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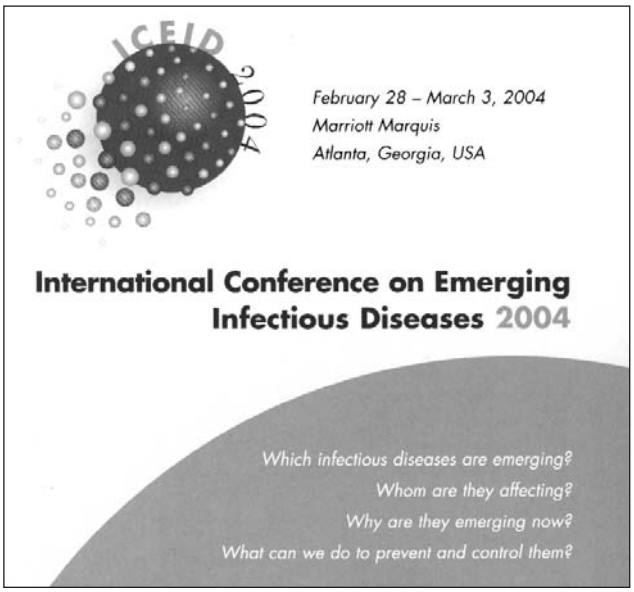

Mycobacterial Aerosols and Respiratory Disease

Joseph O. Falkinham, III*

Environmental opportunistic mycobacteria, including *Mycobacterium avium*, *M. terrae*, and the new species *M. immunogenum*, have been implicated in outbreaks of hypersensitivity pneumonitis or respiratory problems in a wide variety of settings. One common feature of the outbreaks has been exposure to aerosols. Aerosols have been generated from metalworking fluid during machining and grinding operations as well as from indoor swimming pools, hot tubs, and water-damaged buildings. Environmental opportunistic mycobacteria are present in drinking water, resistant to disinfection, able to provoke inflammatory reactions, and readily aerosolized. In all outbreaks, the water sources of the aerosols were disinfected. Disinfection may select for the predominance and growth of mycobacteria. Therefore, mycobacteria may be responsible, in part, for many outbreaks of hypersensitivity pneumonitis and other respiratory problems in the workplace and home.

Hypersensitivity pneumonitis is an occupational hazard of workers in two different industries, automobile manufacturing (e.g., metal working) and leisure (e.g., indoor swimming pools). Pulmonary illness and infection have also been a consequence of exposure to aerosols generated by hot tubs, spas, and coolant baths. Respiratory problems have also been associated with exposure to water-damaged buildings during reconstruction, and mycobacteria isolated from materials from such buildings have been shown to provoke inflammatory reactions. The outbreaks share the common feature of aerosol exposure and respiratory illness. I propose that exposure to aerosols containing mycobacteria is a common feature of the outbreaks and that mycobacteria or their products could be responsible for the respiratory symptoms.

Epidemiologic studies have established that the workers in such outbreaks were exposed to aerosols generated in the workplace from water that was either a work tool (e.g., metalworking fluid) or an integral part of the workplace or household (e.g., swimming pools and hot tubs) (1–7). Outbreaks of respiratory disease occurred in spite of disinfectant treatment of the waters or fluids to reduce the number of microorganisms. Living or working in water-damaged buildings or as a consequence of reconstruction of water-damaged buildings has also been associated with outbreaks of respiratory problems (8,9). Respiratory disease has been associated with mycobacteria in reservoirs, aerosols, or structural material in a number of cases (2,3,6,7,9).

Hypersensitivity Pneumonitis in Workers Exposed to Metalworking Fluid

An estimated 1.2 million workers in the United States are exposed to aerosols generated by metal grinding (10). Metalworking fluids are widely used in a variety of common industrial metal-grinding operations to lubricate and cool the tool and the working surface. Metalworking fluids are oil-water emulsions that contain paraffins, pine oils, polycyclic aromatic hydrocarbons, and heavy metals (10,11). Exposure to metalworking fluid aerosols can lead to hypersensitivity pneumonitis and chronic obstructive pulmonary disease (1,6,12–14). Mycobacteria were recovered significantly more frequently from metalworking fluid samples collected from facilities where hypersensitivity pneumonitis was found; compared to facilities that did not have hypersensitivity pneumonitis (6). In one study, exposure to metalworking fluid mist resulted in hypersensitivity pneumonitis in 10 workers (7). Acid-fast microorganisms identified as mycobacteria were present in the reservoir at 10^7 CFU/mL (7). A mycobacteria in the reservoir was considered to be a likely cause of the hypersensitivity pneumonitis because one patient was infected by a *Mycobacterium* sp. and had antibodies against the reservoir fluid (7).

Hypersensitivity pneumonitis appeared in spite of disinfection of the metalworking fluid with morpholine, formaldehyde, or quaternary ammonium-based disinfectants (1,6,12,13), and mycobacteria were recovered from the metal working fluid (6,14,15). Mycobacteria are resistant to formaldehyde and quaternary ammonium disinfectants (16) and the heavy metals in metalworking fluids (17). Further, mycobacteria can grow on the organic compounds in metalworking fluid, including the paraffins, pine

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oils, and polycyclic aromatic hydrocarbons (18,19) and can degrade the disinfectant morpholine (20). Mycobacteria present in the water (21) can likely grow on the organic compounds in metalworking fluids in the absence of competitors after disinfection. Cleaning would not be expected to eradicate mycobacteria because of their ability to form biofilms (21,22). Adding disinfectant and cleaning the reservoir in one facility did not prevent the reappearance of mycobacteria (7×10^5 CFU/mL by 2 weeks [7]). Further, disinfectant treatment would likely result in selection of mycobacteria remaining after the cleaning.

Hypersensitivity Pneumonitis in Swimming Pool Attendants

Granulomatous pneumonitis has been reported in lifeguards ("lifeguard lung") who worked at an indoor swimming pool that featured waterfalls and sprays (5). Affected lifeguards with symptoms worked longer hours than unaffected lifeguards (5), which demonstrated a dose-response effect. The waterfalls and sprays increased the number of respirable particles fivefold and the levels of endotoxin eightfold (5). Based on the presence of endotoxin in the aerosol samples, endotoxin exposure was suggested as the cause of the pneumonitis in lifeguards (5). However, subsequent data provided evidence of a possible second factor resulting in hypersensitivity pneumonitis; aerosols containing mycobacteria were shown to cause granulomatous lung disease (4). Others have reported high numbers of mycobacteria in swimming pools and whirlpools (23) and in hot tubs (2,3,24). Further, amoebae were reported in the indoor swimming pool where lifeguards reported pneumonitis (5). Mycobacteria, including *M. avium* and *M. intracellulare*, can survive and grow in phagocytic amoebae (25) and protozoa (26). In fact, *M. avium* grown in amoebae or protozoa are more virulent (25; Falkinham JO, unpub. data). Mycobacteria are resistant to chlorine (27) and preferentially aerosolized from water (28).

Mycobacterial Disease after Exposure to Aerosols Generated by Hot Tubs

Hypersensitivity pneumonitis and mycobacterial pulmonary disease has been reported after exposure to hot tubs (2,3,24). The mycobacteria isolated (e.g., *M. avium*) were likely responsible for the infections based on the identity of patient and hot tub mycobacterial isolates by either restriction fragment length polymorphism analysis (24) or multilocus enzyme electrophoresis (2,3). Further, exposure was followed closely by the onset of symptoms, and the extent of symptoms was related to the length of exposure (i.e., time spent in the hot tub) (2,24). Although these reports do not document the use of disinfectants in the hot tubs, the waters had been heated. Mycobacteria are relatively resistant to high temperature (29) and concentrated in hospital hot water systems (30).

Hypersensitivity Pneumonitis in Occupants of Water-Damaged Buildings

Inflammatory reactions—including eye irritation, respiratory infections, wheeze, bronchitis, and asthma—in workers in water-damaged or "moldy" buildings have been associated with the presence of high numbers of microorganisms (8). Mycobacteria were recovered from materials collected from water-damaged buildings, as well as from microorganisms normally associated with building materials (9). During reconstruction, those mycobacteria could be aerosolized in the dust. Although other microorganisms could be responsible for the respiratory problems, both saprophytic (e.g., *M. terrae*) and pathogenic (e.g., *M. avium*) strains isolated from moldy buildings were capable of inducing inflammatory responses in a mouse macrophage cell line (31). The mycobacteria elicited dose-dependent production of cytokines interleukin-6 and tumor necrosis factor-α, nitric oxide, and reactive oxygen species from the murine macrophage (31). Because whole mycobacterial cells were used in the assays (31), whether cell metabolites, which are likely easily aerosolized, were responsible for the induction of inflammatory reactions is not known. Heat-shock proteins from a number of mycobacterial species have been shown to generate Th1-type responses, airway inflammation, and airway hyperresponsiveness (32). This evidence suggests that mycobacteria or their metabolites are possible causes of respiratory disease in persons exposed to water-damaged buildings.

Ecology of Mycobacteria

The unique combination of physiologic characteristics that distinguish the environmental opportunistic mycobacteria make them likely agents for causing respiratory disease in these diverse settings. Mycobacteria are found in a great variety of natural and human-influenced aquatic environments, including treated drinking water (21) and aerosols (33). Mycobacteria in drinking water are associated with the presence of particulates (21). Although these microbes are grown in rich media in the laboratory, they are oligotrophic and capable of substantial growth in low concentrations of organic matter. For example, *M. avium* and *M. intracellulare* can grow in natural and drinking water over a temperature range of 10°C to 45°C (34). Mycobacteria are relatively resistant to high temperatures. For example, 10% of cells of a strain of *M. avium* survived after 1 h at 55°C (29). Mycobacteria are slow growing as a consequence of their fatty acid- and wax-rich impermeable cell wall (35). The resulting cell surface hydrophobicity permits adherence to solid substrates (e.g., pipes and leaves) in aquatic environments, which results in

mycobacteria's persistence and resistance to being washed away at high flow rates (21,22). Further, hydrophobicity is undoubtedly associated with the ability of these bacteria to metabolize a wide variety of nonpolar organic compounds (18–20) that are constituents of metal working fluids (15,16).

Resistance of Mycobacteria to Disinfection

Mycobacteria are very resistant to the disinfectants used in water treatment, including chlorine and ozone (27). For example, *M. avium* is almost 500 times more resistant to chlorine than is *Escherichia coli* (27). Mycobacteria are also quite resistant to agents used for surface and instrument disinfection, including quaternary ammonium compounds, phenolics, iodophors, and glutaraldehyde (16,22,23,36) and can degrade the disinfectant morpholine (20). Hydrophobicity and impermeability are undoubtedly factors contributing to the disinfection resistance of mycobacteria (35). Chemical or enzymatic removal of surface lipid, while not reducing viability, reduces surface hydrophobicity and alters cell charge (37). Because of their inherent impermeability, mycobacteria grow relatively slowly compared to other bacteria. The slow growth is not necessarily a disadvantage because it correlates with increased resistance to antimicrobial agents (35), including chlorine (Falkinham JO, unpub data).

Exposure of a mixed microbial population to disinfectants results in selection of a disinfectant-resistant or tolerant population (38). The persistence and growth of mycobacteria in drinking water systems (21) are due, in part, to their disinfectant-resistance (27) and ability to grow under oligotrophic conditions (21). Disinfection of swimming pools, therapy pools, and spas or hot tubs with chlorine is expected to kill nonmycobacterial flora and to permit the growth of even the slowly growing mycobacteria in the absence of competitors for nutrients. High temperature would also be expected to result in enrichment of mycobacteria (29,30). Resistance to disinfectants could also lead to the proliferation of mycobacterial populations in metal working fluid and coolants after disinfection (6,12,13).

Aerosolization of Mycobacteria

Although *M. tuberculosis* is transmitted between patients through aerosols, little information exists on aerosolization of the environmental opportunistic mycobacteria (e.g., *M. avium* and *M. intracellulare*). Patient-to-patient transmission of environmental opportunistic mycobacteria does not occur (39). *M. avium* and *M. intracellulare* are readily aerosolized from aqueous suspension (28,33). Transfer of mycobacteria occurs as a result of binding of mycobacterial cells to air bubbles and ejection of water droplets after the air bubbles reach the liquid surface (28). Aerosolization can result in >1,000-fold increase in numbers of viable mycobacterial cells per milliliter of water droplets ejected from water (28). Mycobacteria in natural aerosols are found in particles and droplets (i.e., <5 μm) that can enter the alveoli of the human lung (28,33). Cell surface hydrophobicity, not surface charge, is a major determinant of enrichment in ejected droplets (28). Transfer of mycobacteria from water to air is subject to prevailing physiochemical conditions and can be manipulated. Salts (e.g., NaCl) or detergents reduce the rate of transfer of mycobacteria from water to air by ejected droplets (28). The influence of the components of metalworking fluid or of chlorine or other disinfectants in water upon aerosolization mycobacteria is unknown.

Mycobacteria and Immune Responses and Airway Inflammation

Mycobacterial cells and cellular components provoke inflammatory responses. Cells of mycobacterial strains isolated from material collected from water-damaged buildings provoke inflammatory responses in macrophages (31). Mycobacterial heat-shock proteins generate Th1-type responses, airway inflammation, and hyperresponsiveness (32). The mycolic acid-containing glycolipids, mannose-containing phospholipids, glycopeptidolipid mycosides, phenolglycolipid mycosides, and sulfatides that are unique to mycobacteria have all been reported to stimulate immune responses in animals (40). Further, mycobacteria produce a variety of extracellular primary and secondary metabolites (19) that could be aerosolized and trigger immune responses, including hypersensitivity pneumonitis. Some of these immunostimulatory compounds are produced in response to growth on polycyclic aromatic hydrocarbons (18). Unfortunately, the studies of inflammatory responses provoked by mycobacteria have been limited to whole cells grown under a single condition (31) or single proteins (32). The influence of growth conditions (e.g., growth in metalworking fluid or chlorinated water) or cell fractions (e.g., membranes) or metabolites to stimulate inflammatory responses has not been measured.

Conclusion

Contemporary reviews of airway dysfunction all describe the need for information concerning microbial agents of workplace and household exposure (41). Although many more studies are needed, the evidence points to a role of environmental opportunistic mycobacteria in provoking hypersensitivity pneumonitis, respiratory disease, and respiratory infection in both the workplace and home. In addition to the recovery of identical species and types of mycobacteria from reservoirs and patients, physiologic characteristics of mycobacteria are consistent

with their presence in the sources, transmission by means of aerosols, and illnesses. Identifying the factors that influence the presence of mycobacteria in aerosols in these workplaces would have an impact on workers in a variety of occupational settings.

On the basis of several physiologic and ecologic characteristics of mycobacteria, several approaches to reduce the impact of mycobacteria in these settings are possible. Because mycobacteria are associated with particulates (21), their numbers in reservoirs can be reduced by removal of particular matter (e.g., filtration). UV light can be used to reduce mycobacterial numbers. Disinfection of mycobacteria at high temperatures (e.g., 40°C) is more effective at reducing numbers, especially if cells were grown at lower temperatures (e.g., 30°C). Agents or combinations with surfactant or detergent-like and disinfectant activity would increase permeation in cells and biofilms and kill more mycobacteria. Finally, aerosolized or waterborne mycobacteria may be trapped in filters coated with hydrophobic compounds (e.g., paraffin) and thereby intercepted before inhalation or ingestion.

Dr. Falkinham is a professor of microbiology in the Department of Biology at Virginia Polytechnic Institute and State University. His research interests include identifying the genes and physiologic characteristics of *Mycobacterium avium* that are responsible for its ecology, transmission, and virulence.

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Global Screening for Human Viral Pathogens

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We propose a system for continuing surveillance of viral pathogens circulating in large human populations. We base this system on the physical isolation of viruses from large pooled samples of human serum and plasma (e.g., discarded specimens from diagnostic laboratories), followed by shotgun sequencing of the resulting genomes. The technology for concentrating virions from 100-L volumes was developed previously at Oak Ridge National Laboratory, and the means for purifying and concentrating virions from volumes in microliters have been developed recently. At the same time, marine virologists have developed efficient methods for concentrating, amplifying, and sequencing complex viral mixtures obtained from the ocean. Given this existing technology base, we believe an integrated, automated, and contained system for surveillance of the human "virome" can be implemented within 1 to 2 years. Such a system could monitor the levels of known viruses in human populations, rapidly detect outbreaks, and systematically discover novel or variant human viruses.

The traditional process of discovering previously unknown human viruses, or variants of known viruses, is neither rapid nor thoroughly systematic. The time between back-calculated initial infection and final identification is often many weeks, months, or even years. For a totally new agent, the estimated interval between initial infection and detailed characterization is variable and depends on the presence of unusual symptoms, the failure to identify a virus after using all available specific tests, the recognition of a unique problem, and, in the past, the ability to grow the agent in culture.

The idiosyncratic nature of virus discovery contrasts with the broad survey approaches characteristic of genomics and proteomics. Only in the relatively small field of ocean viruses has a more inclusive, cataloging approach been tested. Facilitated by the relative ease with which viruses can be isolated from seawater (using commercial filters), investigators in this area have examined a broad and essentially unbiased population of viral agents at the genome sequence level (including phage) and estimated the number of different genomes present (~5,000) (1–3). One would expect that a comprehensive survey of human viruses, defining what we might term the human "virome" would be, at least conceptually, even more straightforward.

Our proposed approach (Figure), in which large populations are continually monitored for new human-infective

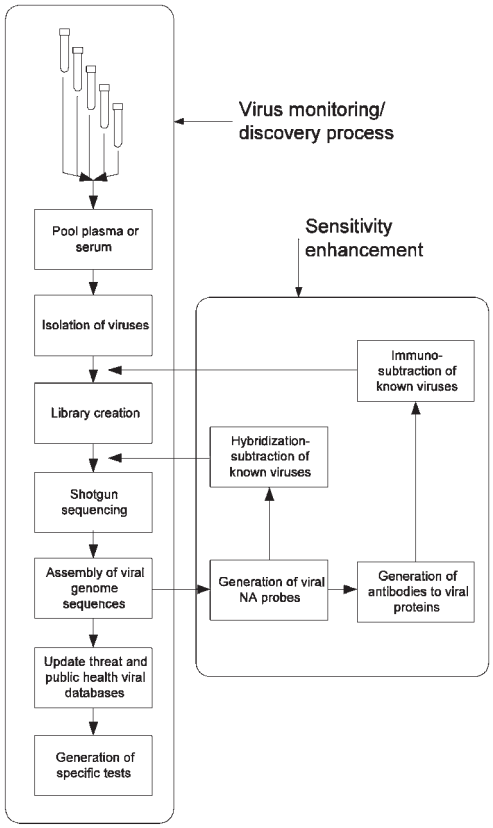

Figure. Schematic representation of a process for systematic discovery of human viruses. The basic process (left vertical series of steps) depends on physical isolation and shotgun sequencing to obtain sequences of frequent and rare viruses. A series of additional steps (right box) can be added to deplete known viruses at two levels, thereby enhancing sensitivity for novel agents.

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viruses, has not been considered technically feasible or medically necessary in the past. For purposes of broad surveillance, we propose using pools of serum or plasma from large numbers of persons, the most likely source of which is excess material collected for routine clinical purposes. These samples would be pooled and processed by using available technology to isolate virus particles en masse, recover viral nucleic acids, produce amplified shotgun libraries, carry out shotgun sequencing of the mixture of viral genomes, and reconstruct these genomes in silico with the techniques originally developed to sequence the entire human genome from random fragments. A central objective is to continually repeat this monitoring process to determine which agents change in abundance over time, find undiscovered agents already present, and detect new viruses when they appear. If successful, we will have for the first time a comprehensive picture of "what is going around." Surprisingly, most of the systems and technology to carry out this process exist in a basic form and have been successfully employed to survey the extremely varied DNA virus population of the oceans. What remains to be done, to create a system applicable to humans, is primarily its integration, optimization, and implementation in a safely contained environment. We briefly explore the components of this process here and suggest that it can be made operational in less than a year.

Availability of Large Pooled Samples

The major commercial diagnostic laboratories in the United States discard approximately 500 L of excess human serum or plasma each week. This material represents a broad cross-section of patients and illnesses. Plasma viral loads as a function of time after onset of illness are not known for most viral diseases, but they appear to be highest in the initial febrile stages. Since one of the first steps in treating a febrile illness of unknown origin is obtaining a blood sample, we expect that current diagnostic networks contain appreciable quantities of virus. Samples from subpopulations enriched for potential viral illness could also be selected. For those viral diseases in which viremia precedes major illness, the inclusion of large numbers of randomly acquired specimens in the pool (i.e., an unselected pool) offers the best chance of detection. Analysis of pooled samples from a large number of persons should raise minimal privacy concerns.

