* or *Naegleria*, two other amebas associated with amebic encephalitis (3).

Three (1.4%) of 215 samples tested were positive for antibodies to *Balamuthia* with titers of 1:128, 1:128, and 1:256. In the course of the study period, serum samples from four additional persons, including serum from one person who had been diagnosed by the Centers for Disease Control and Prevention (CDC), who were not part of California Encephalitis Project were positive. The diagnosis of *Balamuthia* encephalitis was confirmed histologically or by indirect immunofluorescence staining of tissue sections in all seven cases; in one case amebas also were isolated in culture from necrotic brain tissue at autopsy (4). All patients were immunocompetent and of Hispanic American ethnicity, and all died. Case-patients included two adults and three children who were native Californians, a child who had arrived from Mexico the previous year, and a child who was a native of Texas who had been diagnosed by the California Department of Health Services (5). The observation that all were of Hispanic American ethnicity prompted a search through CDC's records (N = 104) to confirm the ethnicity of BAE patients throughout the world (G.S. Visvesvara, unpub. data). Patients were considered to be of Hispanic American ethnicity if they were identified as such in case histories or if they had traditional Hispanic surnames. Specific confirmation of ethnicity was not available in the CDC records, and reliance on surnames to determine ethnicity might be a source of error; some Hispanic American persons may have surnames that are not considered to be ethnically Hispanic, and vice versa. According to the records, approximately 50% of the 50 North American patients, which were confirmed by direct immunofluorescence, histopathology, or both, were Hispanic American. Thirty-six percent of all the BAE cases occurred in Latin America. Eleven cases have occurred in California since the early 1990s, including those described above, and all but two were fatal (6). Eight (73%) of these 11 cases occurred in Hispanic Americans.

BAE is not an insignificant disease in California, with 11 cases and 9 deaths reported in the state in the last decade. By comparison, five deaths from indigenous rabies have been reported in the state since approximately 1990 (7). Furthermore, BAE is likely underdiagnosed because of unfamiliarity with appearance of amebas in tissue sections and nonspecific symptoms. Unless there is a high degree of suspicion, it is unlikely that testing for *Balamuthia* would be conducted. Most cases are diagnosed on autopsy, which is often not allowed by families. Also, BAE develops in a disproportionate number of Hispanic Americans. Hispanic Americans make up 12.5% of the U. S. population (United States Census Bureau statistics for 2000) but represent approximately 50% of the cases of BAE. In California, where Hispanic Americans make up 32% of the state's population, they have 73% of BAE cases ($p = 0.001$, Fisher exact test). In the California Encephalitis Project, Hispanic Americans accounted for approximately 25% of all cases of encephalitis, 26% of serum samples examined for *Balamuthia* antibody, and 21% of cases of viral and bacterial encephalitis, but all BAE patients (n = 3) were in Hispanic Americans (Figure).

Balamuthia lives in soil (4) and can enter through the respiratory tract

or breaks in the skin. Hispanic Americans may be more likely to reside in agrarian settings with increased exposure to soil and opportunities for contamination of cuts and other injuries. Whether caused by environmental factors, genetic predisposition, access to medical care, or other socioeconomic factors and pressures, the reasons for the higher incidence of BAE in Hispanic Americans warrant further study.