Virus Isolation, Sequencing, and Assembly

Methods are required for the routine isolation of all classes of viruses from a pooled sample and for concentrating them by factors of over a million while ensuring that all nonviral nucleic acids have been removed. The concentrates may be dangerously infectious, and sophisticated containment systems will be needed.

The preferred technology for virus concentration from large volumes was developed by the Joint NIH-AEC Zonal Centrifuge Development Project at the Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA, and the Oak Ridge Gaseous Diffusion Plant in the 1960s (4). At the outset, researchers wanted to determine whether viruses as a class differed in a systematic way from all other small particles in nature. When the sedimentation coefficients of then-known viruses were plotted against their isopycnic banding densities, nearly all viruses fell into an otherwise essentially vacant area in the center of the plot, surrounded at higher or lower density and higher or lower sedimentation coefficients by various subcellular organelles and macromolecules. This area was termed "the virus window" (5). Thus, viruses exhibit a unique size and density range and have banding densities that reflect their combined protein and nucleic acid contents. In addition, viral nucleic acids are shielded from attack by nucleases so that contaminating nucleic acid-containing particles (primarily genomic DNA from apoptotic or disrupted cells) can be selectively destroyed by added nucleases (6). The rules of virus isolation are the following: 1) the sedimentation rate (based largely on particle size) falls in a specific range; 2) the banding density in a gradient falls in a specific range; 3) the genome is protected from nuclease attack until the protein (+/- lipid) coat is disrupted; and 4) the major proteins present have sequences that agree with at least part of the genome.

Exploiting the virus window required a two-dimensional separation based on sedimentation rate (S) in one dimension and banding density (rho) in the other, usually carried out in the order: S-ρ. For large-scale isolation and purification, the challenge was to perform these separations continuously and simultaneously in large continuous-flow centrifuge rotors spinning at high speed in a vacuum.

In this scheme, a flowing stream passes inboard of a thin, nonflowing density gradient, held in place against the rotor wall by centrifugal force. The recovered virus forms a narrow band in the gradient, which is recovered after reorienting the gradient to rest at the end of the run. If the flow through two centrifuges is cascaded, the first operating at lower speed than the second, particles having a higher S rate than viruses could be removed from the flowing stream, and the viruses then concentrated and banded in the second higher speed centrifuge, thus providing a large-volume S-ρ separation.

The end result of this work included the design and construction of the K-II large-scale ultracentrifuge (7–10). This device was designed to recover virus in a high state of purity from 100-L batches of crude influenza vaccine in an 8-hour day (11,12) and subsequently was used for the large-scale purification of the hepatitis B (Australia) surface antigen (13,14) from human serum for use as a vac-

cine and for mass isolation of polyhedral inclusion bodies (15). K centrifuges have come into worldwide use for large-scale virus isolation and have been commercially available with little rotor modification for the last 35 years (16,17). Approximately 200 such systems have been constructed.

Methods are now available to further purify components of complex viral mixtures, to sediment the viruses through gradients containing nonsedimenting zones (e.g., of nucleases), and to concentrate them down to tens of microliters (18,19) for sequencing or mass spectrometric analysis.

Ocean Viruses

During the Oak Ridge centrifuge development project, large volumes of test material were required, and seawater, among other sources, was examined as a possible source of virus. The ocean was found to contain examples of almost every known viral form at high titers (20), initiating the exploration of marine virology (1–3,21–30). Recent data in this field suggest that the oceans of the world contains approximately 10^{31} phage particles or virions (27) (c. 22 million metric tons), much of it turning over once per day and including some human pathogens (28). This vast mutation engine, even if one assumes a minimal mutation rate, generates the equivalent of hundreds of new complete human genomes per day. That viruses are ubiquitous in the ocean has been demonstrated by studies on samples recovered in widely separated locations from filtration systems installed on surface ships (29), nuclear submarines (3), and remotely operated vehicles (30). Indeed, the entire ocean has an average viral content in the lower range of the viral loads reported for human plasma from viremic patients. Marine virologists have in fact come closest to implementing a surveillance system such as we propose for humans. On limited budgets, these researchers have developed the means of recovering marine viruses from large volumes by filtration (especially well-suited to such a dilute sample), and for producing shotgun libraries from them by random amplification (1). Marine virologists have also begun to estimate the diversity of marine viruses (1–3,27,30) and are reconstructing large numbers of complete viral genomes. In one study (2), a 200-L sample of surface seawater was concentrated; ~2 x 10^{12} viral particles were recovered; the DNA was randomly sheared and cloned; and 1,934 fragments were sequenced. Data analysis showed that most of the sequences were from previously unknown viruses. Approximately 3.5% of the total sequence samples overlapped, suggesting that the marine viral community was highly diverse. A unique mathematical analysis (2) further suggested that less than 10^4 different viral types were present and that shotgun sequencing of the most abundant could be done, with existing facilities, in 1 month. Although efforts to date have focused on viruses with DNA genomes, most human viral pathogens have RNA genomes. Genomic sequencing libraries will therefore have to be prepared from mixtures of both single- and double-stranded DNA and RNA viruses (the latter generated by reverse transcription).

The feasibility of the genomics assembly and annotation components of this project derives from the demonstration that the entire human genome could be fragmented, the fragments sequenced, and the original sequence reconstructed from overlaps; that abundant sequencing capacity now exists in search of high-value projects; and that marine virologists have succeeded in parallel ventures. The challenge is to shorten the time of the entire process so useful epidemiologic and viroterrorism response data can be rapidly obtained.

Remaining Challenges

Recovering viruses from large pools of human serum and plasma and routinely cloning and sequencing the viral nucleic acids (using established shotgun approaches) appears technically feasible. Consequently, the titers of many known human viral pathogens may be estimated routinely, and new viruses (both pathogenic and nonpathogenic) may be discovered systematically.

The initial choice is between filtration and centrifugation. Seawater contains little contaminating material of the size and density of the virus particles, filtration is simple and efficient, and no free nucleic acids have been reported. Plasma and serum present different problems, complicated by the presence of large amounts of protein, some nonviral particles in the virus window, and variable amounts of soluble nonviral nucleic acids (31) that must be eliminated. An advantage of centrifugal methods is that all separations, down to banding in microliter gradients, can be (and have been) done with the virions in suspension, a process that avoids aggregation that may occur on filter surfaces.

Several key questions remain to be addressed in a practical project: 1) Can this process be carried out rapidly enough to support a timely therapeutic or prophylactic response to a new agent (natural or engineered)? 2) Will the novel virions that originated from one or a very few infected persons be recovered and detected? 3) Can the affected persons be located? 4) Will the prescence of antibodies in the starting samples against most known viruses affect the separations?

Speed of Operation

Samples can be collected weekly, and materials rapidly transported to one or more processing sites. Virus isolation, library construction, and preparation of clones for sequencing require <7 days but the time may be com-

pressed into <4 days with a 24-hour per day operation. Sequencing time will be determined by available capacity, but since capacity is abundant, with large increases in sight, extensive library sequencing (e.g., 10–100 megabases) could be carried out in 2 to 3 days. Less than 1 day would be required to assemble viral genomes as contigs in silico. By the conducting of each step in sequence, turnaround (serum pool to raw sequence data) could be completed in approximately 10 operating days. Additional time would be required for bioinformatics analysis of the data and annotation, but prevalence and novelty conclusions should be available almost immediately. These estimates assume an integrated and fully developed system in continuous operation, analogous in some respects to those monitoring computer viruses.

Sensitivity

The mass of virus ultimately recovered from pooled human plasma is difficult to estimate in advance. If the average virus has a mass of 1.0×10^{-15} g; if the average titer of an infected person is 10^6 virions/mL, and if 0.1% of the samples are from viremic patients, then ~0.5 μg of virus would be recovered from each 500-L pool, substantially more than the few nanograms required to make a large library with current technology (1,2). If the average sample contributed to the pool was 1 mL, and if the final concentrated virus were in 1 mL, the final concentration of a totally new virus would be close to that in the original individual sample. The possibility of detecting all viruses for which polymerase chain reactions (PCR) primers are available, down to contributions from single patients, therefore exists.

Dynamic Range

The problem of dynamic range can be addressed in three ways. First, given large sequencing capacity, one could sequence deeply into the libraries (millions of clones instead of a few thousand), thereby detecting parts-per-million sequences. Second, one could apply antibody-based affinity methods to deplete known viral particles from the initial concentrated viral sample. Third, one could use subtractive hybridization to remove known viral genomic sequences to further enrich libraries in novel genomes. The last two approaches can be progressively extended as viruses are characterized to provide a continuous increase in sensitivity to new agents (Figure).

Identification of Viral Sources

Two approaches can be used, if necessary, to link viruses to patients. In the first approach, viruses would be tracked geographically, first in terms of large regions, and then, sequentially, in terms of smaller areas. Detecting a new agent in large pooled samples would thus be repeated in smaller, localized pools that had been combined hierarchically to generate the larger pool (32).

A potentially more efficient approach involves overlapping subpools designed such that a new viral sequence can be assayed (e.g., by PCR) in the subpools and the affected persons identified in one step. To achieve this result, each sample is added to a series of different pools, the identity of these subpools providing an "address" of the sample (33). This process can be visualized by analogy to a 3-D chessboard, where each position represents a sample, and the subpools are the various planes parallel to the top, front, and side: each sample would contribute to three subpools. In practice, additional pools would be created to provide a relatively unambiguous means of backtracking from the pattern of subpools positive for a specific sequence to one or a few persons.

Viral Pathogens That May Be Missed

Not all human viral pathogens will be detected easily by analyzing plasma or serum samples. Neurotropic viruses such as rabies, for example, are found in cells and tissues and do not appear free in serum or plasma in appreciable amounts. Thus, these viruses would escape the screening system described to this point. Although using rabies for viroterrorism would be unlikely, such viruses are of great public health interest, and efforts should ultimately be made to include them in any global screening system.

The rapid turnover of viruses found in plasma suggests that they are removed into cells, and that appears to be generally true. Centrifugal S-ρ technology was originally developed for cell fractionation with the aim of isolating viruses from tumors, cells, and tissues (5). Trace quantities of virus could be added to tissue homogenates and recovered in a high state of purity. The basic technology therefore exists for isolating viruses from lymphocytes and a variety of different tissues. At a later stage, the proposed approach should be applied to whole blood (with cells lysed before virus recovery), nasal washings, tissues, and other potentially virus-laden samples.

Automation and Containment

To routinely detect new and potentially lethal viruses, researchers may need to create completely automated and contained laboratories that continually search for and sequence viruses from a wide variety of sources to hone skills; demonstrate efficiency; and develop improved systems, methods, and reagents.

Containment was of great concern in the original Manhattan Project to deal with radiologic hazards, and in the Oak Ridge centrifuge project (34) to contain infectious agents. Containment systems have since evolved in two directions. In biological sciences, interest has centered on schemes to allow investigators to work in a safe environ-

ment using essentially the same tools they would use on an open bench. As a result, designs has evolved in which human operators are contained in "space suits." In nuclear programs, in contrast, (where containment systems actually originated), operators are completely isolated from the contents of "hot cells," and operations in these cells are done remotely with specially designed equipment. Given the national urgency for automated systems for virologic studies, completely robotic automated systems should be developed, analogous to those used in nuclear research, because 1) the concentrated samples to be analyzed are potentially extremely dangerous, 2) work must be done without interruption, and 3) speed and precision depend on automation. Although the K-II centrifuge has not thus far been automated for totally remote operation, including cleaning between runs, this is not an overwhelming problem and should involve cascaded centrifuges, as was done for the mass isolation of Tussock moth polyhedral inclusion bodies (15).

False Positives, False Negatives, and the Price of Errors

All current diagnostic tests have the potential for false-negative and false-positive results. In the atmosphere of an actual terrorist attack with a biological agent, however, the consequences of these false outcomes place an enormous strain on public services, as demonstrated by the recent anthrax episodes. A false positive triggers highly disruptive responses, whereas a false-negative result exposes the population to the obvious health concern. The approach described here reduces the possibility of such outcomes and only assumes that the virus has the expected biophysical properties of size and mass and an internalized genome. If a particle with the appropriate biophysical properties coincides with an internal genome that codes for structural proteins that are also found in the same fraction, the possible number of false-positive error is acceptably small and few mechanisms exist by which false evidence for a truly nonexistent viral sequence might emerge from the process described. The level of false negatives depends not only on the overall quality of the analysis but also on its sensitivity to rare events, i.e., the dynamic range. For sequence-based analyses, sensitivity depends on the frequency with which a sequence appears in a fragment library, the number of clones produced, the efficiency of known virus subtraction (if applied), and the number of different clones sequenced.

The number of intentional false positives (duping) is another matter and one that has two aspects. First, intentional introduction of unexpected pathogens or their genes into a global analytical system is itself a terrorist act and one that should be detected and known. To insert a substantial amount of recoverable viral particles into the sample collection system, a person trying to deceive the system would have to engineer and grow these viruses—an act sufficiently close to actual bioterrorist use that it requires detection, whether the agent is a serious human pathogen or not. The second aspect concerns the best response to the suspicion that such duping has occurred. Complete sequencing of the agent(s) involved would be important since one cannot initially distinguish a genuine sample from one used in duping. Only after extensive further studies and the demonstration that an outbreak has not occurred may the sample be determined not to be of patient origin.

Forewarned is forearmed. Given advance notice, even by weeks, of an impending viral outbreak, the hope exists that the tools and imaginations of molecular biology will find the means to prepare some effective biological defense.

Medical Contributions of Global Surveillance

The problem of developing new antiviral agents, especially those specific for one or only a few viral diseases, is circular. Without such treatments rapid agent identification is not necessary, but without such identification no pressing commercial justification for developing specific antiviral agents exists (except for HIV) because they will not be widely used. To be successful, diagnosis and therapy must be linked. This project would assist in forging that link.

Conclusion

Isolating and sequencing the genomes of a wide variety of viruses from pools of the excess human serum and plasma currently collected and discarded by large diagnostic laboratories is now technically feasible. This collection and analysis process could allow new or unknown pathogens to be identified in the first, or at most second, round of infection. Not all human viral pathogens will be present in such mixtures, but they will include a large fraction of all known highly infectious viral agents. Since the core technologies, though varied, are highly developed, we believe that the initial feasibility studies could be completed in 1 year.

Former Senator Sam Nunn and William H. Wulf, president of the National Academy of Engineering, have both proposed setting up a project concerned with bioterrorism, modeled after the Manhattan Project. We believe that the project described could form the nucleus of such an effort and suggest that lessons learned in the Oak Ridge centrifuge project may apply. As noted by Alvin Weinberg, that project was the first (and hopefully not the last) large-scale project in the biological sciences in which facile access to a wide range of technologies was provided, on the model of the original Manhattan Project (35).

In separate articles, we will discuss the possibility of linking rapid detection to rapid responses, including vac-

cine and therapeutic antibody development, in an attempt to abort epidemics caused by new viruses while they are in progress.

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Dr. Anderson was director of the Molecular Anatomy Program at the Oak Ridge National Laboratory, held a similar position at the Argonne National Laboratory, was chief scientist at Large Scale Biology Corporation, and is now president of the Viral Defense Foundation. His chief interests include proteomics, virology, and biophysical separations.

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Salmonella Control Programs in Denmark

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We describe *Salmonella* control programs of broiler chickens, layer hens, and pigs in Denmark. Major reductions in the incidence of foodborne human salmonellosis have occurred by integrated control of farms and food processing plants. Disease control has been achieved by monitoring the herds and flocks, eliminating infected animals, and diversifying animals (animals and products are processed differently depending on *Salmonella* status) and animal food products according to the determined risk. In 2001, the Danish society saved U.S.$25.5 million by controlling *Salmonella*. The total annual *Salmonella* control costs in year 2001 were U.S.$14.1 million (U.S.$0.075/kg of pork and U.S.$0.02/kg of broiler or egg). These costs are paid almost exclusively by the industry. The control principles described are applicable to most industrialized countries with modern intensive farming systems.

Salmonellosis is one of the most common causes of foodborne diarrheal disease worldwide. Most of these infections are zoonotic and are transmitted from healthy carrier animals to humans through contaminated food. The main reservoir of zoonotic *Salmonella* is food animals, and the main sources of infections in industrialized countries are animal-derived products, notably fresh meat products and eggs. In developing countries, contaminated vegetables, water, and human-to-human transmission are believed to contribute to a comparatively larger proportion of the human cases than those in industrialized countries (1). However, the incidence of human salmonellosis increased in most industrialized countries in the 1980s and 1990s. Rapid spread of a limited number of successful *Salmonella* clones in different sectors of food animal production (swine, broiler chickens, and particularly layer hens) has been suggested as the most important cause of this increase (2).

Despite much research and many national and international attempts to implement control strategies, the incidence of human salmonellosis in most countries remains high. One notable exception is Sweden, which remains essentially free from the *Salmonella* problems typical for most other industrialized countries. The background for the Swedish success has been described (3). Unfortunately, other countries cannot apply the Swedish model of *Salmonella* control, which requires near freedom from *Salmonella* in domestic food animal production from the onset. In the European Union, the Zoonosis Directive (4) was an attempt to initiate a European Union–wide control effort against foodborne zoonoses, particularly *Salmonella* in broiler chickens and layer hens. Most European Union countries found that they either could not or would not implement the directive, which did not permit use of vaccines, antimicrobial drugs, or both as elements in the control program of *Salmonella* in broiler chickens or layer hens. This constraint was seen as an obstacle by some countries. Recently a new directive has been formulated, which is awaiting final approval by the European Union Parliament.

In Denmark, the incidence in human salmonellosis increased rapidly in the second half of the 1980s because of the spread of *Salmonella* in broiler chickens. This increase led to the initiation of a targeted national control program (5). Subsequent spread of *Salmonella* in swine and layer hens has also led to increases in human disease incidence and subsequently to the development and implementation of targeted control efforts (6–8). We review Denmark's *Salmonella* control programs and the effect on *Salmonella* in food animals, food, and humans. We also evaluate and discuss control costs and public health economy aspects.

Control of *Salmonella* in Broiler Chickens

Objectives, Program, and Effects

The initial aim of the program was that <5% of broiler flocks would be infected with *Salmonella*. The program was successful and was gradually revised towards assurance of complete freedom from *Salmonella* in broiler production.

The program is based on the principle of top-down eradication, ensuring freedom from *Salmonella* from the top of the broiler-breeding pyramid down. Infected flocks of breeding animals are destroyed, and infected birds are

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processed for slaughter. The testing program has developed gradually to adjust to higher food safety objectives. As progress stalled, more intensive serologic and bacteriologic testing was developed and applied (5,9–11). The current testing scheme is shown in Table 1.

Birds from infected flocks are slaughtered on separate slaughter lines or late in the day to avoid cross-contamination. Farmers get a better price for birds from *Salmonella*-free flocks, and slaughterhouses can use the label "*Salmonella*-free" for birds that meet criteria determined by the authorities. No decontaminants, such as organic acids or chlorine, are used during carcass processing.

The proportion of *Salmonella*-infected broiler flocks has been markedly reduced since the initiation of the control program. Figure 1 shows that >65% of broiler flocks tested positive for *Salmonella* during the first year of the program, 1988–89, versus <5% in 2000. This decrease in *Salmonella* has led to a concomitant reduction in the proportion of infected broiler carcasses after slaughter and at retail.

The Danish government and the European Union equally compensate owners of destroyed breeding stock for their losses. In 1993, a major Danish retailer (COOP-Denmark) stopped the marketing of broiler chicken, which exceeded a 5% target. Danish chicken could not meet this target at that time, so producers suffered severe losses because they had to export their chicken to lower priced markets.

Salmonella can be effectively reduced (nearly eliminated) from broiler chickens by intensive flock-level testing and top-down eradication. Essential to success is a sufficiently sensitive testing program in the breeding and rearing flocks as well as in the hatcheries, i.e., one that involves intensive sampling and a combination of serologic and bacteriologic testing methods (Table 1). Bacteriologic testing alone is not sufficiently sensitive to achieve control, especially if *S.* Enteritidis infections are present. Removal of all organic material, thorough cleaning and disinfection of the poultry house, and an empty resting period of 10–14 days between flocks can effectively eliminate residual infections. In Denmark, most infections appear to be vertically transmitted (nearly always traceable to an infected hatchery or parent flock), whereas horizontal transmission from the environment and wild fauna appear to play a minor role. Competitive exclusion cultures, vaccines, or antibiotics have not been used in the Danish control program.