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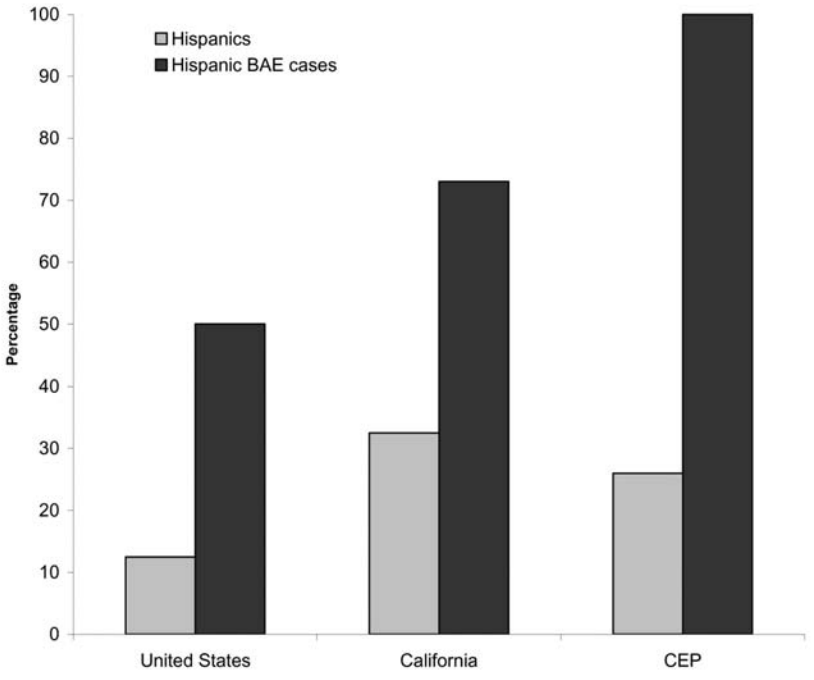

Figure. The graph compares Hispanic American populations and Hispanic American *Balamuthia amebic* encephalitis (BAE) cases in the United States, California, and those samples tested for *Balamuthia* antibody in the California Encephalitis Project (CEP). In each of the three groups, the percentage of Hispanic Americans in the population is compared to the percentage of BAE cases in Hispanic Americans.

SARS Alert Applicability in Postoutbreak Period

To the Editor: Since its emergence early in 2003, the epidemic of severe acute respiratory syndrome (SARS) has been characterized by its rapid spread among healthcare workers. On August 14, 2003, the World Health Organization (WHO) issued an alert concerning SARS and recommended a staged approach to surveillance (1). Because occupational transmission has been a feature of the SARS outbreak, WHO recommends surveillance for clusters of alert cases among healthcare workers in low-risk areas (i.e., cases not reported, only imported cases reported, or local cases with limited transmission potential reported). A SARS alert is identified when two or more healthcare workers in the same healthcare unit meet the clinical case definition of

SARS with onset of illness in the same 10-day period.

To determine the value of routinely collecting worker absence data as part of this kind of surveillance and to assess a threshold level of possible alert cases, directors of six major Italian hospitals were asked for the number of cases that fit the alert definition in 2003. (In Italy, the hospital director is a physician who is in charge of nosocomial and occupational infection control.) The facilities involved were three general hospitals, two university hospitals, and one research hospital; each has an infectious and respiratory tract diseases unit. Three of four patients with imported cases of probable SARS observed in Italy during the 2003 epidemic (2) were treated in two of these hospitals.

No hospitals were able to immediately provide the requested data; in all hospitals in Italy, information on sickness certificates is recorded only for administrative purposes, and certificates are not generally used for medical surveillance. The European Union Council Directive 89/391 directs all participating countries to introduce measures to improve worker safety and health and to provide a designated service that will protect workers, prevent occupational risks, including hazards from biological agents, and conduct health surveillance. In the hospital, these activities are coordinated by the hospital director. When a worker has a transmissible disease, the attending physician for the infected patient recommends that the patient stay home from work for the duration of the infectivity period. If the illness is included in the list of notifiable infectious diseases, the case must be reported to the local public health authority so infection control measures can be implemented. However, neither the attending physician nor public health personnel usually supervise home isolation, and adherence to the recommendations relies on the patient.

Sickness certificates are generally provided by the physician and sent by the worker to the hospital administration within 3 days of illness onset. The certificate indicates the prognosis (i.e., recommended number of days absent from work) but does not report the diagnosis because of privacy concerns. In case of hospital admission, the worker can send the hospital certificate (attesting to the duration of the hospital stay), followed by a physician's certificate for the recommended length of convalescence, if any.

To determine how the sickness certification system in other European Union countries operates and assesses the feasibility of the WHO alert surveillance, we interviewed specialists in infectious diseases or public health in France (seven imported cases of SARS, two in healthcare workers), Spain (one case), and Denmark (no cases) (2) by electronic mail. According to their answers, the situation in those countries is not substantially different from that in Italy.