Control of *Salmonella* in Layer Hens

Objectives, Program, and Effects

All shell eggs from commercial layer flocks should be free from *S.* Enteritidis and *S.* Typhimurium. Control of layer breeders in Denmark is essentially identical to the control program for broiler breeders (Table 1). Blood and fecal samples of rearing flocks are tested (8,11), and infected flocks are destroyed. All commercial flocks of layer hens in production are tested routinely every 9 weeks by a combination of serologic testing of egg yolk and bacteriologic testing of environmental samples (Table 1, Figure 2).

Table 1. *Salmonella* surveillance of the broiler and egg production, Denmark, 2000

Stage of production	Age or frequency	Samples taken	Method
Central rearing stations, broiler and egg sector	Day-old chickens	10 samples of crate material, 20 dead or destroyed chickens[a]	Bacteriologic
	1 wk	40 dead chickens	Bacteriologic
	2 wks	2 pairs of sock samples	Bacteriologic
	4 wks	60 fecal samples[a]	Bacteriologic
	8 wks	2 pairs of sock samples	Bacteriologic
	2 weeks before moving	60 fecal samples and 60 blood samples[ab]	Bacteriologic, serologic
Breeders (hatching egg production)-broiler and egg sector	Every 2 wks	50 dead chickens or meconium from 250 chickens taken from the hatchery[ac]	Bacteriologic
	Every wk	2 pairs of sock samples[d]	Bacteriologic
Hatchery	After each hatching	Wet dust	Bacteriologic
Rearing egg production	Day-old chickens	10 samples of crate material and 20 dead chickens	Bacteriologic
	3 wks	5x2 sock samples in floor production units or 300 fecal samples	Bacteriologic
	12 weeks	5x2 sock samples in floor production units or 300 fecal samples, and 60 blood samples[b]	Bacteriologic, serologic
Egg production	Every 9th wk for eggs sold to authorized egg-packing centers	2 pairs of sock samples in floor production units or fecal samples and egg samples	Bacteriologic, serologic
	Every 6 mo for eggs sold at barnyard sale	2 pairs of sock samples or fecal samples and egg samples	Bacteriologic, serologic

[a]Requirements of the European Union Zoonosis Directive (92/117/EEC).

[b]Samples taken by the district veterinary officer.

[c]Samples taken by the district veterinary officer every 8 weeks.

[d]Samples taken by the district veterinary officer every 3 months.

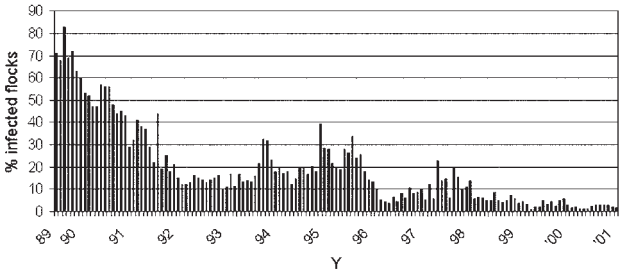

Figure 1. *Salmonella* in Danish broiler flocks as determined by bacteriologic testing of every flock 2–3 weeks before slaughter (N>4,000 flocks/year).

All eggs from suspect or confirmed-positive layer flocks are pasteurized. All shell eggs are distributed in a cold chain (not exceeding 12°C) and kept refrigerated at retail; eggs are generally refrigerated in private homes.

The government and the European Union equally compensate owners of destroyed breeding stock for their losses. The proportion of layer flocks infected with *Salmonella*, notably *S.* Enteritidis, has been markedly reduced since the initiation of the control program. Figure 3 shows that >7% of layer flocks tested positive for *Salmonella* in the first year of the program, 1998, versus <2% in 2001. The level of *Salmonella*-contaminated shell eggs has not been measured from the initiation of the control program. However, a year before the program began, a study of 13,000 eggs from different types of production determined the level to be 1 per 1,000 eggs (20% of the contaminated eggs harbored *S.* Enteritidis) (12).

Top-down eradication of *S.* Enteritidis has effectively reduced the level of *Salmonella*, notably *S.* Enteritidis, in Danish commercial layer flocks. The program has been effective in free range, deep litter, organic, and caged birds.

Figure 2. A) Receipt of 60 eggs per producer every 9 weeks (barcode indicating producer is shown). B) The "eggbreaker" punches a hole in 30 eggs at a time. C) Withdrawal of egg yolk from 30 eggs and transfer to microtiter tray. D) Enzyme-linked immunosorbent assay analysis, reading, and transfer of results to central database.

Frequent testing by a combination of serologic and bacteriologic testing methods is essential to achieve adequate sensitivity in the monitoring program. Control of residual infections in poultry houses can be conducted with a success rate of nearly 70% by thorough cleansing and disinfection of the depopulated house (removal of all organic material, disinfection of surfaces, and resting of the empty house for 2 weeks). Day-old chicks for rearing must be antibiotic free. Competitive exclusion cultures and vaccination are not used in the Danish program. Vaccination cannot, at present, be used in combination with serologic testing because of problems of cross-reaction.

Control of *Salmonella* in Pork

Objectives, Program, and Effects

Denmark is the only country with a nationwide control program of *Salmonella* in pork that is integrated from "feed-to-food." The program is based on routine testing and classification of slaughter pig herds and subsequent slaughter of pigs according to the inherent risk, as measured by the continual test program (Figure 2; Table 2). The program has been described in detail elsewhere (7,8,13).

Pre-Harvest Control

Pigs from breeding and multiplying herds are tested monthly by serologic testing of blood samples. If a specific cutoff level is reached, bacteriologic confirmatory testing is carried out. Further, if the serologic reactions exceed a specific high level, all movement of animals is restricted. Slaughter pig herds are monitored continuously by serologic testing of "meat juice" (drip fluid released from meat after freezing and thawing) (14). Meat samples for testing are collected at the slaughter line, and the number of samples and frequency of sampling are determined by the size of the herd. Approximately 700,000 slaughter pigs are cur-

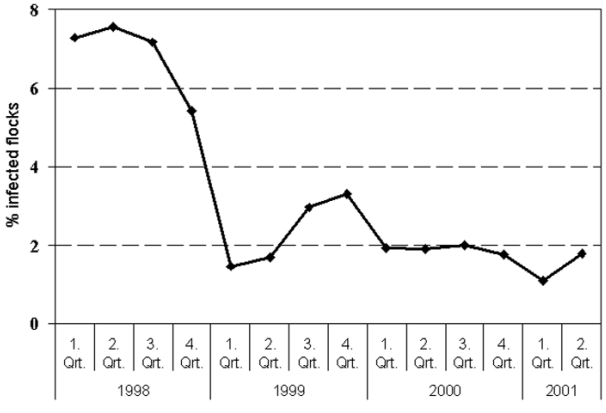

Figure 3. *Salmonella* in Danish layer flocks as determined by serologic and bacteriologic testing of each commercial flock in week 9 of production.

Table 2. *Salmonella* surveillance in pig and pork production, Denmark, 2001

Type of production	Sample	No. and frequency	Response
Breeding and multiplying herds	Blood	10 times per mo	Confirmatory bacteriologic testing and restrictions on the movement of animals if above predetermined level
Pig herds	Feces	100 in 20 pools of five collected on indication	*Salmonella* reduction plan implemented
Slaughter pig producers producing >200 pigs per year	Meat juice	Depending on herd size (60–100 samples per yr). Samples are collected continuously and semi-randomly	Confirmatory bacteriologic testing (20 pools of 5 fecal samples). Herds are assigned to one of three levels depending on serology. Level 1: no sanctions; level 2: implementation of *Salmonella* reducing actions in the herd; and level 3: same as level 2 and obligatory slaughter of pigs under special hygienic precautions, including postslaughter microbial testing and potential heat treatment of all meat products
Carcass after slaughter	Surface swab	Swabs of five carcasses are pooled into one sample. One sample per day in each slaughterhouse.	Slaughterhouses exceeding a predetermined number of positive swabs in a 3-months period are obliged to implement corrective actions

rently tested each year (Figure 4). Herds sending <200 pigs to slaughter each year are not tested, leaving 1.6% of the slaughter pigs outside the monitoring scheme (13). The herds are categorized in three levels based on the proportion of seropositive meat juice samples during the last 3 months. Owners in level 2 and 3 are encouraged to seek advice on how to reduce the *Salmonella* problem in the herd (e.g., feeding, hygiene, and management). Furthermore, payment from the slaughterhouse is reduced by 2% and 4%, respectively.

The postharvest surveillance program has been described (8,15,16). Pigs from herds in levels 1 and 2 are slaughtered traditionally without any special precautions. Pigs from level 3 herds can only be slaughtered in special slaughterhouses under special hygienic precautions. Carcasses from level 3 herds are tested for bacteria after slaughter, and if the level of contamination exceeds a certain limit all carcasses from the particular herd have to undergo heat treatment or other risk-reducing processing. All slaughterhouses do routine bacteriologic testing of carcasses according to a sampling plan, which ensures that testing is random and representative of the national swine production (>30,000 samples/year). Slaughterhouses that exceed a certain predetermined level of *Salmonella* in the routine monitoring of carcasses are obliged to investigate and reduce the contamination problem to an acceptable level.

The prevalence of swine herds in level 2 and 3 respectively, has been steadily reduced since the program began (Figure 5). Bacteriologic testing has indicated that the herd infection level was reduced by 50% (from 14.7% to 7.2% in small herds and 22.2% to 10.4% in large herds) from 1993 (when the program was implemented) to 1998 (17). In the same period, the level of *Salmonella* contamination in pork products, as determined by the routine monitoring program, was reduced from 3% to <1% (Figure 6).

As *Salmonella* eradication in swine herds is difficult because of the continual nature of the production system, reducing the infection level should be the aim of a control strategy. The low infection level in the herds and contamination in the products can likely be reduced further in Denmark. As the contamination level goes below 1%, testing for contamination requires increasingly large numbers of samples and consequently becomes very expensive, which is one reason the sampling plan of herds and products has become more sensitive in recent years. This change in testing sensitivity makes it difficult to compare current and past levels of infection and contamination but is nevertheless a necessity for the continued improvement of the program.

A combination of serologic and bacteriologic testing is essential for the success of the program. Nearly 1 million serologic samples are tested each year. Testing this large number of samples would not be possible because of financial and logistic constraints if the program were to rely on bacteriologic testing alone.

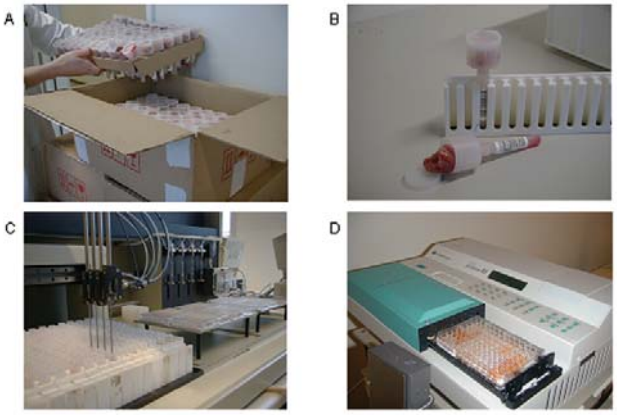

Figure 4. A) Receipt of pork samples from the slaughterhouse. Each tube is labeled with a barcode, indicating herd of origin. Samples are frozen overnight. B) The tube is entered in a rack with the barcode facing outward. Meat juice is sieved into the tube from the container during thawing. C) Withdrawal of meat juice from tube and transfer to microtiter tray. D) Enzyme-linked immunosorbent assay analysis, reading, and transfer of results to central database.

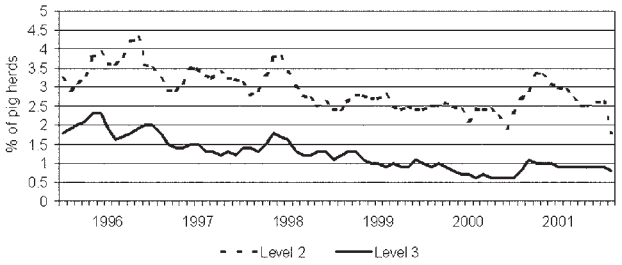

Figure 5. Prevalence of *Salmonella* in Danish pig herds as determined by continuous serologic testing of all commercial pig herds (N >700,000 samples tested/year). Herds are categorized in three levels based on the proportion of seropositive meat juice samples during the last 3 months. Owners in level 2 and 3 are encouraged to seek advice on how to reduce the *Salmonella* problem in the herd (e.g., feeding, hygiene, and management). Furthermore, pigs from level 3 herds can only be slaughtered in special slaughterhouses under special hygienic precautions. Data from the Danish Veterinary and Food Administration.

Feeding strategy (e.g., increased coarseness of feed and wet feeding) and improved management (e.g., sectioning and all-in all-out production) and hygiene standards are important elements in the preharvest control efforts. Using commercial prebiotic cultures is not necessary; natural microflora in the feed, especially wet fermented feed, appear to have a protective effect (18).

A reduction of *Salmonella* in slaughter pig herds has been attained without *Salmonella*–free breeding herds. However, to ensure the highest degree of consistency in the program, the levels of *Salmonella* in breeding herds should be kept as low as possible, and infected breeding herds should not be sold to producers of herds of a superior *Salmonella* status.

Determination of Public Health Impact

To better explain the mechanisms in the occurrence of *Salmonella* infections in humans, the Danish Zoonosis Centre has previously described a method that estimates the number of human cases attributable to each of the major animal-food sources (19,20). Using this method, we compared *Salmonella* types isolated from animals and foods with *Salmonella* types isolated from humans. In brief, subtypes of *Salmonella* that are almost exclusively found in a particular food animal reservoir or food type (unique types) are used as anchor points for the distribution of subtypes occurring in several reservoirs and sources. All human infections caused by the unique types are associated with the indicated food type or derived from the indicated food animal reservoir (e.g., pork, beef, chickens, or eggs). *Salmonella* types, which occur in several reservoirs, are distributed relative to the prevalence of unique types in each reservoir or food type. Detailed knowledge of the distribution of *Salmonella* types in all relevant food animals and food types, generated through intensive and continuous monitoring, is a prerequisite for the analysis. Recently, a stochastic model based on the principles of the previous method was developed and applied. This model allows us to consider the uncertainty around the estimated parameters (21).

Figure 7 shows the human salmonellosis incidence associated with the three major sources of human salmonellosis in Denmark from 1988 to 2001. The year that a control program was launched for a specific food animal production system is also indicated. The control programs have been successful in achieving the main objective, a reduction of the incidence of human salmonellosis. The broiler-associated salmonellosis incidence (cases/100,000) has been reduced by >95.0, from 30.8 in 1988 to 0.5 in 2001; the pork-associated salmonellosis incidence has been reduced by >85, from 22.0 in 1993 to 3.0 in 2001; and the egg-associated salmonellosis incidence has been reduced by nearly 75, from 57.7 in 1997 to 15.5 in 2001. Trends in the animal and food-specific disease incidence estimates show a high degree of agreement with the trends in prevalence of *Salmonella* in specific food animals and the corresponding animal-derived food products. These trends serve as an indirect validation of the estimates because these estimates do not rely on prevalence data.

Economy of *Salmonella* Control

Costs of *Salmonella* Control

The Audit Office of Denmark has evaluated the government spending in relation to the national *Salmonella* control efforts (22). From 1994 to 1999, the control program for broiler chickens and layer hens involved government finances of a total of 188.1 million Danish kroner (DKK) (U.S.$26.5 million) (U.S.$1.00 = 7.1 DKK). A total of 109.7 million DKK (U.S.$15.45 million) was paid to compensate farmers for destroyed animals; most of the remaining costs were associated with establishing and running the surveillance program. These costs were highest in the initial phase of the layer hen control program in 1997 but

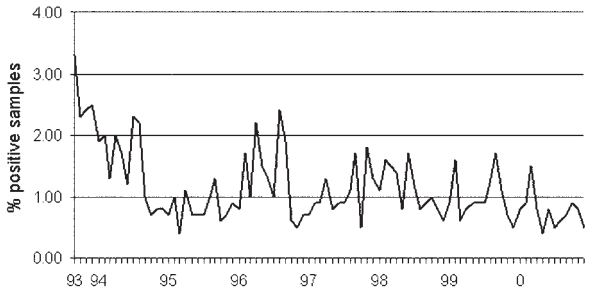

Figure 6. *Salmonella* detected in pork, as determined by continuous randomized sampling of pork end-products from all major national pig slaughterhouses (N >30,000 samples/year).

have been reduced considerably since then. In 2001, all costs associated with running the program were assumed by the poultry industry (with the exception of governments' compensation for flocks destroyed according to the European Union Zoonosis Directive). The current control costs for layer hens and broiler chickens are estimated to be in the range of US$4.2 million per year (for 344 broiler producers, producing 135 million broiler chickens year, and 392 shell-egg producers, producing 1 billion shell-eggs per year).

In the initial phase, the *Salmonella* control program of pigs and pork cost the industry and government a total of U.S.$14 million per year. With the recent revision of the program, the responsibility has been taken over solely by the industry, and operational costs have been reduced to approximately U.S.$8.5 million per year (for 21,000 producers, producing 21–22 million slaughter pigs each year) (B. Nielsen, pers. comm.).

Public Health Economy

Direct health costs (e.g., hospitalization, consulting a physician, and laboratory testing) as well as the costs of lost labor (e.g., loss of production per day away from work) in relation to a case of salmonellosis in Denmark were evaluated as part of a multidisciplinary task force (Korsgaard and Wegener, pers. comm.). For 2001, foodborne salmonellosis cost the Danish U.S.$15.5 million. The estimate is based on an incidence of 54.6 cases per 100,000, and approximately 10% of cases are laboratory confirmed. Assuming that 5% or 20% of cases are laboratory-confirmed changes the estimate to U.S.$25.5 million and U.S.$10.4 million, respectively.

Costs and Benefits

Assuming that salmonellosis associated with each of the major sources would have remained at the precontrol program incidence (and not increased further) (i.e., if no action had been taken to curb the problem), we calculated a hypothetical "no-control" salmonellosis incidence. This incidence would have been 137.5 (pork 22, broiler chickens 30.8, eggs 57.7, and average residual, 27). The societal costs, in the absence of the existing control programs, would thus have been U.S.$41 million per year (assuming 10% of cases are laboratory confirmed). Thus, in 2001, Denmark saved U.S.$25.5 million by controlling *Salmonella.* The estimated annual *Salmonella* control costs from 2000 and onwards are approximately U.S.$14.1 million. These costs are borne almost exclusively by the animal producers and the food industry, which suggests that the costs are passed on to consumers through higher food prices. Based on the figures above and data on annual production (23), control costs amount to approximately U.S.$0.075/kg of pork and U.S.$0.02/kg of broiler or egg.

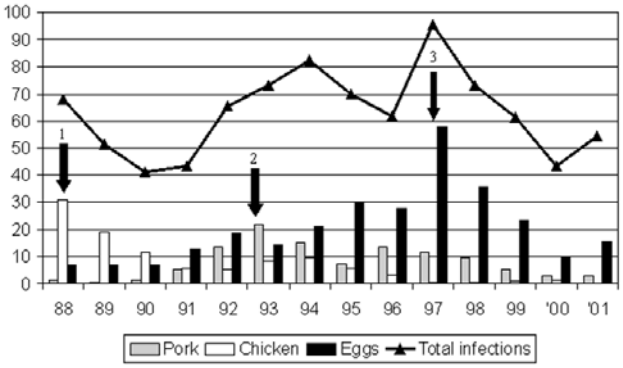

Figure 7. Effects of *Salmonella* control programs as indicated by incidence of human infection attributable to the different major sources of human salmonellosis in Denmark; 1) *Salmonella* control program for broiler chickens implemented, 2) *Salmonella* control program implemented for pigs and pork, 3) *Salmonella* control program implemented for layer hens and eggs The three sources account for approximately 50% to 75% of *Salmonella* each year. Remaining cases are attributable to beef, imported food products, infections acquired while traveling abroad, and unknown sources. Incidence (cases/100.000 inhabitants).

Discussion

Danish *Salmonella* control efforts have been successful in achieving their objective; reduction of human salmonellosis. These efforts illustrate that with a focused and integrated programs, including a strong element of preharvest control, and based on a public-private partnership, *Salmonella* can be reduced. At the same time, the industries involved have remained profitable and internationally competitive (approximately 75% of the chicken products and 85% of the pork are exported).

Initially, the programs have received some government funding, primarily for research, development, and compensation for destroyed animals. After the initial implementation and clean-up phase of the programs, the responsibility for running and funding the programs have been nearly completely taken over by the industries involved. The government, however, maintains access to all relevant information and data through a central database managed by the Danish Zoonosis Centre, and food safety objectives continue to be determined by the Danish government.

A proactive and collaborative approach to food safety by food industry and government ensures consumers' confidence in the domestic food production. For example, when the bovine spongiform encephalopathy (BSE) crisis hit Europe, the beef industry in most countries was adversely affected by reduced consumer demand. In Denmark, the sale of beef remained nearly unaffected by the crisis even after the first positive BSE findings occurred in Danish cattle. These steady beef sales are likely attributable to a high degree of consumer confidence in the public and private control systems.

The success of the programs supports the effectiveness of a preharvest control approach to *Salmonella*. Monitoring and intervention at the farm and in the food animal breeding systems are feasible means to achieve lasting control of the *Salmonella* problem. Development and application of a two-tiered detection system based on a combination of serologic testing and bacteriologic confirmation have been essential for the success of the programs. Serologic testing enables semi-automated mass screening of animals and eggs at a low price and with good, and in some cases superior, sensitivity. Bacteriologic testing serves to compensate for the sub-optimal specificity of a serologic-based monitoring system. The programs could not have been operated solely on the basis of bacteriologic testing because of the higher costs involved and logistical problems (i.e., screening nearly 2 million samples per year by bacteriologic testing is unrealistic). Preharvest control tools, such as vaccines, antibiotics, or competitive exclusion, are not used to control *Salmonella* in Denmark; these tools might be counterproductive, as they mask the *Salmonella* problem rather than aid in its reduction or eradication.

Evaluating the costs and benefits of the national *Salmonella* control efforts is difficult; estimating the public health and societal costs in the absence of the control program is impossible. However, this conservative estimate suggests that efforts have been cost beneficial and those benefits are likely to increase with time.

Dr. Wegener is a professor of zoonoses epidemiology and head of the Danish Zoonosis Center. His main research interests are the epidemiology of foodborne zoonosis and antimicrobial resistant bacterial in the food chain. He is involved in the coordination of the World Health Organization Global Salmonella Surveillance Program.

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Disease Surveillance and the Academic, Clinical, and Public Health Communities

Robert W. Pinner,* Catherine A. Rebmann,* Anne Schuchat,* and James M. Hughes*

The Emerging Infections Programs (EIPs), a population-based network involving 10 state health departments and the Centers for Disease Control and Prevention, complement and support local, regional, and national surveillance and research efforts. EIPs depend on collaboration between public health agencies and clinical and academic institutions to perform active, population-based surveillance for infectious diseases; conduct applied epidemiologic and laboratory research; implement and evaluate pilot prevention and intervention projects; and provide capacity for flexible public health response. Recent EIP work has included monitoring the impact of a new conjugate vaccine on the epidemiology of invasive pneumococcal disease, providing the evidence base used to derive new recommendations to prevent neonatal group B streptococcal disease, measuring the impact of foodborne diseases in the United States, and developing a systematic, integrated laboratory and epidemiologic method for syndrome-based surveillance.

During the 1980s, clinicians added newly recognized infectious diseases, such as toxic shock syndrome and AIDS, to their differential diagnoses when evaluating previously healthy young adults with severe illness. More recently, clinicians in the United States found themselves considering the possibility of inhalational anthrax among patients with influenzalike illnesses and adding West Nile virus infection to their workup of posttransfusion fevers (1–3). The existence of these and dozens of other emerging and reemerging infectious diseases, naturally or intentionally transmitted, has removed any doubt about the interdependence of clinical medicine and public health. Clinicians are sentinels for detection of new or reemerging diseases and may benefit from information acquired through public health surveillance and research projects, which helps to place the quantitative risks of these new diseases in perspective amidst the media attention that often accompanies the latest medical mysteries.

In 1992, the Institute of Medicine (IOM) articulated the concept of emerging infections, discarding the naive view that infectious diseases were problems of the past and cautioning against complacency about public health preparedness for infectious diseases (4). By defining emerging infectious diseases as "new, reemerging, or drug-resistant infections whose incidence in humans has increased within the past two decades or whose incidence threatens to increase in the near future," IOM recognized the broad scope of these diseases. The IOM report also cited factors that influence the emergence of infectious diseases: changes in human demographics and behavior; advances in technology and changes in industry practices; economic development and change in land-use patterns; increased volume and speed of international travel and commerce; microbial adaptation and change; and breakdown of public health capacity at the local, national, and global levels. The intentional release of anthrax in the United States in 2001 emphasized the need to add intentionally inflicted harm to the list of factors that influence the emergence of infectious diseases and to suspect the unexpected.

In response to the IOM report, Addressing Emerging Infectious Disease Threats to Health: A Prevention Strategy for the United States was developed by the Centers for Disease Control and Prevention (CDC) (5). A key recommendation of the plan called for establishing population-based centers to complement and support local, regional, and national surveillance and research efforts. This concept was realized through Emerging Infections Programs (EIPs), a network of state health departments (Figure 1) coordinated by CDC. EIPs are intended to be a national resource for surveillance and epidemiologic research by conducting work that goes beyond the routine public health department functions; by fostering collaborations between the public health, academic, and clinical communities; and by maintaining an infrastructure flexible enough to address new infectious diseases challenges as they emerge. An updated plan released in 1998 described the important role assumed by EIPs in addressing emerging infections and identified several high-priority target areas (6), which include: antimicrobial drug resistance, foodborne and waterborne diseases, vector-borne and zoonotic diseases, chronic diseases caused by infectious

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Figure 1. Distribution of Emerging Infections Programs (EIPs), a network of 10 state health departments and their collaborators in local health departments, academic institutions, and clinical settings, coordinated by the Centers for Disease Control and Prevention. *New Mexico was added as the 10th EIP site in late 2002 and will begin EIP activities during 2003.

agents, diseases transmitted through blood transfusions or products, vaccine development and use, diseases of pregnant women and newborns, diseases of persons with impaired host defenses, and diseases of travelers, immigrants, and refugees. We describe EIP accomplishments and future directions.

EIP Methods

The principal functions of EIPs are to perform active, population-based surveillance for infectious diseases; conduct applied epidemiologic and laboratory research; implement and evaluate pilot prevention and intervention projects; and provide capacity for flexible public health response. EIPs also develop and evaluate public health practice and transfer what is learned to the public health and medical communities.

These programs are supported through cooperative agreements between CDC and state health departments, who engage collaborators in local health departments, hospitals, and academic institutions. Additional funding for certain EIP activities comes from other sources; for example, the U.S. Department of Agriculture and the Food and Drug Administration provide support for activities involving foodborne illnesses, and the National Vaccine Program Office has provided support for postlicensure vaccine evaluations.

The population base for EIP activities is approximately 36 million persons, though the base varies by project. This population represents an approximation of the U.S. population with respect to demographic characteristics such as age, gender, race, and urban residence, as well as health indicators such as population density and percentage of persons at or below the poverty level (7). EIPs are geographically dispersed throughout the country (Figure 1).

Active, laboratory-based surveillance is the foundation of two core EIP projects conducted at all sites: Active Bacterial Core Surveillance (ABCs) and Foodborne Disease Active Surveillance (FoodNet) (Table 1). These active surveillance projects generate reliable estimates of the incidence of certain infections and provide the foundation for a variety of epidemiologic studies to explore risk factors, disease spectrum, and prevention strategies (8,9). For example, the total impact of foodborne illnesses in the United States has been estimated by combining FoodNet active surveillance data with other data sources and results from FoodNet surveys of the general population (to learn about the frequency of diarrhea in the general population and to determine what proportion of persons with diarrhea seeks medical care), physicians (to determine the frequency of stool-culturing by physicians), and clinical laboratories (to determine the frequency of culturing for selected foodborne pathogens) (9–11). These data provide estimates of the overall occurrence of diarrheal illness (0.7 illnesses/person-year), as well as the likely degree of underreporting for specific infections under surveillance (10).

Other projects are conducted by EIPs, depending on local priorities and expertise. The Unexplained Deaths and Critical Illness (UNEX) project, a prospective study that uses epidemiologic and laboratory methods to detect and investigate unexplained illnesses with clinical features suggesting infectious diseases, has been in place at four states with EIPs since the inception of the program (12,13). The Connecticut EIP conducts active surveillance for emerging tick-borne diseases that are transmitted by a single tick vector (*Ixodes scapularis*) in the state (14). EIPs also strive to maintain the flexibility to meet new challenges effectively. For example, in 1996 four EIP sites conducted active surveillance for variant Creutzfeldt-Jakob

Table 1. Surveillance and focus area for two core projects conducted at all Emerging Infections Program sites[a]

Projects	Type of surveillance	Focus
Active Bacterial Core Surveillance	Active, laboratory-based	Invasive disease (isolated from a normally sterile site such as blood or cerebrospinal fluid) caused by group A streptococcus, group B streptococcus, *Haemophilus influenzae, Neisseria meningitidis,* and *Streptococcus pneumoniae*
FoodNet/Foodborne Disease Active Surveillance	Active, laboratory-based	Disease (first isolation from a person) caused by *Campylobacter, Listeria, Salmonella, Shigella, Yersinia, Vibrio,* Shiga toxin–producing *Escherichia coli*, including O157:H7, *Cryptosporidium,* and *Cyclospora*

[a]Intended to generate reliable estimates of the incidence of certain infections and provide the foundation for a variety of epidemiologic studies to explore risk factors, disease spectrum, and prevention strategies.

Disease (CJD) and physician-diagnosed CJD cases. This study contributed to surveillance methods by confirming that death certificate reviews are a sensitive method for detecting CJD deaths while providing some assurance that variant CJD was not occurring in these states (15).

Impact of a New Pneumococcal Vaccine

Through ABCs, we are evaluating the effect of the pneumococcal conjugate vaccine on the epidemiology of invasive pneumococcal disease in the United States. *Streptococcus pneumoniae* (pneumococcus), which is an important cause of serious illness among young children, is the leading cause of bacterial pneumonia and meningitis in the United States. For many years, immunization against pneumococcus with a 23-valent polysaccharide vaccine was recommended for persons ≥2 years of age who are at high risk and for all adults ≥65 years of age. Although disease incidence is highest in the first 2 years of life, the polysaccharide vaccine was poorly immunogenic in this group. In February 2000, a protein-polysaccharide pneumococcal conjugate vaccine for seven pneumococcal serotypes (Prevnar, Wyeth Pharmaceuticals, Pearl River, NY) was licensed for use in infants and children (16). This conjugate vaccine is now recommended in the United States for all children <2 years of age, with catch-up vaccination schedules suggested for children 2 to 4 years of age. In clinical trials, the vaccine was efficacious against invasive disease in infancy and reduced nasopharyngeal colonization by vaccine-type strains, an indication of potential for herd immunity.

One method used by ABCs is to collect available isolates from identified cases. Serotyping data were analyzed to learn about the epidemiology of *S. pneumoniae* in the pre-conjugate vaccine era and to predict the potential impact of the conjugate vaccine (17). Of pneumococcal cases identified by ABCs from 1995 to 1998, at least 82% in children <2 years of age were caused by serotypes included in the 7-valent pneumococcal conjugate vaccine. These population-based ABCs data were used to formulate the original pneumococcal conjugate vaccine schedules and provide recommendations for administering the vaccine to infants and children. When a vaccine shortage became evident in 2001, ABCs data were again used by public health officials to weigh alternative strategies for delivering available doses (18). Surveillance is now focused on evaluating changes in disease impact after the conjugate vaccine was introduced, including whether it interrupts transmission of antibiotic-resistant pneumococci. Analysis of ABCs data shows a substantial decline in disease caused by serotypes in the vaccine formulation among children in the age group for whom the vaccine is recommended. More modest declines also occur in selected adult groups (19).

ABCs will continue to evaluate the impact of the recently introduced pneumococcal conjugate vaccine, including whether vaccine shortages have slowed the initial steep decline in disease occurrence. Other goals are measurement of vaccine efficacy, assessment of whether the vaccine is interrupting transmission, and evaluation of the distribution of serotypes causing disease (to determine if decline in disease because of serotypes included in the vaccine has been counterbalanced by emergence of invasive disease caused by nonvaccine serotypes). While this "replacement disease" phenomenon was recognized for otitis media and colonization in the prelicensure vaccine trials, no evidence of replacement invasive disease has thus far been recognized.

Clinicians were challenged by the emergence of multidrug-resistant pneumococci during the 1990s, when new treatment guidelines were developed for meningitis, otitis media, and pneumonia (20). Vaccines, in concert with campaigns to promote appropriate use of antibiotics, provide opportunities to transform the problem of drug-resistant pneumococci from a treatment dilemma to a prevention success story (21).

Revised Recommendations for Preventing Perinatal Group B Streptococcal Disease

Data developed through ABCs provided a basis for revising recommendations for the prevention of perinatal group B streptococcal (GBS) disease. Since its emergence in the 1970s, GBS disease has been the leading invasive bacterial infection associated with illness and death among newborns in the United States. Surviving infants may have long-term developmental disabilities, such as mental retardation or hearing and vision loss. Newborns at increased risk for GBS disease are those born to women who are colonized with GBS in the genital or rectal areas. Although the use of intrapartum prophylaxis has led to a 70% decline in the incidence of GBS disease during the 1990s (Figure 2) (22,23), early-onset GBS disease (in infants <7 days old) remains a leading cause of illness and death among newborns. Guidelines issued in 1996 recommended either screening pregnant women for GBS colonization by means of prenatal cultures (screening approach) or assessing obstetric risk factors intrapartum (risk-based approach) to identify candidates for intrapartum antibiotic prophylaxis.

An EIP population-based, retrospective cohort study compared the effectiveness of prenatal screening for GBS with the risk-based approach for preventing early-onset GBS sepsis (24). The analysis, which combined ABCs population-based active surveillance data on GBS cases with a sample survey representing >600,000 deliveries, showed that infants born to women who had been screened for GBS before delivering had less than half the risk for

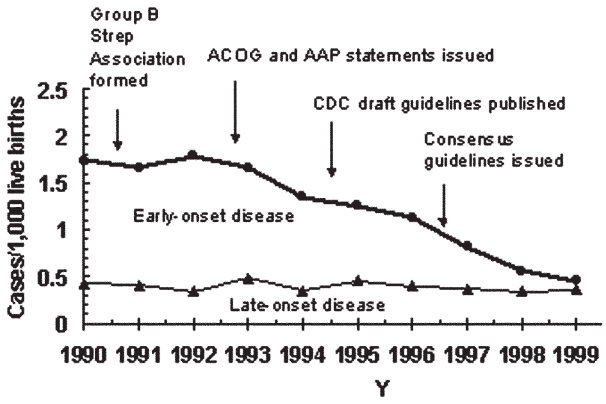

Figure 2. Incidence of early- and late-onset invasive group B streptococcal disease in three active surveillance areas (California, Georgia, and Tennessee), 1990–1998, and activities for the prevention of group B streptococcal disease (22). CDC, Centers for Disease Control and Prevention. ACOG, American College of Obstetricians and Gynecologists; AAP, American Academy of Pediatrics.

early-onset GBS compared to infants of women who had not been screened, after adjustments were made for potential confounders. The protective effect of the screening approach resulted mainly from broader coverage of the population at risk because many early-onset GBS cases in the preprevention era occurred in GBS-colonized women without obstetric risk factors. The evidence for updated prevention recommendations from key health organizations (i.e., American College of Obstetricians and Gynecologists, American Academy of Pediatrics, American College of Nurse-Midwives, and CDC) was based on the finding that routine screening for GBS during pregnancy more effectively prevents cases of early-onset disease than the risk-based approach (25). Through ABCs, CDC will continue to monitor GBS disease trends to understand the impact of the new recommendations and detect potential adverse consequences of intrapartum antibiotic use such as emergence of sepsis caused by other organisms or new patterns of antimicrobial resistance (26,27).

Decrease in Bacterial Foodborne Diseases

FoodNet documented a decrease in bacterial foodborne illnesses from 1996 to 2001. Many infections are transmitted through food and can cause illness ranging from mild gastroenteritis to severe illness requiring hospitalization. Foodborne pathogens cause an estimated 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year (11). Clinicians treating patients with acute gastroenteritis are principally focused on whether empiric antimicrobial agents are warranted and the value of diagnostic evaluation. However, the task of providing accurate information on trends in specific foodborne pathogens capable of causing this syndrome, as well as probable sources of infection, has historically fallen to public health authorities.

Data from FoodNet documented recent declines in the occurrence of several major bacterial foodborne illnesses (9,28); preliminary surveillance data for 2001 were compared with 1996–2000 data (28). Significant declines occurred in major bacterial foodborne illnesses, including infections caused by *Yersinia* (49%), *Listeria* (35%), *Campylobacter* (27%), and *Salmonella* (15%) (Figure 3). The combined estimated incidence of infections caused by *Listeria*, *Campylobacter*, *Salmonella*, and *E. coli* O157 in 2001 was 21% lower than in 1996, on the basis of a multivariate regression model.

The factors influencing the occurrence of foodborne illnesses are complex. However, the observed declines in foodborne disease incidence did occur in the context of several control measures, including the U.S. Department of Agriculture's Food Safety Inspection Service's implementation of the Pathogen Reduction/Hazard Analysis and Critical Control Point regulations in meat and poultry slaughter and processing plants, egg-quality assurance programs for *Salmonella* Enteritidis, and increased consumer education in food safety (28).

FoodNet will continue to monitor the occurrence of foodborne diseases. In 2003, FoodNet will also conduct studies of the consequences of and risk factors for illness caused by *S.* Enteritidis, *S.* Newport, and illness in infants caused by *Campylobacter* and *Salmonella*. Other activities include a project to improve collection and transport of specimens during outbreaks so that a cause is identified in a higher percentage of outbreaks.

Rapid identification of a cause for cases of infectious diarrhea and appropriate reporting of cases of foodborne illnesses to state or local public health authorities are important not only in identifying and controlling outbreaks but also for more precise assessments of the local, region-

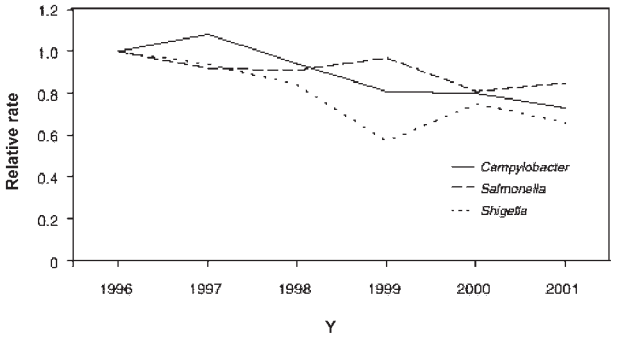

Figure 3. Relative rates compared with 1996, adjusted for sites, of laboratory-diagnosed cases of *Campylobacter*, *Salmonella*, and *Shigella*, by year, FoodNet, United States, 1996–2001 (28). Bacterial pathogens with highest incidences of the 10 studied diseases are shown.

al, and national trends in foodborne illnesses (29). In turn, such estimates can inform clinicians of likely causes, probable sources, and prognostic factors for episodes of illness in persons under their care.

Unexplained Deaths and Critical Illnesses Project

Many clinicians have treated patients with puzzling situations, in which the acute onset of a critical illness suggestive of an infectious origin occurred in otherwise healthy young people for whom diagnostic tests failed to identify an etiologic agent. Occasionally, such episodes are retrospectively diagnosed many years later with the recognition of a new infectious disease and testing of stored clinical specimens. For example, hantavirus pulmonary syndrome was first recognized and described in the United States in 1993 by an alert clinician during an outbreak in the Southwest (30); retrospective reviews of fatal illnesses showed that unrecognized cases of hantavirus pulmonary syndrome had preceded the 1993 outbreak by at least 15 years (31). Similarly, cases of legionellosis and AIDS were recognized in hindsight years after they had occurred (13). These observations, coupled with the new laboratory techniques for pathogen identification, particularly methods that do not rely on culture, suggested that an effort to prospectively identify pathogens causing unexplained syndromes might yield useful information (12,13); this was the beginning of the UNEX project. Laboratory evaluation of cases includes traditional serologic and in vitro culture diagnostic methods as well as molecular techniques. This combined epidemiologic and laboratory approach is a hallmark feature of other EIP projects that study hepatitis, acute respiratory diseases, and encephalitis (32).