In view of the increasing concern related to the emergence and reemergence of transmissible diseases, surveillance efforts focused on groups likely to be first affected by the reemergence of SARS have been strongly encouraged (3,4). Possible alternatives similar to the SARS alert system have been proposed, based on healthcare workers' sickness absenteeism, when other illnesses are concerned. For example, the effectiveness of enforced monitoring of pneumonia in healthcare workers requiring hospitalization should be evaluated in the context of a wider syndromic surveillance strategy (5).

Although the current healthcare worker sickness reporting system cannot be fully representative and generalizable, Italy and several other European Union countries (e.g., France, Spain, and Denmark) do not support initiating the WHO recommendation and do not have the capacity to detect and respond to SARS, should it reemerge. To overcome barriers to early detection of cases and clusters of severe unexplained respiratory infections that might signal the reemergence of SARS, regulatory changes are necessary, and efforts should be made to balance the need for protecting the privacy of persons with the need for an effective surveillance system.

To identify clusters of occupational diseases among healthcare workers and provide prompt response to any alert, an expanded sickness information system should be implemented. For example, an active confidential assessment of diagnosis could be performed in selected circumstances when healthcare workers are absent. We plan to evaluate the feasibility of this kind of surveillance by focusing on workers with absences with longer than a week and on workers with onset of illness in the same 10-day period.

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SARS Outbreak in Taiwan

To the Editor: The article by Hsieh et al. analyzed the daily case-report data for severe acute respiratory syndrome (SARS) from May 5 to June 4, 2003, posted on the Web site for the Taiwan Center for Disease Control, to show how this disease had rapidly spread in the 2003 outbreak (1). Hsieh et al. suggested that infection in hospitalized patients who were classified erroneously as suspected SARS case-patients was a major factor in the rapid spread of the disease in hospitals. Slow classification and delayed placement of these patients in negative-pressure isolation rooms contributed to the high percentage (73%) of nosocomial infection in Taiwan (1).

During the outbreak period (stage II), three teams were responsible for classifying SARS cases (2). The team included infectious disease specialists, respiratory specialists, and epidemiologists recruited from major teaching hospitals throughout Taiwan and was organized by the Taiwan Center for Disease Control and the National Health Insurance Bureau. The team met daily and reviewed the clinical data, travel and contact history, and chest radiographic scans of the reported case-patients obtained (by email or fax) from the patients' attending physicians. The same protocol (Figure) was used by all team members to classify the case-patients as having suspected or probable SARS. All hospitals that treated patients with suspected SARS either had their own committee to classify patients according to World Health Organization guidelines or followed the protocol for classification or reclassification of reported cases by the team members (3).

Although official reclassification might have taken 12.5 days as suggested by Hsieh et al., the conclusion that inadequate isolation of infected patients during this period led to a higher rate of nosocomial transmission cannot be based on the data available to these authors. From the first day that suspected cases were reported to the Taiwan Center for Disease Control, the patients were placed in negative-pressure isolation rooms when available. Suspected case-patients may have been less likely than probable case-patients to be placed in negative-pressure isolation rooms when these were in short supply; however, all other available isolation precautions were used to treat suspected case-patients before they were reclassified. The notion that increased infection transmission occurred despite these isolation precautions is not consistent with the literature suggesting the central role of gloves, gowns, and surgical masks in preventing transmission (4). Thus, the process of reclassification was not associated with the timing of isolation measures shown to have the greatest impact in preventing infection transmission.