The UNEX project has developed methods for evaluating severe syndromes indicating infection, including non-culture-based methods to identify etiologic agents. From May 1, 1995, to December 31, 1998, 137 illnesses meeting the UNEX case definition were reported to participating EIPs. After adjustments for age and race were made, this number translates to an estimated 920 U.S. cases per year; the overall annual incidence rates did not change during this time. No differences were observed in the seasonal distribution of cases of unexplained illnesses, nor did cases cluster by time or place. The largest proportion of cases was treated as a neurologic syndrome (29%), followed by respiratory (27%) and cardiac (21%) syndromes. Diagnostic testing through UNEX identified a cause in 34 (28%) of 122 cases from which specimens were available (Table 2).

Two recent outbreaks demonstrate the usefulness of the approach developed for UNEX. During a 1999 outbreak of

Table 2. Infectious causes and explanations for unexplained deaths and critical illnesses cases, 1995–1998, California, Oregon, Connecticut, and Minnesota (n=34)[a,b]

Syndrome	Etiologic agent (n)	Tests (n)
Neurologic (n=15)	*Neisseria meningitidis* (4)	16S rDNA PCR (2), PCR (1), EIA IgM (1)[a]
	Bartonella hensaelae (1)	PCR, IFA, IgG
	Bartonella spp. (2)	IFA, IgG
	Chlamydia pneumoniae (1)	MIF, IgG
	Mycoplasma pneumoniae (1)	EIA, IgM/IgG
	Cytomegalovirus (1)	EIA and IFA,IgG
	Coxsackie B virus (1)	EIA, IgM, viral culture
	Enterovirus (1)	EIA, IgM
	Epstein-Barr virus (1)	IFA, IgG (VCA and EA)
	Human herpesvirus 6 (1)	IFA and EIA (IgM and IgG)
	Mumps virus (1)	IFA IgM, IFA and EIA, IgG
Respiratory (n=13)	*Chlamydia pneumoniae* (2)	MIF IgG (2), IFA, IgM
	Mycoplasma pneumoniae (4)	PCR (blood), EIA, IgM/IgG
	Streptococcus pneumoniae (2)	16S rDNA PCR (pleural fluid)
	Legionella spp. (1)	PCR (from lung)
	Adenovirus (1)	EIA and IFA, IgG
	Influenza B virus (1)	EIA and IFA, IgG
	Influenza A virus (1)	EIA and IFA, IgM, EIA (IgG)
	Human parainfluenza virus types 1 and 3 (1)	EIA and IFA, IgG
Cardiac (n=3)	*Borrelia burgdorferi*/*Ehrlichia chaffeensis* (1)	EIA/IFA flagella, IgG, Western blot (IgG and IgM)
	Enterovirus (1)	EIA IgM
	Legionella spp. (1)	PCR (heart)
Multisystem (n=3)	*Neisseria meningitidis* (1)	PCR (cerebrospinal fluid)
	Adenovirus (1)	PCR (blood)
	Enterovirus (1)	IgM, EIA

[a] PCR, polymerase chain reaction; EIA, enzyme immunosorbent assay; IFA, indirect immunofluorescent assay; Ig, immunoglobulin; EA, early antigens; VCA, viral capsid antigens; MIF, microimmunofluorescence.
[b]Reference 12.

West Nile encephalitis in the northeastern United States, which was recognized by an alert clinician (33), and during an outbreak of unexplained illness among injecting drug users in Scotland and Ireland (34), initial reports of illness were received and initial laboratory testing performed through the laboratory infrastructure established for the UNEX project.

The frequency and distribution of the syndromes identified through this project undoubtedly reflect both the distribution of their occurrence and gaps in our ability to diagnose causes of neurologic and respiratory syndromes in particular. Although novel pathogens have not yet been discovered through the UNEX project, this systematic approach improves chances of recognizing infectious disease causes earlier than in the past and lays the groundwork for the development of improved diagnostic tools. Moreover, concerns about bioterrorism have put a premium on the early detection of an intentional release or infectious or chemical agents; this syndrome-based surveillance, which seeks early identification and diagnosis, can contribute to public health preparedness for such events.

Future Directions of EIPs

Since the release of the plan that launched the EIPs, these programs have made substantial contributions to the practice of U.S. public health. Using domestic EIPs as a model, CDC has begun developing a network of international EIPs (IEIPs) in collaboration with Ministries of Health and other international partners. The first IEIP was established in Thailand during 2001, and a second IEIP is being established in Kenya. Collaborations between EIPs and IEIPs will provide valuable opportunities for training. In addition, the new U.S. EIP in New Mexico will feature work along the U.S.-Mexico border and also promises to enhance international collaborations.

Opportunities presented by new laboratory and information technologies, as well as challenges posed by potential bioterrorism, will influence the evolution of the EIPs over the next several years. EIP work will build on experience gained through the combined epidemiologic and laboratory evaluation of syndromes to enhance bioterrorism preparedness and develop the capacity for identifying previously unrecognized pathogens. However, even as new technologies are found, knowledgeable and engaged clinicians will remain a vital element in efforts to detect, respond to, and prevent emerging infectious diseases.

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Acute Flaccid Paralysis and West Nile Virus Infection

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Acute weakness associated with West Nile virus (WNV) infection has previously been attributed to a peripheral demyelinating process (Guillain-Barré syndrome); however, the exact etiology of this acute flaccid paralysis has not been systematically assessed. To thoroughly describe the clinical, laboratory, and electrodiagnostic features of this paralysis syndrome, we evaluated acute flaccid paralysis that developed in seven patients in the setting of acute WNV infection, consecutively identified in four hospitals in St. Tammany Parish and New Orleans, Louisiana, and Jackson, Mississippi. All patients had acute onset of asymmetric weakness and areflexia but no sensory abnormalities. Clinical and electrodiagnostic data suggested the involvement of spinal anterior horn cells, resulting in a poliomyelitis-like syndrome. In areas in which transmission is occurring, WNV infection should be considered in patients with acute flaccid paralysis. Recognition that such weakness may be of spinal origin may prevent inappropriate treatment and diagnostic testing.

Most human infections with West Nile virus (WNV), a flavivirus within the Japanese encephalitis virus antigenic complex, are clinically inapparent (1,2). Mild febrile illness develops in approximately 1 in 5 infected persons; more severe neurologic disease, mostly meningitis or encephalitis, occurs in and 1 in 150 (1–4). Less frequently, acute WNV infection has been associated with acute flaccid paralysis, which has been attributed to Guillain-Barré syndrome, motor axonopathy, or axonal polyneuropathy (4–6). However, these reports describe clinical and laboratory features that seem inconsistent with such diagnoses, and the exact cause of acute flaccid paralysis has not been thoroughly assessed with rigorous electrophysiologic, laboratory, and neuroimaging data. Brief descriptions of six patients have suggested that this flaccid paralysis is due to anterior horn cell involvement with a resultant poliomyelitis-like syndrome (7–9). Because understanding the clinical characteristics and underlying etiology of WNV-induced acute flaccid paralysis is critical for therapeutic decisions as well as prognosis, we describe the detailed clinical, laboratory, and electrophysiologic findings from these six patients and from one additional patient.

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Patients and Methods

Seven patients were detected through WNV surveillance conducted by the Mississippi Department of Health and the Louisiana Office of Public Health. For each patient, a standardized questionnaire, including demographics, medical history, initial signs and symptoms, risk factors, and treatment, was completed; a standardized neurologic examination was performed by a single neurologist (JJS). Electrodiagnostic studies were performed by neurologists (AAL and JAVG) specializing in electrodiagnostic medicine.

Cerebrospinal fluid (CSF) and acute- or convalescent-phase serum specimens (or both) from each patient were tested for antibody to WNV by immunoglobulin (Ig) M antibody-capture enzyme immunoassay (10) or plaque reduction neutralization assay (11). The initial specimen for one patient (patient 5, Table 1) was tested with a slightly modified IgM antibody assay at a commercial laboratory (12). IgM assays were considered positive if the optical density ratio of the patient and negative control samples (P/N ratio) was greater than three. For patient samples, a P/N ratio for WNV at least three times that for St. Louis encephalitis virus indicated WNV infection (13). A plaque reduction neutralization test result of at least 10 was considered positive.

All seven patients had serologic evidence of WNV infection (Table 1). On the basis of serologic data, three of the patients were classified as confirmed case-patients (patients 4, 6, and 7) and four as probable case-patients (patients 1–3, 5), according to the national case definition (14).

Case 1

On July 1, 2002, a previously healthy, 56-year-old, male Mississippi resident was hospitalized with a 1-week

Table 1. Serologic results for West Nile virus (WNV)–specific antibodies in patients with acute flaccid paralysis associated with acute WNV infection[a]

				IgM-capture enzyme immunoassay		Plaque reduction neutralization assay	
Case no.	Onset	Collection	Sample	SLEV	WNV	SLEV	WNV
1	6/24	7/12	Serum	3.5	22.3	320	5,120
2	7/12	7/16	Serum	8.0	22.7	80	1,280
3	7/26	8/1	Serum	2.79	24.9	<10	640
4	7/29	8/3	Serum	1.1	14.1	<10	80
4	7/29	8/3	CSF	3.3	39.2		
4	7/29	8/13	Serum	4.4	23.5	40	2,560
5	8/11	8/15	Serum		2.02		
5	8/11	8/29	Serum	3.4	25.7		
6	8/13	8/16	CSF	6.1	23.8		
6	8/13	8/16	Serum	1.0	5.7	<10	40
7	9/1	10/24	Serum	2.8	10.6	10	320
7	9/1	9/6	CSF	Not performed	7.4		

[a]IgM, immunoglobulin M; SLEV, Saint Louis encephalitis virus; CSF, cerebrospinal fluid.

history of fever, chills, night sweats, myalgias, and acute encephalopathy. Neurologic examination showed profound weakness in both arms, asymmetric weakness in the legs with a right foot drop, and acute respiratory distress (Table 2). Sensory test results were normal. Although computed tomography (CT) and magnetic resonance imaging (MRI) of the brain showed normal results, heparin was administered for suspected evolving stroke. Admission laboratory values (Table 3) showed serum leukocytosis, and cerebrospinal fluid (CSF) obtained on day 3 showed elevated protein. By day 8, Guillain-Barré syndrome was suspected, and intravenous immunoglobulin (IVIG) was administered. Electrodiagnostic studies performed that day were interpreted as showing a proximal neuropathy or myopathy. A deltoid muscle biopsy for suspected inflammatory myopathy showed mild type 2 fiber atrophy but no obvious necrosis or marked inflammatory response. On day 30, the patient was transferred for rehabilitation with flaccid, areflexic paralysis in the right leg and variable weakness and diminished reflexes in all other limbs. Neck flexors were normal in strength, and sensation was preserved in all limbs; loss of bladder function was evident. MRI of the cervical spine showed normal results. Electrodiagnostic studies showed widespread but variable denervation, reduced compound muscle action potentials (CMAPs), and normal sensory nerve action potentials (SNAPs), consistent with a severe, asymmetric process affecting anterior horn cells or motor axons. Myopathy, demyelinating polyneuropathy, and diffuse axonal polyneuropathy were not evident.

Case 2

On July 15, 2002, a 57-year-old male Mississippi resident with a remote history of prostate cancer and glucose intolerance was hospitalized with a 3-day history of fever, chills, nausea, vomiting, and headache. Neurologic examination showed encephalopathy and asymmetric weakness in all limbs (Table 2). Results of a brain MRI weree normal. Admission laboratory studies showed a CSF pleocytosis with elevated protein (Table 3). On day 5, acute respiratory distress developed, and the patient required mechanical ventilation. Upon extubation 2 weeks later, the patient had continued extremity weakness and was aspirating fluids; a modified barium swallow study showed oropharyngeal dysphagia. Upon transfer to a rehabilitation center on day 30, the patient had asymmetric weakness in the legs and right arm and moderate weakness in neck flexors and facial muscles. Hypotonia and areflexia were noted in all limbs. Sensation was slightly diminished to vibration and proprioception in toes bilaterally but preserved to light touch, pinprick, and temperature. Sensory testing was normal in the upper limbs. Urinary incontinence was noted.

Table 2. Initial clinical signs and symptoms in patients with acute flaccid paralysis associated with acute West Nile virus infection

Case no.	Fever (≥38.5°C)	Headache	Nuchal rigidity	Altered mental status	Tremor	Distribution of weakness[a]
1	+	+	+	+	+	Upper and lower limbs, R > L
2	+	+	-	+	-	Upper and lower limbs, R > L
3	+	-	-	-	+	Lower limbs, R > L
4	+	+	+	+	+	R upper limb
5	-	+	-	-	-	R upper limb
6	+	+	-	-	+	Lower limbs, R > L
7	+	+	+	-	-	Upper and lower limbs, L > R; bulbar muscles

[a]R, right; L, left.

Table 3. Initial laboratory findings in patients with acute flaccid paralysis associated with acute West Nile virus infection

Case no.	Leukocytes ($x10^3/mm^3$)	Hematocrit (%)	CSF WBC ($/mm^3$)	CSF RBC ($/mm^3$)	CSF protein (mg/dL)	CSF glucose (mg/dL)
1	17.6	38.0	3	1,778	89	54
2	3.6	38.2	2,600	87	204	99
3	11.8	44.4	140	40	234	74
4	9.5	37.8	143	4	116	119
5	7.9	45.6	Not performed	Not performed	Not performed	Not performed
6	13.0	45.4	329	7	75	66
7	10.3	Not performed	182	9	37	79

[a]CSF, cerebrospinal fluid; WBC, leukocyte count; RBC, erythrocyte count.

Electrodiagnostic studies showed widespread denervation, reduced CMAP amplitudes in all nerves of the lower limbs and right upper limb, and normal SNAP responses, consistent with a severe, asymmetric process affecting anterior horn cells or motor axons. Myopathy, demyelinating polyneuropathy, and diffuse axonal polyneuropathy were not apparent.

Case 3

On July 24, 2002, a low-grade fever, nausea, and vomiting, followed by shaking chills and sweats, developed in a 56-year-old male Louisiana resident with a history of hypertension and coronary artery disease. The next day, asymmetric weakness developed in the lower extremities, with no pain or numbness. Upper extremities were normal. No bowel or bladder dysfunction was present. The patient was hospitalized on July 29, and neurologic examination showed a flaccid, areflexic right lower extremity and a weak left lower extremity with diminished reflexes. Results of strength and reflex testing of the upper extremities were normal. Sensory examination results were normal except for a mild decrease in sensitivity to pinprick, temperature, touch, and vibration in a stocking-and-glove distribution (i.e., distal arms and legs). A coarse bilateral upper extremity action tremor was noted. The patient had no headache, neck stiffness, or alteration of mental status (Table 2). Admission laboratory values showed leukocytosis and CSF pleocytosis (Table 3). Results of other diagnostic tests were unremarkable. Postviral demyelination syndrome and viral-induced polyradicultis were considered, and IVIG**,** dexamethasone, and antibacterial and antiviral medications were administered without patient improvement. On day 15, the patient was discharged to a skilled nursing facility for rehabilitation.

MRI of the cervical, thoracic, and lumbosacral spine obtained during rehabilitation was notable for showing mild cervical and lumbosacral spinal stenosis and foraminal restrictions from C3 through C7 and homogeneous enhancement of the nerve roots of the cauda equina consistent with meningitis. Electrodiagnostic studies showed denervation in thoracic and lumbosacral myotomes, with no muscle activation in the right leg and reduced muscle activation in the left leg. CMAPs in the right leg were absent; SNAPs were normal. Electrodiagnostic findings suggested a severe, asymmetric process affecting anterior horn cells or motor axons. Diffuse axonal polyneuropathy was not evident, despite a slight sensory loss in the distal extremities.

Case 4

On August 2, 2002, fever, headache, and neck stiffness developed in a 69-year-old female Louisiana resident with a history of diabetes and degenerative disc disease; the next day acute weakness occurred in the right arm without pain, numbness, or paresthesias. She was hospitalized on August 4. On admission, physical examination documented fever, vomiting, encephalopathy, nuchal rigidity, and a bilateral rash on the lower extremities. Neurologic examination displayed a flaccid and areflexic right arm. Her legs and left arm exhibited normal strength, reflexes, and coordination, with normal sensation in all limbs. A coarse tremor was noted in the chin, left arm, and legs (Table 2). Laboratory findings included CSF pleocytosis (Table 3). Differential diagnoses included meningoencephalitis with associated motor polyradiculopathy and monoplegia secondary to stroke. The patient was treated with antibacterial and antiviral medications. Results of CT and MRI of the brain were normal. MRI of the cervical spine showed multilevel degenerative disc disease. The patient remained lethargic until day 13, when mental status abruptly improved; right arm weakness persisted. On day 19, she was transferred to a rehabilitation facility. Electrodiagnostic studies showed absent CMAPs and profound denervation with no voluntary activation in muscles of the right arm. Scattered denervation was also seen in the other three limbs. SNAPs had borderline amplitudes and conduction velocities bilaterally. The results were most consistent with a severe, asymmetric process affecting anterior horn cells or motor axons. The patient was subsequently transferred back to intensive care because her respiratory function was deteriorating, but she was not intubated. After Guillain-Barré syndrome was diagnosed, she was started on IVIG but had no improvement in weakness.

Case 5

On August 11, 2002, severe nausea, vomiting, headache, and diarrhea in the absence of fever developed in a 50-year-old male Mississippi resident with a history of alcohol abuse; the next day, progressive right arm weakness developed. He was hospitalized on August 14. Neurologic examination on admission showed flaccid paralysis of the right arm and mild weakness of the right leg, with normal sensation in all limbs (Table 2). Laboratory values are shown in Table 3; a lumbar puncture was not performed. Acute stroke was diagnosed, and the patient was treated with heparin. Mental status changes, dysarthria, and dysphagia subsequently developed but resolved. Upon transfer to a rehabilitation center on day 12, the patient had paralysis and areflexia limited to the right arm, with normal sensation and diffuse tremor in all limbs. Brain MRI results were normal; cervical spine MRI displayed mild multilevel foraminal stenosis on the left. Electrodiagnostic studies showed markedly reduced motor responses in the right arm with normal sensory responses, consistent with a severe asymmetric process affecting anterior horn cells.

Case 6

On August 16, 2002, a 46-year-old male Louisiana resident with a history of coronary artery disease was hospitalized with fever, headache, fatigue, and leg weakness of 3 days' duration. He reported no nuchal rigidity or mental status changes, although family members described him as intermittently confused. Neurologic examination showed a plegic and areflexic right leg and mild left leg weakness; sensation was intact throughout. A bilateral tremor of the upper extremities and jaw was noted (Table 2). Laboratory abnormalities included a CSF pleocytosis (Table 3). He was diagnosed with Guillain-Barré syndrome and started on IVIG. Brain CT and MRI results were normal. Results of an enhanced MRI of the spine suggested meningitis involving the conus medullaris and cauda equina. Electrodiagnostic studies performed on day 4 demonstrated early denervation and absent activation in muscles of the right leg and reduced activation of muscles in the right arm. CMAPs and SNAPs in the right arm and leg were normal. These findings were consistent with a severe, asymmetric process affecting anterior horn cells or motor axons. He was transferred to a rehabilitation facility on day 6 with no improvement of weakness.

Case 7

On September 1, 2002, a previously healthy, 39-year-old male Louisiana resident had onset of fever, headache, and nuchal rigidity followed the next day by dysphagia and bilateral arm and leg weakness that was worse on the left. He was hospitalized on September 6 for acute respiratory failure and intubated. Neurologic examination showed normal cognition, asymmetric flaccid paralysis of the left arm and leg with absent reflexes, hyporeflexic weakness of the right arm and leg, and weakness of bulbar muscles (Table 2). A partial supranuclear gaze palsy, cogwheel rigidity, and bilateral Babinski signs were also evident. Admission laboratory findings showed peripheral leukocytosis and CSF pleocytosis (Table 3). Brain MRI showed increased T2 signal in the periaqueductal gray matter, substantia nigra, and trigeminal motor nuclei. Electrodiagnostic studies performed on day 15 showed diffuse denervation in all myotomes, reduced CMAPs (worse on the left), and preserved SNAPs. On day 25, he was transferred to a long-term care facility with no improvement of limb weakness.

Discussion

The clinical and electrodiagnostic findings in these patients with WNV infection suggest involvement of spinal cord gray matter, specifically anterior horn cells, and a resulting acute poliomyelitis-like syndrome. All patients exhibited features typical for polio, including acute flaccid paralysis without paresthesias or sensory loss, marked asymmetric weakness, diminished or absent deep tendon reflexes in the affected limbs, and weakness that developed during an acute infectious process. Other typical features of poliomyelitis included CSF pleocytosis in five of six patients with CSF examination, acute respiratory distress in four, and acute changes in bowel or bladder function in two. In addition, electrodiagnostic findings showed asymmetric muscle denervation, reduced CMAPs, and preserved SNAPs. No patients had evidence of demyelinating polyneuropathy or myopathy. The absence of new sensory abnormalities localizes the disease process to the anterior horn cells or motor axons. Although muscle denervation and reduced CMAP amplitudes do not distinguish loss of anterior horn cells from loss of motor axons (15), these patients' clinical features can be explained only by anterior horn cell disease, since no known infectious processes limited to motor axons produce widespread, asymmetric paralysis without sensory involvement. While MRI signal abnormalities in the anterior spinal cord have been noted in patients with poliomyelitis (16,17), these findings are inconsistent (18,19), and the absence of such changes in our four patients in which imaging was performed does not preclude a diagnosis of a poliomyelitis-like syndrome.

Since immunization has eradicated wild-type poliovirus from the developed world, most cases of paralytic polio-like conditions in the United States have been linked to other RNA viruses, including echoviruses, enteroviruses, and coxsackieviruses (20). Case reports have documented a poliomyelitis-type syndrome associat-

ed with other flaviviruses (21–23), as well as anterior myelitis associated with WNV infection (24).

The assertion that WNV infection involves anterior horn cells and causes a polio-type syndrome has a pathologic basis. The neuropathology of experimental WNV infection in monkeys was most pronounced in the cerebellum, medulla, and the cervical and lumbar regions of the spinal cord (25). Anterior horn cells showed degeneration and neuronal cell death; conversely, no changes were seen in the oligodendroglia or peripheral nerves. Similarly, WNV-infected horses displayed multifocal polioencephalomyelitis, with involvement of the ventral and lateral horns of the thoracic and lumbar spinal cord (26,27). WNV antigen was mainly localized within the gray matter of the spinal cord, with no lesions apparent in peripheral nerves or ganglia. In WNV-infected birds, lesions and viral antigen were most prominent in the cerebellum and the gray matter of the spinal cord (28).

Previous case studies have attributed WNV-associated acute flaccid paralysis to Guillain-Barré syndrome, motor axonopathy, or severe axonal polyneuropathy (4–6). The clinical signs and symptoms and electrodiagnostic findings reported in those cases, and those described here, are most consistent with a polio-like condition, and would be atypical for Guillain-Barré syndrome or other peripheral nerve disorders. Although acute poliomyelitis and polio-like conditions may occasionally simulate Guillain-Barré syndrome (29), our cases had several clinical, laboratory, and electrodiagnostic features that differed from typical Guillain-Barré syndrome (30–32; Table 4).

In Guillain-Barré syndrome, electrodiagnostic findings generally suggest peripheral nerve demyelination or, less commonly, a combined demyelinating and axonal process (30,31). The cases reported here displayed reduced or absent CMAPs with preserved SNAPs, no evidence of demyelination, a neurogenic pattern of recruitment, and widespread denervation; combined with the clinical picture of an asymmetric paralysis, these findings are typical for a polio-like condition and uncommon for Guillain-Barré syndrome. A pure axonal variant of Guillain-Barré syndrome has been described (33) and may be confused with poliomyelitis and polio-like conditions; however, such cases are generally characterized by distally prominent weakness and show subclinical sensory nerve involvement on electrodiagnostic testing. Thus, in the context of WNV infection, electrodiagnostic studies previously interpreted as motor axonal polyneuropathy or motor axonopathy without sensory nerve involvement (4–6) are more suggestive of anterior horn cell loss than of Guillain-Barré syndrome.

Three of the seven patients had acute flaccid paralysis without other findings, suggestive of severe central nervous system involvement caused by WNV infection. Physicians should suspect WNV infection in patients from areas where WNV is being transmitted and who have acute, painless, asymmetric weakness, even if unaccompanied by fever or apparent meningoencephalitis. Diagnostic studies should include testing for WNV-specific IgM antibody in CSF or acute- and convalescent-phase serum samples. In patients from such areas who have acute flaccid paralysis, CSF analysis, thorough electrodiagnostic studies, and spinal imaging should be considered before initiating diagnostic evaluations or therapies directed at Guillain-Barré syndrome, stroke, inflammatory myopathies, or other peripheral inflammatory processes. These therapies are ineffective for polio-like syndromes and can produce serious sequelae (34–37).

Continued surveillance and investigation of WNV-infected patients are needed to fully define the scope of clinical illness and determine the incidence of acute flaccid paralysis. In addition to assessing clinical outcome, the identification of risk factors and the pathologic confirmation of anterior horn cell involvement in patients with WNV-associated acute flaccid paralysis remain important public health goals.

Table 4. Clinical characteristics of patients with West Nile virus–associated acute flaccid paralysis compared with patients with typical Guillain-Barré syndrome (25–27)[a]

Characteristic	West Nile virus–associated flaccid paralysis	Guillain-Barré syndrome
Timing of onset	Acute phase of infection	1–8 weeks after acute infection
Fever and leukocytosis	Present	Absent
Weakness distribution	Asymmetric; occasional monoplegia	Generally symmetric; proximal and distal muscles
Sensory symptoms	Absence of numbness, paresthesias, or sensory loss; occasional myalgias	Painful distal paresthesias and sensory loss
Bowel/bladder involvement	Often present	Rare
Concurrent encephalopathy	Often present	Absent
CSF profile	Pleocytosis and elevated protein	No pleocytosis; elevated protein (albuminocytologic dissociation)
Electrodiagnostic features	Anterior horn cell/motor axon: reduced/absent CMAPs, preserved SNAPs; asymmetric denervation	Demyelination: marked slowing of conduction velocity; conduction block, temporal dispersion; reduced SNAPs

[a]CSF, cerebrospinal fluid; CMAPs, compound muscle action potentials; SNAPSs, sensory nerve action potentials.

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West Nile Virus in Farmed Alligators

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Seven alligators were submitted to the Tifton Veterinary Diagnostic and Investigational Laboratory for necropsy during two epizootics in the fall of 2001 and 2002. The alligators were raised in temperature-controlled buildings and fed a diet of horsemeat supplemented with vitamins and minerals. Histologic findings in the juvenile alligators were multiorgan necrosis, heterophilic granulomas, and heterophilic perivasculitis and were most indicative of septicemia or bacteremia. Histologic findings in a hatchling alligator were random foci of necrosis in multiple organs and mononuclear perivascular encephalitis, indicative of a viral cause. West Nile virus was isolated from submissions in 2002. Reverse transcription-polymerase chain reaction (RT-PCR) results on all submitted case samples were positive for West Nile virus for one of four cases associated with the 2001 epizootic and three of three cases associated with the 2002 epizootic. RT-PCR analysis was positive for West Nile virus in the horsemeat collected during the 2002 outbreak but negative in the horsemeat collected after the outbreak.

West Nile virus (WNV) has been reported in a variety of species but primarily endotherms. Arboviruses have been reported to affect ectotherms, and in some cases ectotherms are thought to serve as a reservoir (1–4). The mode of transmission of the arbovirus to ectotherms has often been presumed to be through ingestion or a bite from the insect carrier (5).

During the fall of 2001 and 2002, two epizootics occurred among captive alligators on a south Georgia alligator farm that houses over 10,000 animals. Approximately 250 alligators died between November and December 2001, and >1,000 alligators died in 2002. These epizootics tended to occur approximately 2 weeks after the first abrupt drop in ambient temperature, which occurred both years in mid-October and was characterized by minimum temperatures between 0°C and 8°C and maximum temperatures between 10°C and 18°C for a period of 1 to 3 days.

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Methods

Animals and Housing

Animals were housed in six barns that were divided into 10 pens; each pen contained approximately 100–200 alligators. The nursery animals are obtained either as eggs from Florida or as hatchlings from onsite breeders.

All pens are cleaned in the morning starting at 6 a.m. An automatic flushing system is used to drain the pens, flush them, and fill them with clean water. Well water is chlorinated with an automated system that injects chloride gas into the water. The water is then piped into a central collecting area and heated. The water temperature is maintained at 32.2°C year-round, and the buildings are kept dark to reduce environmental stress on the animals. The reduced stress and warm environment allow continued growth (i.e., growth of ≥1 m per year rather than 0.30 m per year).

Alligators are fed in the mid- to late afternoon. The diet consists of 95% ground raw horsemeat (obtained frozen from a source in Pennsylvania) to which vitamins and minerals are added in a pelleted alligator diet carrier. The ingredients are thoroughly mixed in a large commercial mixer. The source of the horsemeat has remained constant since 1985. The source of the vitamins and minerals has varied, based upon availability.

The breeding population is maintained in a separate fenced enclosure on the premises. This enclosure is a native swampland and therefore subjected to ambient weather conditions. A rookery was recently established in the breeding area by native birds. Attempts to depopulate the rookery (using U.S. Department of Agriculture–approved methods) have been unsuccessful. The alligators eat fledglings and older birds that fall from the nests and branches or otherwise get within reach. Alligators do not nest under the rookery. No mosquito control is practiced on the farm.

Tissue Collection

Animals were seen moribund or dead upon arrival at the laboratory. Blood was collected from the occipital

sinus or caudal vein of live animals. Gross observations were made, and the animals were humanely euthanized. Tissues were collected from the eye, thyroid gland, lymph node, lung, heart, brain, spinal cord, kidney, liver, spleen, pancreas, adrenal gland, gallbladder, tonsil, trachea, stomach, intestines, and reproductive tract. Fresh tissue specimens were submitted for virus isolation, reverse transcription-polymerase chain reaction (RT-PCR), and bacterial culture. Tissues were also collected in 10% buffered formalin, processed, and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin and eosin and viewed by light microscopy. Tissues opportunistically collected from an adult clinically normal, free-ranging alligator served as a control.

Multiple aliquots (totaling 1 g) of the ground raw horsemeat (without additives) that was being fed during the 2002 epizootic (October and November) were collected and processed for RT-PCR. Subsequent aliquots from postepizootic horsemeat shipments (in December and January) were similarly processed.

Virus Isolation

A 10% homogenate in Earle's minimal essential media (MEM) containing gentamicin was made of each specimen. The homogenate was centrifuged for 10 min at 2,000 RPM and 4°C. The supernatent was filtered and spread onto a preformed monolayer of Vero cells. In 2001, fathead minnow (FHM), white sturgeon skin (WSS), epithelioma papillosum caprini (EPC), and channel catfish ovary (CCO) cells were used instead of the Vero cells. Inoculated cells were incubated in a 5% CO_2 atmosphere at 37°C. Cells were examined each day for viral cytopathic effect (CPE). If no CPE was observed, aliquots of the first passage were transferred to a second preformed monolayer of Vero cells (FHM, WSS, EPC, and CCO cells in 2001) on day 7. If no CPE was observed after a second 7 days of passage, the culture was considered negative. Monolayers demonstrating viral CPE were passaged to chambered slides. The slides were fixed in cold methanol, and a West Nile fluorescent-antibody test was conducted to confirm the isolate.

Fluorescent-Antibody Testing

Mouse anti–WNV-specific polyclonal antibody (Centers for Disease Control and Prevention [CDC], Division of Vector-Borne Infectious Diseases, Fort Collins, CO) was applied to the chamber and the slide incubated in 5% CO_2 at 37°C for 30 min. The slide was rinsed two times for 5 min in a sodium carbonate/bicarbonate buffer (pH 9.3). The slide was then air-dried, followed by an anti-mouse fluorescein-conjugated antibody, and incubated as before for 30 min. The slide was washed twice in carbonate buffer, followed by 5 min in 0.5% Evans blue counter stain. Slides were dipped in distilled water, and a glycerin/water mounting media and coverslip was added. Slides were examined with a fluorescent microscope. All isolates were tested for WNV. All isolates were also tested for Eastern equine encephalomyelitis virus (EEEV) by using a similar protocol. We tested for EEEV because of its known prevalence within the geographic area. The EEEV-specific monoclonal antibody (CDC, Atlanta, GA) was prepared against the New Jersey 1960 strain of EEEV.

RNA Extraction

RNA was extracted from various specimens (fresh tissue, virus isolation homogenate or cell culture lysate, and formalin-fixed paraffin-embedded tissue). For extraction from fresh specimens, approximately 1 g of tissue was placed in a whirlpack bag and homogenized by using a Stomacher Lab Blender 80 (Tekmar Co., Cincinnati, OH) with three times the tissue volume of phosphate-buffered saline (PBS). Three milliliters of the tissue homogenate was processed with a Rneasy Midi kit (QIAGEN, Inc., Valencia, CA) per manufacturer's directions. If a virus isolation homogenate or cell culture lysate in Earle's MEM was used, approximately 4 mL of the homogenate or lysate was washed with 5 mL of PBS, the supernatant removed, and the pellet processed with the Rneasy Midi kit. For paraffin sections, several 5-µm sections from paraffin blocks were cut and deparaffinized with xylene. The xylene was removed, and samples were washed two times with 100% ethanol for 10 min, once with 95% and once with 70% ethanol. Samples were incubated overnight at 56°C in 80 µL of proteinase K with 5 mL of Buffer RLT from the Rneasy Midi kit and then processed per manufacturer's directions.

RT-PCR

RT-PCR for WNV was performed on the tissues according to the procedure described by Kuno (6) and using the RT-nested primer sets described by Johnson et al. (7). In brief, a RT-PCR mixture was prepared by using the outside primer set (P1401 – ACCAACTACTGTGGAGTC and P1845 – TTCCATCTTCACTCTACACT) to amplify a 445-bp product. Forty microliters of the RT-PCR mixture and 10 µL of sample were dispensed into a 0.2-mL thin wall PCR tube, and 10 µL of Rnase-free water was added for a final volume of 50 µL. With the use of a model PTC-200 thermal cycler (MJ Research, Inc., Waltham, Massachusetts), cycling conditions for the RT-PCR were as follows: 53°C for 30 min, followed by 40 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 1 min, and then held at 4°C. Ten microliters of RT-PCR first-round product was used for the nested PCR (nPCR). The nPCR mixture was prepared by using 40 µL of PCR mixture (now with

the inside primer set [P1485 – GCCTTCATACACACTAAAG and P1732 – CCAATGCTATCACAGACT]) to amplify a 248-bp product. The cycling conditions for the nPCR were as described above, but the first ramp was omitted (53°C for 30 min). A 10-μL aliquot of each reaction with 1 μL of loading buffer added was loaded onto a 1.5 % agarose gel in Tris-borate-EDTA (TBE) buffer and run at 70 V for approximately 1.5 h.

This protocol was repeated on all samples with primer sets for EEEV and St. Louis encephalitis virus (SLEV). For the 262-bp EEEV genomic fragment, an outer set of forward (P4 (EEE-4) - CTAGTTGAGCACAAACACCGCA) and reverse (P7 (cEEE-7) - CACTTGCAAGGTGTCGTCTGCCCTC) primers, followed by a nested set of forward (P5 (EEE-5) - AAGTGATGCAAATCCAACTCGAC) and reverse (P6 (cEEE-6) - GGAGCCACACGGATGTGACACAA) primers, was used (8). The RT-PCR mixture was similar to that described by Kuno (6). The thermal cycling parameters varied from those of WNV as follows: 94°C for 90 s followed by 30 cycles of 94°C for 20 s, 65°C for 35 s, 72°C for 17 s, and then a final elongation step of 72°C for 4 min. A single RT-PCR procedure was used for SLEV. The 393-bp genomic fragment was generated by using forward (SLE727 – GTAGCCGACGGTCAATCTCTGTGC) and reverse (SLE119c - ACTCGGTAGCCTCCATCTTCATCA) primers and using parameters as for WNV (9).

Bacterial Culture

Swabs of individual tissues were streaked onto 5% bovine blood agar (BBA), Wilkins-Chalgren anaerobe agar, mycoplasma agar, Lowenstein-Jensen agar slant, and Hektoen Enteric agar (HE) agar (intestines only). Blood was inoculated into thioglycolate broth and streaked onto BBA. Inoculated media were incubated at 30°C with duplicate blood agar plates incubated in the presence or absence of 5% CO_2, with the exception of the anaerobic cultures, which were incubated at 37°C. The thioglycolate broth was subcultured onto BBA after 24 h. Plates were examined each day for growth and subcultured onto BBA as needed. Bacterial colonies selected from pure cultures were Gram stained. Cultures were injected into Sensititre (Trek Diagnostic Systems, Westlake, OH) gram-negative AP80 or gram-positive AP90 autoidentification plates and the antibiotic sensitivity plate CMVIECOF and allowed to incubate for 18 h at 37°C before automated reading of the reactions per the manufacturer's directions. Any isolates that failed to be identified by the Sensititre system were identified by using the RapID NF Plus System (Remel, Norcross, GA) or the API20E system (API Analytab Products, Plainview, NY).

Results

Clinical Findings

The affected alligators appeared to "star gaze" in the water just before death, suggesting neurologic lesions (10). Alligators sometimes became stranded in the dry part of the pen with loss of leg control and neck spasms. No long-term signs of stress were noted, and most animals were eating well until a few days before death. The hatchlings (approximately 30-cm long at the time of the epizootic) and juveniles (1–2 m long) seemed to be more severely affected.

A specific pattern of transmission was not noted in 2001. However, in 2002, the alligator deaths initially occurred in one building and spread throughout the building in the opposite direction from that taken to feed and clean the animals. At least one interruption of chlorine addition to flush water occurred before the 2002 epizootic. Deaths were not incurred in the breeding colony, and no deaths were reported in birds that inhabited the rookery.

Gross Findings

2001

Both Florida and Georgia stock animals were affected, but, in general, the Florida stock was affected first. Initially, three juvenile alligators were sent for necropsy during the 2001 epizootic. In general, the alligators were in good to excellent body condition. One alligator had approximately 25 mL of serosanguinous fluid in the pericardial sac and 50 mL yellow serous fluid in the peritoneal cavity. Two of the three had yellow-tan, caseous necrosis of the palatine tonsils and multiple caseous yellow-tan plaques, 2- to 10-mm in diameter, on the mucosal surfaces of the esophagus, corpus, and pars pylorica. Only scant ingesta were noted throughout the gastrointestinal (GI) tract, and the intestinal mucosa was hemorrhagic in rare instances. The liver and spleen of one alligator had multiple 1- to 3-mm tan foci scattered throughout the parenchyma. One alligator was in poor to moderate body condition and had scattered bronchiectasis, no ingesta throughout the GI tract, and mild multifocal serous atrophy of fat. No other gross lesions were noted.

Approximately 2 months after the 2001 epizootic began, another juvenile, live alligator was submitted to our laboratory. The gross lesions were similar to those described above but with numerous 1- to 3-mm tan foci in the parenchyma of the liver, spleen, and kidneys.

2002

Three alligators were examined from the fall 2002 epizootic, two juveniles and a hatchling. The two juveniles had lesions similar to those described in the previous year.

The liver and kidneys of the hatchling were pale and mottled tan/brown. Ingesta were scant throughout the GI tract. The free-ranging alligator was in excellent body condition. No significant gross changes were noted in its tissues.

Light Microscopic Findings

2001

Tissues of the alligators from the 2001 epizootic were examined and were similar in two of the three alligators. In the brain, rare glial nodules that contained occasional heterophils were present (Figure 1). The spleen was congested with moderate diffuse reticuloendothelial hyperplasia and moderate numbers of heterophils. The tonsil had severe multifocal coalescing areas of caseous necrosis and heterophilic inflammation with reactive lymphoid follicular hyperplasia. In the esophagus, a focally extensive, mixed ulcerative, and proliferative lesion was present; it had a marked mixed but predominantly mononuclear inflammation, colonies of bacteria, and extensive fibrin deposition. In the liver, multifocal lymphoplasmacytic aggregates and heterophilic granulomas were present, consisting of caseous necrotic foci with degenerate heterophils surrounded by an outer layer of macrophages, lymphocytes, and heterophils. The lungs were congested with mild diffuse or patchy lymphoplasmacytic and heterophilic interstitial infiltrates. The kidney had multifocal heterophilic granulomas. The pars pylorica region of the stomach had multifocal mucosal abscesses and moderate diffuse lymphoplasmacytic and heterophilic infiltrates of the lamina propria. The small intestine had moderate, diffuse mucosal and submucosal infiltrates of lymphocytes, heterophils, and plasma cells and multifocal areas of acute necrosis associated with bacteria. The remaining tissues appeared within normal limits. Special stains for fungi and acid-fast bacteria were negative. A population of primarily gram-negative and fewer gram-positive bacteria was observed in the heterophilic granulomas.