The high proportion of patients with nosocomial SARS infection in Taiwan is consistent with the observations of Lingappa et al. (5) and others who have noted that the hospital setting was the primary amplifier of SARS transmission, with significant community transmission occurring in only the largest outbreaks. The high

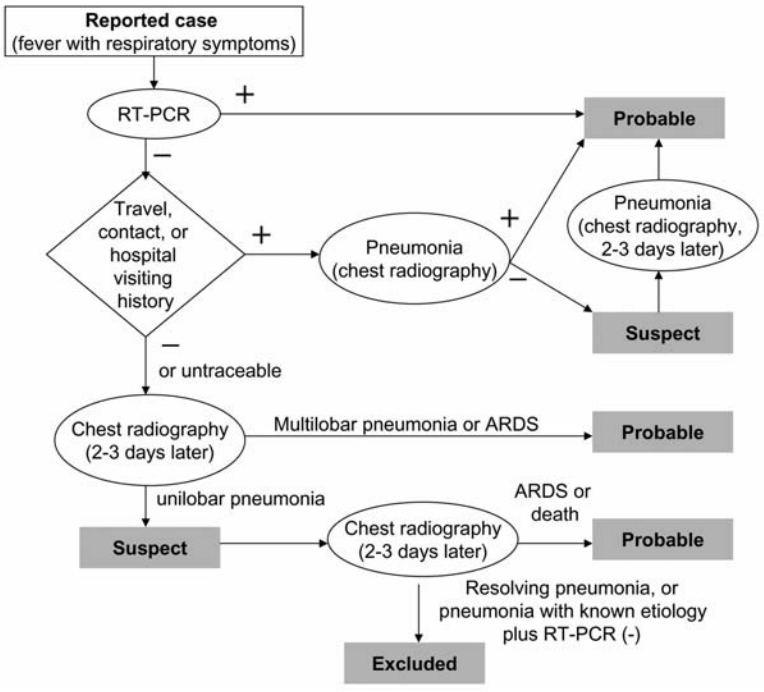

Figure. Flowchart of classification for severe acute respiratory syndrome (SARS) revised on May 1, 2003. ARDS, acute respiratory distress syndrome.

proportion of nosocomial cases suggests that containment measures instituted in Taiwan were ultimately successful in preventing a much larger outbreak. Multiple factors were associated with the nosocomial outbreaks in Taiwan, including inadequate infection control infrastructure and triage screening that led to delayed detection of several highly contagious index cases.

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In Reply: Hsueh and Yang (1) correctly described the case classification procedure in Taiwan during the 2003 severe acute respiratory syndrome (SARS) outbreak as being conducted simultaneously by three teams of local experts in the northern, central, and southern parts of Taiwan; however, they failed to mention that this procedure was implemented only after May 10 (2). More precisely, before May 9, the relevant medical records of all reported SARS case-patients were reviewed by a SARS Advisory Committee at the Taiwan Center for Disease Control, whose members included respiratory specialists, infectious disease physicians, and epidemiologists. After May 10, because the dramatic increase in the number of new cases attributed to the hospital cluster outbreaks in Taipei in late April (3), the SARS Advisory Committee at the Taiwan Center for Disease Control in Taipei could no longer effectively provide care for the rapidly increasing case load. Consequently, three regional offices of the Bureau of National Health Insurance (BNHI) north, central, and south of Taiwan took over the responsibility of case review and used standard operating procedures for case evaluation (2). Local SARS expert committees were established in all three regions, with each committee consisting of the relevant experts. This policy change provides irrefutable evidence that the authorities expedited the case classification process, which was deemed too slow, because the backlog of cases waiting to be reviewed was mounting.

In a subsequent, related study (Hsieh et al., unpub. data), retrospective statistical analysis of the laboratory–confirmed case data conducted with a two-sample *t* test indicated that the mean time from initial diagnosis of patients with suspected SARS to reclassification as probable SARS, improved significantly after May 10. The estimated mean time from diagnosis to reclassification was 12.56 days from May 5 to June 4 (3). Final classification was substantially delayed in the suspected SARS cases that were reclassified as probable SARS cases in the days after the new procedure was implemented. However, they were well represented in our mean estimation result.