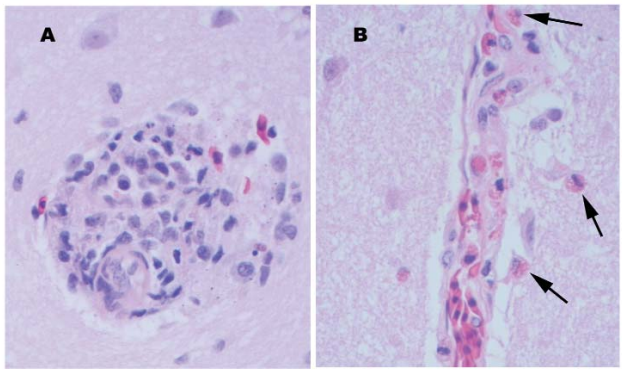

Figure 1. Perivascular changes observed within the brain of alligators infected with West Nile virus (400x). A. Perivascular infiltrates were composed of primarily lymphocytes, plasma cells, and macrophages in the hatchling alligator. B. Perivascular infiltrates were composed of primarily heterophils (arrows) in juvenile alligators.

The third alligator had primarily pulmonary changes. The airways contained moderate numbers of heterophils, occasional mucous plugs with degenerate inflammatory cells, and scattered bacterial colonies. The remaining tissues were as described for the first two alligators.

Tissues from the alligator seen 2 months after the epizootic had similar findings to those of the first two alligators with the addition of rare, small caseating granulomas within the lungs. The granulomas contained numerous large macrophages and multinucleated cells. Acid-fast stains demonstrated low numbers of slender, beaded, acid-fast positive bacilli consistent with mycobacteria.

2002

Multiple tissues from the two juvenile alligators from the 2002 epizootic were examined. The tissue changes were similar to those described for the 2001 epizootic except that the inflammatory component was primarily heterophils. The meninges within the brain and all spinal cord sections except those from the sacral spinal cord had stasis of heterophils within the blood vessels and perivascular infiltration of mild numbers of heterophils (Figure 1). One alligator had a small focus of macrophages and heterophils noted within the endocardium.

Multiple tissues were examined from the hatchling alligator, and lesions differed from the previous submissions on the basis of cellular composition of the inflammatory cell infiltrates. Lymphoplasmacytic perivascular cuffs were present throughout the brain and meninges (Figure 1). Rarely, heterophils were admixed within the cuffs. Similar changes were not seen within the spinal cord. Random foci of necrosis were seen within the liver, pancreas, and tonsil. Mild to moderate perivascular infiltrates of lymphocytes, plasma cells, and heterophils were seen within the kidney and heart, and similar but fewer numbers of infiltrates were seen within the pulmonary interstitium. The heart had multiple, random foci of patchy vacuolar degeneration of the myocytes and random aggregates of lymphocytes, plasma cells and heterophils. Mild numbers of mixed inflammatory cells were seen within the intestinal lamina propria. The remaining tissues were unremarkable. Major pathologic changes were not observed by light microscopy in the tissues from the free-ranging alligator.

Virus Isolation/RT-PCR

Virus isolation was negative for all animals from the 2001 epizootic. WNV was isolated from tissues from all animals in the 2002 epizootic. Additionally, all animals from the 2002 epizootic and one animal from the 2001 epizootic were positive for WNV by RT-PCR from fresh or

formalin-fixed, paraffin-embedded tissues (Figure 2). In general, liver was the most likely tissue to yield positive results. Positive results were not obtained from any of the tissues from the free-ranging alligator. All tissues tested negative by RT-PCR for EEEV and SLEV. Retrospective attempts to culture WNV at both 37°C and room temperature on FHM, CCO, EPC, and WWS cells were negative.

Aliquots from the horsemeat that was being fed during the 2002 epizootic tested positive for WNV by RT-PCR (Figure 2). Aliquots of the horsemeat from two postepizootic shipments were negative for WNV by RT-PCR.

Bacterial Culture

Aeromonas sobria and *Edwardsiella tarda* were consistently cultured from the intestines. These organisms and occasionally others (*Escherichia coli*, *Pseudomonas fluorescens*, α- and β-hemolytic *Streptococcus*) were isolated from various tissues (liver, lung, and kidney) from the alligators dying during the 2001 epizootics and the juveniles from the 2002 epizootics. *Alcaligenes* spp. were isolated from a tonsil swab in one of the animals in 2001. *Salmonella* Group D was isolated from the intestines of the hatchling alligator submitted in 2002.

Discussion

The histologic findings from the hatchling alligator were most suggestive of a viral etiology, whereas those of the older alligators were most suggestive of a primary bacterial cause. Given that both the RT-PCR and virus isolation were positive for WNV, that virus is suspected to be the underlying cause of both epizootics. Contaminated horsemeat is the presumed source of the outbreak. We speculate that the WNV infection led to the alligators' immune systems' becoming immunocompromised, which resulted in the animals being more susceptible to various environmental stressors and subsequent invasion by opportunistic pathogens. Failure to isolate virus from the alligators in 2001 may have been due to the inability of the virus to propagate in the four cell lines used (FHM, CCO, EPC, and WWS cells), as determined by retrospective culture attempts, rather than absence of virus.

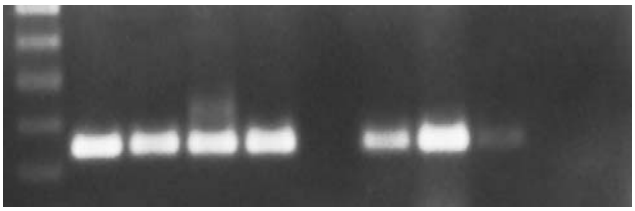

Figure 2. West Nile virus (WNV) reverse transcription-polymerase chain reaction results from epizootic die-offs in farm-raised alligators. The expected amplicon is 248 bp. Lane 1, a 100-bp molecular weight ladder. Lane 2, the positive WNV control. Lane 3, fresh tissue samples from a juvenile alligator in the 2002 epizootic. Lane 4, virus isolation cell homogenate from a juvenile alligator in the 2002 epizootic. Lane 5, horsemeat that was being fed to alligators during the 2002 epizootic. Lane 6, initial postepizootic horsemeat shipment. Lanes 7, 8, and 9, formalin-fixed, paraffin-embedded tissues of juvenile alligators in 2001 and 2002. Lane 10, fresh tissue from a wild alligator. Lane 11, negative WNV control.

Two important points to examine further are time of year and age of affected animals. Both epizootics occurred in the late fall to early winter. Although the epizootics appeared to be correlated with the first abrupt drop in environmental temperature, this finding was likely coincidental, especially given that the animals were housed in environmentally controlled barns. The most likely factor in the time of year is correlation with the occurrence of WNV infection in horses. Historically, horses become infected with WNV during the mosquito season (summer through early fall). Undiagnosed WNV-infected animals sold for food would most likely end up in the food supply during the late summer and early fall months. As was found in this study, deaths traced to consumption of contaminated food would taper off in late fall or early winter as the food supply was less likely to contain virus. Furthermore, all animals have equal potential for viral exposure through consumption because individual packages of horsemeat are combined before mixing with the vitamin supplements and being divided between all barns. In general, reptiles achieve immunocompetence at an early age (often in a matter of days), but this immunocompetence may be temperature dependent until the animals are several months of age (11). This fact may partially explain why the hatchling alligators tended to die from the viral infection, whereas the juveniles tended to die from infections caused by secondary invaders.

Extrinsic stressors may have increased certain animals' susceptibility to the virus or opportunistic pathogens. For example, the pens where the epizootics originated tended to be the first to be washed out at 6 a.m., the coolest time of the day. During the first abrupt drop in environmental temperature, the first wash water was possibly cooler because of colder water in the line between the boiler and the pens. This cold stressor would serve as a shock to the animals' systems. During the 2002 outbreak, an additional stress was internal construction, undertaken 2 weeks before the epizootic within the initially affected building. The environmental (temperature and darkness) control of the building was maintained during this time, but silence was not maintained. Additionally, sanitation-related stress may have occurred during periods of intermittent flushing, such as over weekends and during pen renovation activities.

Whether brood stock source had an affect on the susceptibility of the animals is not clear. Although Florida stock animals were those initially affected, this finding was likely coincidental because of their location in the pens. The pens that were more exposed to external stres-

sors contained Florida animals. Additionally, most animals in the production unit are from Florida brood stock.

Several management recommendations were suggested to the producer. The primary recommendation was to stop feeding horsemeat and switch to another food source such as beef or fish. We also recommended that the water temperature be reduced to 29.4°C in an attempt to reduce the stress of rapid growth and perhaps produce an environment less conducive for viremia. To date, neither of these recommendations has been implemented, but subsequent horsemeat shipments have tested negative. Future investigation will include the testing of the eggs from the brood stock, clinically healthy animals, rookery birds, and free-ranging alligators to explore the epidemiology of this virus in ectotherms.

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Emergence and Global Spread of a Dengue Serotype 3, Subtype III Virus

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Over the past two decades, dengue virus serotype 3 (DENV-3) has caused unexpected epidemics of dengue hemorrhagic fever (DHF) in Sri Lanka, East Africa, and Latin America. We used a phylogenetic approach to evaluate the roles of virus evolution and transport in the emergence of these outbreaks. Isolates from these geographically distant epidemics are closely related and belong to DENV-3, subtype III, which originated in the Indian subcontinent. The emergence of DHF in Sri Lanka in 1989 correlated with the appearance there of a new DENV-3, subtype III variant. This variant likely spread from the Indian subcontinent into Africa in the 1980s and from Africa into Latin America in the mid-1990s. DENV-3, subtype III isolates from mild and severe disease outbreaks formed genetically distinct groups, which suggests a role for viral genetics in DHF.

Arthropod-borne viruses are responsible for the emergence of unexpected diseases in humans, as illustrated by the identification of West Nile virus encephalitis in the American hemisphere in 1999 (1). The emergence of a new disease is often attributable to the transport of a pathogen (as in the case of West Nile virus) or changes in the evolution or ecology of a native pathogen that hitherto caused mild or no disease in humans (2,3). We studied unexpected outbreaks of dengue hemorrhagic fever (DHF) in Sri Lanka, East Africa, and Latin America caused by dengue serotype 3 (DENV-3) virus.

Most persons infected with dengue viruses are asymptomatic or develop dengue fever (DF). DHF and dengue shock syndrome (DSS), which can be fatal, develop in a minority of infected persons. The pathogenesis of DHF is poorly understood, although factors such as age and previous exposure to dengue infections increase the risk for severe disease (4). Epidemiologic studies point to particular DENV strains being more virulent than others (5–8). For example, the dengue genotypes endemic to Central and South America have caused mild disease, while the Asian genotypes introduced to the region have led to DHF epidemics (9–16). Similarly, outbreaks of DHF in some Pacific islands have been traced to the introduction of Southeast Asian dengue strains (17). DENV-2 subtypes associated with mild and severe disease epidemics have distinct mutations in the E gene and 5′ and 3′ untranslated segments of the viral genome, although whether these mutations directly contribute to pathogenesis is unproven (18).

The distribution of DHF and DSS in Asia has been particularly puzzling. Before 1989, DHF was common in Southeast Asia but rare in the Indian subcontinent despite the circulation of all four serotypes in both regions. After 1989, this pattern of disease changed and regular epidemics of DHF were reported from several countries in the Indian subcontinent (19). Sri Lanka, in particular, experienced a dramatic and persistent increase in DHF cases (20). Epidemiologic studies of dengue in Sri Lanka have demonstrated that the intensity of virus transmission, as well as the relative abundance of each serotype, remained constant before and after the emergence of DHF (21). Thus, DHF did not emerge in Sri Lanka because of an overall increase in virus transmission or shift in serotype.

Although all four serotypes of dengue circulate in Sri Lanka, persons who have the severe form of the disease are most frequently infected with DENV-3 (20,22). Lanciotti et al. characterized the genetic relatedness of DENV-3 isolates from regions throughout the tropics and subtropics and identified four geographically distinct subtypes (23). All Sri Lankan isolates were classified as subtype III, which also includes isolates from East Africa and India, as well as recent isolates from Latin America. Because DENV-3 isolates from Sri Lanka isolated before and after 1989 (when DHF emerged) formed separate groups within subtype III, Lanciotti and colleagues postulated that a genetic shift in DENV-3 may have been responsible for the emergence of DHF (23).

In the current study, using phylogenetic methods, we analyzed DENV-3 viruses isolated from Sri Lanka for up to 10 years after the emergence of DHF to confirm the

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establishment of a new genotype and evaluate the roles of virus evolution and transport in establishing a new genotype. DENV-3, subtype III was introduced into Latin America in 1994 (11), and the virus has subsequently been isolated from DF and DHF outbreaks throughout Central and South America (12–16). We also examined the genetic relationships between DENV-3, subtype III isolates from Latin America, East Africa, and the Indian subcontinent. On the basis of our results, we describe the most likely scenario of events that led to the emergence of DENV-3 –associated DHF in the Indian subcontinent and the Americas.

Materials and Methods

Virus Strains

The dengue virus strains sequenced for this study as well as sequences obtained from GenBank for this study are listed in Table 1. The virus isolates were obtained from the Centers for Disease Control and Prevention,

Table 1. Dengue virus type 3 sequences used[a]

Strain	Y	Location	Name	Subtype	Sequence source	GenBank accession no.
D1266	1983	Sri Lanka	83SriLan1	III	This study	AF547225
D1306	1983	Sri Lanka	83SriLan2	III	This study	AF547226
D1307	1983	Sri Lanka	83SriLan3	III	This study	AF547227
D1336	1983	Sri Lanka	83SriLan4	III	This study	AF547228
D1440	1984	Sri Lanka	84SriLan1	III	This study	AF547229
073	1985	Sri Lanka	85SriLan	III	This study	AF547241
D2783	1989	Sri Lanka	89SriLan1	III	This study	AF547230
D2863	1989	Sri Lanka	89SriLan2	III	This study	AF547231
D2803	1989	Sri Lanka	89SriLan3	III	This study	AF547232
D3197	1990	Sri Lanka	90SriLan1	III	This study	AF547233
D5231	1993	Sri Lanka	93SriLan1	III	This study	AF547234
D9397	1994	Sri Lanka	94SriLan1	III	This study	AF547235
L57	1997	Sri Lanka	97SriLan1	III	This study	AF547242
K1	1998	Sri Lanka	98SriLan	III	This study	AF547243
1557	1985	Mozambique	85Mozamb1	III	This study	AF547236
1558	1985	Mozambique	85Mozamb2	III	This study	AF547237
1559	1985	Mozambique	85Mozamb3	III	This study	AF547238
251991	1991	Kenya	91Kenya	III	This study	AF547239
SOM079	1993	Somalia	93Somalia	III	This study	AF547240
32267	1994	Nicaragua	94Nicara1	III	This study	AF547244
6845	1998	Nicaragua	98Nicara1	III	This study	AF547245
7431	1998	Nicaragua	98Nicara2	III	This study	AF547246
7071	1998	Nicaragua	98Nicara3	III	This study	AF547262
BC 96/94	1994	Panama	94Panama1	III	This study	AF547247
032231	1994	Panama	94Panama2	III	This study	AF547248
BC 13/96	1994	Panama	94Panama3	III	This study	AF547249
BC 20/97	1996	Mexico	96Mexico1	III	This study	AF547250
BC 172/97	1996	Mexico	96Mexico2	III	This study	AF547251
BC 184/97	1996	Mexico	96Mexico3	III	This study	AF547252
BC173/97	1996	Mexico	96Mexico4	III	This study	AF547253
17605	1995	Costa Rica	95CostaR1	III	This study	AF547254
17608	1995	Costa Rica	95CostaR2	III	This study	AF547255
322473	1995	Costa Rica	95CostaR3	III	This study	AF547256
322488	1995	Costa Rica	95CostaR4	III	This study	AF547257
20/8	1997	Guatemala	97Guatem1	III	This study	AF547263
366-781	1998	Puerto Rico	98PuertoR1	III	This study	AF547258
400-996	2000	Puerto Rico	00PuertoR1	III	This study	AF547264
MK	1998	El Salvador	98ElSalv1	III	This study	AF547259
612210	2001	Venezuela	01Venezue1	III	This study	AF547260
VEN03	2001	Venezuela	01VEN03	III	This study	AF547261
Ref. 18	1981–91	Sri Lanka	81,85,89,91 SriLanA	III	GenBank	L11431,L11436–L11438
Ref. 18	1984	India	84IndiaA	III	GenBank	L11424
Ref. 18	1986	Samoa	86Samoa	III	GenBank	L11435
Ref. 18	1962–86	Thailand	62,73,86,86 Thailand	II	GenBank	L11440–L11442,L11620
Ref. 18	1983	Philippines	83Philipp	I	GenBank	L11432
Ref. 18	1989	Tahiti	89Tahiti	I	GenBank	L111619
Ref. 18	1992	Fiji	92Fiji	I	GenBank	L11422
Ref. 18	1973–85	Indonesia	73,78,85 Indones	I	GenBank	L11425,L11426,L11428
Ref. 18	1974–81	Malaysia	74,81 Malaysi	I	GenBank	L11429,L11427
Ref. 18	1956	Philippines	D3H-87	I	GenBank	L11423
Ref. 18	1963–77	Puerto Rico	63,77 PuertoR	IV	GenBank	L11433,L11434
Ref. 18	1965	Tahiti	65 Tahiti	IV	GenBank	L11439

[a]Includes original identifier for strain, year of isolation, taxa name used in this paper, dengue virus 3 subtype, source of viral sequence, and GenBank accession numbers.

Dengue Branch, Puerto Rico, and Division of Vector-Borne Infectious Diseases, Ft. Collins, Colorado; Medical Research Institute, Colombo, Sri Lanka; School of Public Health, Berkeley, California; Walter Reed Army Institute for Research, Washington, D.C.; and University of Massachusetts Medical Center, Worcester, Massachusetts.

RNA Extraction

QiaAmp Viral RNA Mini Kit (QIAGEN, Valencia, CA) was used to extract viral RNA from both the mosquito grind supernatants and infected tissue culture media following the manufacturer's protocol. Extracted RNA was stored at –70°C or immediately subjected to reverse transcription–polymerase chain reaction (RT-PCR).

RT-PCR

DENV-3 RT-PCR was carried out as described by Lanciotti (23). Primers were designed to amplify and sequence a 966-bp fragment from positions 179–1,144, encompassing part of Capsid, all of PreM, and part of the E gene sequences. The reverse primer (DEN3/735) hybridized to positions 1,189–1,171 (5′-ctcctcaggcaaaaccgct-3′) and the forward primer (D1 consensus) hybridized to positions 132–159 (5′-tcaatatgctgaaacgcgcgagaaaccg-3′). The reverse primer DEN3/735 was added to extracted RNA, incubated at 85°C for 90 s, and allowed to cool to room temperature. RT was carried out for 45–60 min in 20 μL of reaction mix containing 25 U avian myeloblastosis virus reverse transcriptase (Roche, Nutley, NJ), deoxynucleoside tripophosphate, $MgCl_2$, and RT buffer. PCR was performed by adding a 30-μL cocktail containing D1 consensus primer, PCR buffer, and EXPAND polymerase (Roche) to the 20-μL RT reaction. PCR conditions were 4 min at 94°C, 30–35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 90 s with 5 s/cycle added to elongation step after the first 10 cycles. We separated 5 μL of the reaction products on 2% agarose gels and visualized it by ethidium bromide staining. When necessary, target bands were excised and purified by using the Qiagen QIAquick Gel Extraction kit (QIAGEN) following manufacturer's instructions. All remaining PCR reaction products were purified by using the Qiagen PCR Purification kit following the manufacturer's protocol.

DNA Sequencing

Purified PCR products were sent to the automated DNA sequencing facility at the University of North Carolina, Chapel Hill, NC. The DENV-3 sequences used in this manuscript included 40 newly determined sequences, which have been submitted to GenBank (accession nos. AF547225–AF547264).