Another issue raised by Hsueh and Yang concerns the evidence of nosocomial infections. From May 5 to June 4, the suspected SARS patients in Taiwan were placed in negative pressure chambers, when available, as soon as they were diagnosed. However, the operative word here is "when available." In National Taiwan University Hospital, the most established and well-equipped hospital in Taiwan, swift and efficient isolation was accomplished. Only 31 SARS cases, a small fraction of the Taiwan case data, occurred through exposure in the emergency room at the National Taiwan University Hospital Hospital, which culminated in the temporary shutdown of emergency services on May 12 (3,4). Other hospitals in Taiwan had cluster infections on wards as late as the end of May (5,6). Multiple factors were associated with the nosocomial outbreaks in Taiwan. Our modeling result merely suggested that the slow classification process, which was effectively rectified with the policy change on May 10, had been one of the contributing factors, and the change was subsequently instrumental in the quick containment of the outbreak. The intervention efforts helped prevent SARS infection transmission in medical facilities from spreading into the community. Nonetheless, a more proactive and constructive approach is to learn from this experience and to minimize the opportunity for nosocomial infections to occur in the future.

Finally, we stressed that "with more and better data, one could perhaps estimate the parameters over smaller periods of interest during the complete progression of the epidemic, if not the parameter values for each time n" (3). In the last 2 decades, the academic literature contains abundant evidence of how mathematical modeling can provide insights into infectious diseases (7). The purpose of mathematical modeling is to recon-

struct the epidemic events of importance from the data that are available at the time. Our modeling was conducted during the summer before the end of the epidemic and only the data available from various Web sites was used, which did not allow us to compare the difference in classification time before and after May 10. With the laboratory-confirmed SARS case data now available, we are able provide more definitive and detailed results in a manuscript under review (Hsieh et al., unpub. data), as well as in an ongoing modeling of the SARS outbreak in Taiwan that encompasses intervention measures and behavior change of the general public.

With the current void of precise knowledge regarding the chains of infections that led to the in-hospital and inter-hospital infections in Taiwan, mathematical modeling gives the best hope of understanding exactly how the cluster infections occurred, so we can better meet the challenges of future epidemics. Such knowledge is possible only with the interface of detailed epidemiologic and molecular data of the SARS cases with mathematical modeling. During this past winter season, a second wave of the SARS epidemic was averted. Hopefully, retrospective modeling studies such as ours will better prepare us for the emergence of any infectious diseases in the future.

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Ebola and Marburg Viruses: A View of Infection Using Electron Microscopy

Elena I. Ryabchikova and Barbara B.S. Price

Batelle Press, Columbus, Ohio
ISBN: 1-57477-131-0
Pages: 211, including index
Price: US $56.00

More than 25 years have passed since the discovery of a filovirus, Marburg virus, which caused an epidemic of hemorrhagic fever among laboratory workers in Marburg, Germany, in 1967. The persons affected had contact with the blood or tissues of monkeys or with other infected persons. Marburg virus has reappeared only three times since its discovery, with the largest and most recent outbreak occurring in 1999 in Durba, Democratic Republic of the Congo. Ebola virus, another filovirus, was first described in 1976 during two hemorrhagic fever epidemics in Zaire and Sudan. Since then, Ebola virus has caused large hospital outbreaks of hemorrhagic fever in Kikwit, Zaire, in 1995, and Gulu, Uganda, in 2000. Ebola virus has also been implicated in small chains of transmission among persons with direct contact with intermediary hosts, mostly nonhuman primates in the central African countries of Gabon and Republic of the Congo.

The reservoirs for both viruses are still unknown, and the rarity of outbreaks and the remote location of human outbreaks make it difficult, if not impossible, to study the pathogenesis of the human disease. Thus, animal models have been the best, and often only, approach available for studying the progression of disease caused by Marburg and Ebola viruses. Dr. Ryabchikova, the principal author of this book, and her laboratory group have studied the pathogenesis of filoviruses for several decades by using animal models and electron microscopy, a unique approach that has made her one of the few filovirus experts in the world. This book is a compilation not only of her work but of all the information available on Marburg and Ebola viruses.

The first three chapters of the book provide a general review of filovirus history, laboratory methods (with an emphasis on electron microscopy), viral structure, morphology, and replication. Chapters 4 and 5 provide more specific details on infection of the target cells (macrophages and reticuloendothelial system) in different organs and during the course of filoviral infection. In chapter 6, the authors deal with the "hemorrhagic" side of Ebola and Marburg virus infection. Not all patients infected with these viruses bleed, and when bleeding disorders do occur, no correlated infection of endothelial and hematopoietic cells occurs. Dr. Ryabchikova has found that changes in the microcirculation system, such as the appearance of hemorrhages, clotting, and fibrin deposits, vary by virus and by animal species. Chapter 7, which describes pathologic changes in the organs during the course of filoviral infection, could have been combined with chapter 5. Likewise, the last chapter, which covers immunopathology, appears more like a discussion of the previous chapters.

Much of the data have already been published in the Russian or Western literature. However, this book provides one source for all information available on Marburg and Ebola viruses and has a great advantage over other sources. The number and the quality of the illustrations are impressive, and a comprehensive index is provided. The book will prove useful to clinicians and researchers interested in understanding the pathogenesis of hemorrhagic fevers, and it will provide researchers working with other viruses a lesson in the benefits of using electron microscopy technology.

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Emily Carr (1871–1945). Big Raven (1931)
Oil on canvas, 87.3 cm x 114.4 cm, Vancouver Art Gallery, Emily Carr Trust

North American Birds and West Nile Virus

"Prophet!" said I, "thing of evil!—prophet still, if bird or devil!
Whether tempter sent, or whether tempest tossed thee here ashore,
Desolate, yet all undaunted, on this desert land enchanted—
On this home by horror haunted—tell me—tell me I implore!"
Is there—is there balm in Gilead?—tell me—tell me I implore!"

Edgar Allan Poe "The Raven"

"Not far from the house sat a great wooden raven mounted on a rather low pole; his wings were flattened to his sides.… His mate…had rotted away long ago, leaving him moss-grown, dilapidated and alone…these two great birds had been set, one on either side of the doorway of a big house that had been full of dead Indians who had died during a smallpox epidemic. Bursting growth…grew up round the…raven, sheltering him from the tearing winds now that he was old and rotting...." wrote Emily Carr in Klee Wyck, the best-selling book of short stories about her many visits to Native villages near Victoria, Canada, where she was born (1).

Artist, author, and passionate advocate of trees and birds, Carr drew inspiration and focus from decaying aboriginal artifacts that littered the wilderness of her beloved British Columbia. Many of her works seem haunted by these artifacts' legacy of epidemics and death. With a "smothering darkness," descended perhaps from her own Anglo-Victorian culture's fear of the primeval forest, she conveyed the frailty of human efforts against the power of the woods and the spirits in them (2).

Carr pursued an artistic career from age 16 and attended the California School of Design in San Francisco. She taught art; traveled to England, France, and the wilds of Canada's Pacific Coast in search of personal style; and exhibited widely, in spite of financial constraints and ill health from heart disease and frequent bouts of depression. Her dedication to the natural world and her belief in the mystical and spiritual connection between all things culminated during the latter part of her life (1933–1936) in landscapes of "exceptional spontaneity and expressiveness" (2).

The late 19th century witnessed sweeping cultural changes. Existing values were questioned in science, philosophy, and the arts, at the individual and social levels. This was the era of, among countless greats, Robert Koch, Louis Pasteur, Charles Darwin, Marie Curie, Albert Einstein, Fredrich Nietzsche, Sigmund Freud, George Eliot, Walt Whitman, Mary Cassatt. Artists were moving away from descriptive likeness toward visual impression of objects. During her travels to Europe, Carr explored modernism and pondered its "big ideas" in the context of the "big land" of her childhood, adopting new styles, transcending her own experience, creating potent landscapes for the world (3).

Like fellow North American artists Georgia O'Keeffe and Frida Kahlo, Carr turned for authenticity to nature and to her own and Native cultures. The wilderness of British Columbia and southern Alaska and the work of Pacific Coast communities roused her artistic imagination. She came to view nature as anthropomorphic and trees, stars, rocks, and all natural forms as symbolic reality with which she could identify—once, in a rare self-portrait, she painted herself in the form of a tree (4).

Carr continued to paint decaying tribal artifacts as she experimented with modern techniques. And by adapting the structuring influence of cubism to paintings of Pacific

Coast tribal art, she did more than preserve this art from extinction. She brought history full circle by reviving and reformulating artifacts whose kin, the tribal art of Africa and South Pacific, had greatly influenced the development of cubism in France (2).

Big Raven evolved from the watercolor image of a Haida totem pole Carr had painted at Cumshewa, Queen Charlotte Islands, almost 20 years earlier. "I want to bring a great loneliness to this canvas and a haunting broodiness, quiet and powerful," the artist wrote in her journal (4). Broodiness notwithstanding, Big Raven is full of energy and movement. The sky and landscape are sculptured, as solid and heavy as the raven itself, yet their interlocking elements are spirited. They heave and swell, their scalloped edges undulating in a powerful swirl around the massive bird.

This remnant of a vibrant household struck down by the plague of its time stands a lonely symbol of passing plagues in Carr's green sea of anthropomorphic nature. Perched low, impassive, silent, and seemingly unmoved, it feigns obscurity and anonymity, but the upward avian thrust, grave countenance, and ghastly glare label it prophet of doom.

A single bird like Carr's lonely oracle sends proper warning. A population of birds in distress or dying is a far more useful sentinel; watchful tracking of their predicament informs the ecology and dispersal of disease. Animals turn sentinels as their deaths presage human illness on the epidemic curve. Dying prairie dogs signal human plague in the American Southwest (5). Horses dying of eastern equine encephalomyelitis point to increased spread of virus in a community (6). When an "Old World epizootic strain," West Nile virus, made its way across North America, from the Atlantic to the Pacific Coasts, from Canada into tropical regions and the Caribbean (7), unexplained avian deaths sometimes occurred weeks before human West Nile virus encephalitis cases. And dying birds of the crow family, including ravens, foretold human infection in the New World as they likely did in ancient Babylon (8).

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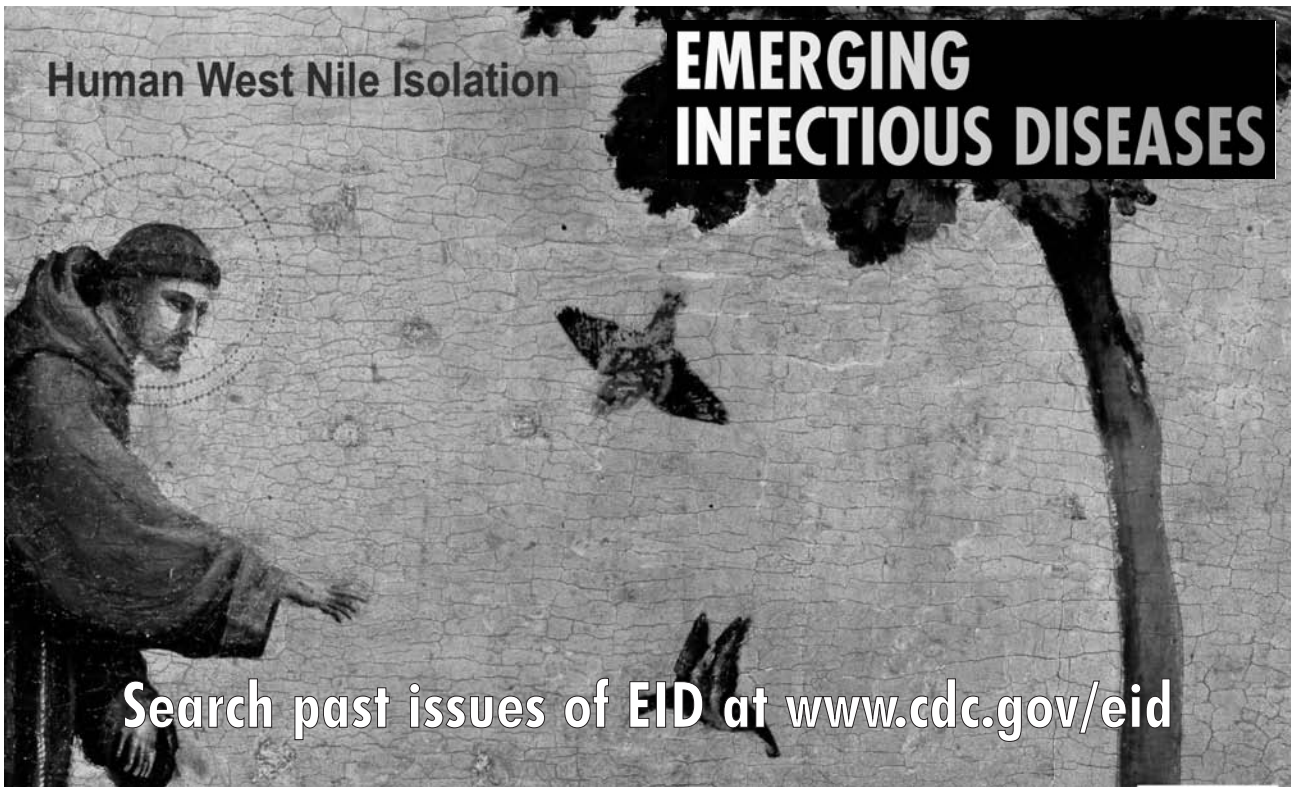

EMERGING INFECTIOUS DISEASES

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Genotyping, Orientalis-like *Yersinia pestis*, and Plague Pandemics

Methicillin-resistant *Staphylococcus aureus* in Europe, 1999–2002

Anaplasma phagocytophilum DNA in *Ixodes* ticks from Madeira Island and Mainland Portugal

Year-round West Nile Virus Activity, Gulf Coast Region, Texas and Louisiana

Complete list of articles in the September issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 6–8, 2004
FACES 2004 Encephalitis Conference
Enfield, CT, USA
http://www.encephalitisglobal.com

August 7–10, 2004
59th Annual Meeting, International Conference on Diseases in Nature Communicable to Man
Beverly, MA, USA
Contact: John Gawoski, 1-781-744-8935 or john.m.gawoski@lahey.org

September 19–23, 2004
Extremophiles 2004
American Society for Microbiology
Cambridge, MD
http://www.asm.org/Meetings/index.asp?bid=19177

September 23–26, 2004
JPGM Gold Con
Parel, Mumbai, India
Contact: Atul Goel, Editor, Journal of Postgraduate Medicine, 91-22-24129884
goldcon@jpgmonline.com
http://www.jpgmonline.com/goldcon.asp

October 30–November 2, 2004
44th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)
Washington, DC, USA
Contact: 202-737-3600
ICAAC@asmusa.org
http://www.icaac.org

November 7–11, 2004
53rd Annual Meeting, American Society of Tropical Medicine and Hygiene
Miami, FL, USA
Contact: 847-480-9592 or astmh@astmh.org

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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

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

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

EMERGING INFECTIOUS DISEASES

EID Online www.cdc.gov/eid

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Vol.10, No.7, July 2004

Search EID Online www.cdc.gov/eid

Disease Emergence

CDC

Editorial Policy and Call for Articles

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

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

Emerging Infectious Diseases is published in English Chinese, French, and Spanish translations of some articles can be accessed at http://www.cdc.gov/eid/ncidod/EID/trans.htm. The journal features the following types of articles: Perspectives, Synopses, Research Studies, Policy and Historical Reviews, Dispatches, Commentaries, Another Dimension, Letters, Book Reviews, and News and Notes. The requirements of each type of article are described in detail below and at http://www.cdc.gov/eid/ncidod/EID/instruct.htm. To expedite publication, we post journal articles on the Internet as soon as they are edited.

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases website (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. This section includes letters that present preliminary data or comment on published articles. Letters (500–1,000 words) should not be divided into sections should contain no more than one figure or table. References (no more than 10) may be included.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only (effective January 2005). Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.