Viral Sequence Analysis

Overlapping individual nucleic acid sequences were assembled with the aid of VECTOR NTI ContigExpress (InforMax, Inc., Bethesda, MD). Sequences were aligned and analyzed by using the following software: Clustal X (available from: URL: http://inn-prot.weizmann.ac.il/software/ClustalX.html), PAUP* (available from: URL: http://www.sinauer.com), PHYLIP (available from: URL: http://evolution.genetics.washington.edu/phylip.html), and MEGA II (available from: URL: http://www.megasoftware.net). Genetic distances were calculated by using Tamura-Nei distance algorithm with 1,000 bootstrap replicates; the trees were generated by using the Minimum Evolution method. The phylogenetic tree in Figure 1 is based on a 708-base segment, positions 437–1,145, spanning pre-M/M and a portion of the E gene. The phylogenetic tree presented in Figure 2 is based on 966-base region spanning positions 179–1,145 on the viral genome, capturing a portion of the C gene, all of pre-M/M gene, and a portion of the E gene.

Results and Discussion

Many investigators have used viral nucleotide sequence data and phylogenetic methods to understand genetic relationships between viruses, as well as the epidemiology of viral disease. Phylogenetic studies have shown that dengue viruses can move long distances between continents (24) as well as short distances between neighboring countries (25). Our goal was to use a phylogenetic approach to understand recent DHF outbreaks caused by DENV-3 infections in the Indian subcontinent and Latin America.

Previous phylogenetic analysis of DENV-3 has principally relied on complete or partial sequences of the pre-M/M and E genes (13,16,17,23). Our analysis used a 708-base segment, positions 437–1,145, spanning pre-M/M and a portion of the E gene, coding for 236 amino acids. This region was selected because it both conserved the original phylogenetic relationship identified by Lanciotti et al. and, in preliminary analysis with previously established sequences, captured 44% of the variable sites within DENV-3, subtype III Sri Lankan sequences. No insertion/deletion mutations and no hypervariable regions were detected in this span.

A total of 40 DENV-3 sequences, including 21 sequences available from GenBank and 19 newly determined Indian subcontinent and African sequences (Table 1) were compared. Dates of isolation ranged from 1963 to 1998. With the exception of 63PuertoR, all sequences were from low-passage (<4) virus cultures. Several approaches to phylogenetic analysis, including maximum likelihood, parsimony, and distance methods, were compared. All approaches yielded identical or nearly identical

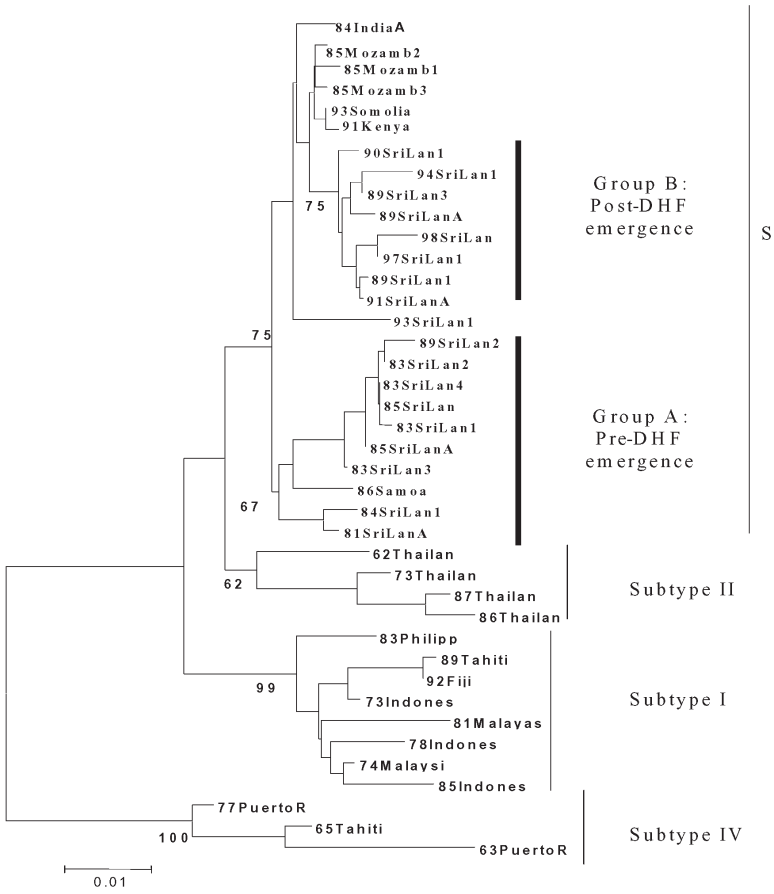

Figure 1. Phylogenetic tree of established dengue virus 3 (DENV-3) subtypes (23) and the relationship of Sri Lanka pre– and post–dengue hemorrhagic fever DENV-3 isolates to the established subtypes. This tree is based on a 708-base segment, positions 437 to 1145, spanning pre-M/M and a portion of the E gene. Scale bar shows number of substitutions per bases weighted by Tamura-Nei algorithm. Horizontal distances are equivalent to the distances between isolates. Numbers at nodes indicate bootstrap support values for the branch of the tree inferred at that node. The origin of the viruses and sequences used are listed in Table 1. The amino acid substitutions conserved within each DENV-3 subtype are listed in Table 2. DHF, dengue hemorrhagic fever.

topologies. Results presented here used the Tamura-Nei algorithm to calculate genetic distances and the minimum evolution method to create the trees (Figure 1). The tree identifies four distinct lineages that correspond to the region of isolation, reproducing the same evolutionary relationship first described by Lanciotti, et al. (23). Subtype I includes isolates from Southeast Asia and the South Pacific islands; subtype II consists of isolates from Thailand; subtype III is comprised of isolates from the Indian subcontinent, East Africa, and a single isolate from Samoa; and subtype IV includes Puerto Rico and Tahiti. Similarity within subtypes was high, with subtype III showing the greatest mean similarity (98.4%), followed by subtypes I, II, and IV (Table 4).

All 24 Sri Lankan, Indian, and East African strains fell into subtype III (Figure 1). The circulating virus genotypes within this region have remained closely related over the relatively long period of 18 years (1981–1998), indicating that countries bordering the western Indian Ocean form a geographically distinct region with regard to DENV-3 viruses. DENV-2 viruses in the regions also form a subtype with a similar geographic distribution (26,27). Frequent trade between East Africa, Western Indian Ocean islands, and the Indian subcontinent may have been responsible for the movement of dengue viruses throughout the region (26,28). Rico-Hesse, for example, demonstrated the introduction of DENV-2 to Africa from islands in the Indian Ocean (26). The earliest subtype III virus on record is an isolate from India in 1966; this virus occupies a node that is ancestral to all the subsequent Asian and African isolates (R.S. Lanciotti, pers. comm.), suggesting that the DENV-3, subtype III viruses have their origin in the Indian subcontinent and have subsequently spread out of the region.

In Sri Lanka, regular epidemics of DHF have been observed only after 1988. DENV-3 is responsible for many of the infections that progress to DHF (20,22). DENV-3 isolates obtained before and after the emergence of DHF are very closely related and belong to subtype III, indicating that the emergence of DHF on the island is not due to the introduction of a new subtype from outside the region. However, within subtype III, most Sri Lankan isolates (except for 93SriLan1) from before and after the emergence of DHF segregated into two distinct clades, designated groups A and B (Figure 1). Group A, with nine isolates from 1981 to 1989, consists of viruses collected up to the year epidemic DHF emerged in Sri Lanka but contains no isolates from later than 1989. Group B includes eight isolates from 1989 to 1998 but none from before 1989.

Table 2. Amino acid substitutions conserved within each dengue virus 3 subtype for the isolates used to create the phylogenetic tree in Figure 1[a]

		Position							
Subtype	Name	31	55	57	128	135	148	188	234
Outgroup	56Philipp	I	H	T	L	I	L	D	I
I	73Indones	–	L	–	F	–	–	–	V
I	74Malaysi	–	L	–	F	–	–	–	V
I	78Indones	–	L	–	F	–	–	–	V
I	81Malaysi	–	L	–	F	–	–	–	V
I	83Philipp	–	L	–	F	–	–	–	V
I	85Indones	–	L	–	F	–	–	–	V
I	89Tahiti	–	L	–	F	–	–	–	V
I	92Fiji	–	L	–	F	–	–	–	V
II	62Thailan	–	–	A	–	–	W	–	–
II	73Thailan	–	–	A	–	–	–	–	–
II	86Thailan	–	L	A	–	–	–	–	–
II	87Thailan	–	L	A	–	–	–	–	–
III	85Mozamb1	–	–	–	–	–	–	–	–
III	85Mozamb2	–	–	–	–	–	–	–	–
III	85Mozamb3	–	–	–	–	–	–	–	–
III	84IndiaA	–	–	–	–	–	–	–	–
III	91Kenya	–	–	–	–	–	–	–	–
III	93Somolio	–	–	–	–	–	–	–	–
III	81SriLanA	–	–	–	–	–	–	–	–
III	83SriLan1	–	–	–	–	–	–	–	–
III	83SriLan2	–	–	–	–	–	–	–	–
III	83SriLan3	–	–	–	–	–	–	–	–
III	83SriLan4	–	–	–	–	–	–	–	–
III	84SriLan1	–	–	–	–	–	–	–	–
III	85SriLanA	–	–	–	–	–	–	–	–
III	85SriLan	–	–	–	–	–	–	–	–
III	89SriLan2	–	–	–	–	–	–	–	–
III	89SriLanA	–	–	–	–	–	–	–	–
III	89SriLan1	–	–	–	–	–	–	–	–
III	89SriLan3	–	–	–	–	–	–	–	–
III	90SriLan1	–	–	–	–	–	–	–	–
III	91SriLanA	–	–	–	–	–	–	–	–
III	93SriLan1	–	–	–	–	–	–	–	–
III	94SriLan1	–	–	–	–	–	–	–	–
III	97SriLan1	–	–	–	–	–	–	–	–
III	98SriLan1	–	–	–	–	–	–	–	–
III	86Samoa	–	–	–	–	–	–	–	–
IV	63PuertoR	T	–	–	F	L	M	E	–
IV	65Tahiti	T	–	–	F	L	M	E	–
IV	77PuertoR	T	–	–	F	L	M	E	–

[a]Reference strain 56Philipp is the highly passaged laboratory strain H87. Positions are numbered sequentially from the first position in the pre-M/M protein

Temporally, the two groups are continuous, by virtue of sharing isolates in 1989. Group A includes isolate 89SriLan2, while group B contains 89SriLan1, 89SriLan3, and 89SriLanA. However, the groups do not form a continuous lineage; they share a common ancestor only at the node for subtype III (Figure 1). Group B shares ancestral nodes with isolates from India and East Africa. Because the Indian and East African isolates overlap temporally with group A (all isolates are from the 1980s), group A and group B lineages likely diverged sometime before 1981 and followed distinct evolutionary pathways.

We propose two likely scenarios that led to the emergence of group B viruses in Sri Lanka. One possibility is that the group B viruses were introduced from India or East Africa into Sri Lanka because Indian and East African isolates from the mid-1980s are closely related to Sri Lankan group B viruses (Figure 1). Of the two regions, India is the more likely source because of geographic proximity to Sri Lanka, although the East African viruses could be the direct ancestors of the group B viruses. Another possibility is that both groups co-circulated in Sri Lanka in the early 1980s, with group B being a minor population. Some

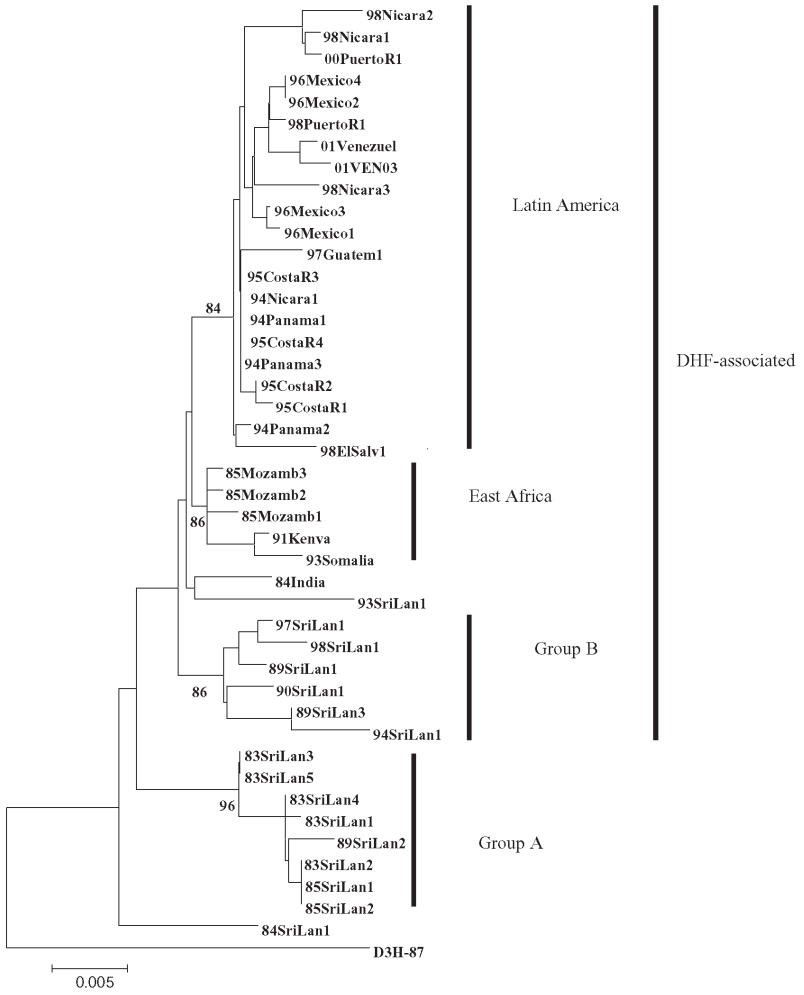

Figure 2. Phylogenetic tree of dengue virus 3, subtype III group A, group B, East Africa, and Latin America. Tree is based on 966-base region spanning positions 179–1,145 on the viral genome, capturing a portion of the C gene, all of pre-M/M gene and a portion of the E gene. Nucleotide substitutions conserved within each dengue virus 3, subtype III group (group A, B, East Africa, and Latin America) are listed in Table 3. DHF, dengue hemorrhagic fever.

selective force operating in the late 1980s may have shifted the balance in favor of group B viruses. In either case, group B viruses emerged in Sri Lanka because a subtype III variant already established in the greater region became more common in Sri Lanka and not because a novel virus evolved and emerged de novo on the island.

DENV-3, subtype III was detected in the Americas during DF and DHF outbreaks in Nicaragua and Panama in 1994 (11). Subsequently, the virus has spread to many countries in Latin America, and DENV-3–associated DHF was confirmed in several countries (13,14,16,29–31) (Figure 3). To establish the relationship of recent Latin American DENV-3 isolates to each other and to the previously identified Indian subcontinent and East African subtype III isolates, we sequenced and analyzed a 966-base region spanning positions 179–1,145 on the viral genome, capturing a portion of the C gene, all of pre-M/M gene, and a portion of the E gene. This region adds 288 positions to the 5′ end of the sequences initially presented in this study.

Forty-three isolates were sequenced (21 from Mexico and Central and South America, 16 from Sri Lanka, 1 from India, and 5 from East Africa) (Table 1). The D3H-87 belonging to DENV-3, subtype 1 was used as an outgroup. Years of isolation ranged from 1983 to 2001, an 18-year span. Except for the D3H-87 outgroup, all other isolates were low-passage clinical isolates. Most of the nucleotide mutations were silent: only 12 amino acid positions showed any variability and only 2 positions showed variability in more than one isolate. Consequently, the evolutionary relationships observed in this analysis likely reflect the results of genetic drift and are unlikely to have been influenced by host-specific selection events on this portion of the genome (26).

All sequences included in this analysis fell within subtype III (data not shown). Several approaches to phylogenetic analysis were compared, and all approaches yielded identical or nearly identical topologies. We used the Tamura-Nei algorithm to calculate genetic distances and the minimum evolution method to create the trees (Figure 2). Bootstrap values are shown at critical nodes. Despite the high overall similarity of the isolates in this analysis, geographically and temporally distinct groups formed sep-

Table 3. Nucleotide substitutions conserved within dengue virus 3, subtype III groups

Group	Strain	Position																			
		338	429	503	566	653	686	695	707	728	734	749	791	821	866	896	902	978	1010	1019	1056
Reference	H87	A	A	C	G	G	T	T	G	C	C	C	A	T	C	T	T	C	C	T	C
Group A	83SriLan1	-	G	T	A	-	-	C	-	-	T	T	-	-	T	C	C	T	T	-	-
Group A	83SriLan2	-	G	T	A	-	-	C	-	-	T	T	-	-	T	C	C	T	T	-	-
Group A	83SriLan3	-	G	T	A	-	-	C	-	-	-	T	-	-	T	-	C	-	T	-	-
Group A	83SriLan4	-	G	T	A	-	-	C	-	-	T	T	-	-	T	C	C	T	T	-	-
Group A	84SriLan1	-	G	-	-	-	-	-	-	-	-	T	-	-	-	C	-	-	-	-	-
Group A	85SriLan1	-	G	T	A	-	-	C	-	-	T	T	-	-	T	C	C	T	T	-	-
Group A	89SriLan2	-	G	T	A	-	-	C	-	-	T	T	-	-	T	C	C	T	T	-	-
unclassified	84India	G	G	-	-	A	-	-	-	-	-	T	-	-	-	-	-	-	-	G	-
unclassified	93SriLan1	G	G	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
East Africa	85Mozamb1	G	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
East Africa	85Mozamb2	G	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
East Africa	85Mozamb3	G	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
East Africa	91Kenya	G	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
East Africa	93Somalia	G	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	
Group B	89SriLan1	G	G	-	-	A	-	-	A	T	-	-	-	C	-	-	-	-	-	G	-
Group B	89SriLan3	-	G	-	-	A	-	-	A	T	-	-	-	C	-	-	-	-	-	G	-
Group B	90SriLan1	G	G	-	-	A	-	-	A	T	-	-	-	C	-	-	C	-	-	G	-
Group B	94SriLan1	-	G	-	-	A	-	-	A	T	-	-	-	C	-	-	-	-	-	G	-
Group B	97SriLan1	G	G	-	-	A	-	-	A	T	T	-	-	C	-	C	-	-	-	G	-
Group B	98SriLan1	G	G	-	-	A	-	-	A	T	-	-	-	C	-	C	-	-	-	G	-
L. America	94Nicara1	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	94Panama1	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	94Panama2	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	94Panama3	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	CostaRica1	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	CostaRica2	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	CostaRica3	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	CostaRica4	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	96Mexico1	G	G	-	-	A	C	C	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	96Mexico2	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	96Mexico3	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	96Mexico4	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	97Guatem1	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	98Nicara1	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	98Nicara2	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	98Nicara3	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	98ElSalv1	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	01Venezue1	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	01VEN03	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	98PuertoR1	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T
L. America	00PuertoR1	G	G	-	-	A	C	-	-	-	-	-	G	C	-	-	-	-	-	G	T

[a]Reference strain is the highly passaged laboratory strain H87. Positions are numbered sequentially from the first nucleotide position at the 5′ end of the genome.

arate lineages. Generally, two separate lineages formed within subtype III. The first consists of group A viruses isolated from 1981 to 1989 in Sri Lanka. These viruses have been associated only with DF. The second is composed of Sri Lankan group B, Indian, East African, and all of the isolates from Mexico and Central and South America.

Within group A, members are closely related, with a nucleotide mean similarity of 99.4% (Table 5). Within the expanded group B and related viruses, three distinct clades exist: a group of closely related Sri Lankan isolates from 1989 to 1998, 5 East African isolates from 1985 to 1993, and 21 isolates from 1994 to 2001 from Latin America. Isolates 84India and 93SriLan1 are less closely related to the other geographically distinct isolates in the larger second lineage.

The isolates from Latin America all emerge from a common node on the tree, suggesting a single introduction

Table 4. Summary of within- and between-subtype nucleotide mean similarity for dengue virus 3 isolates shown in Figure 1[a]

Subtype	Within subtype similarity (%)	Between subtype similarity (%)		
		I	II	III
I	98.1			
II	97.7	94.9		
III	98.4	95.6	96.3	
IV	97.6	92.3	92.5	92.7

[a]Mean similarities were calculated with the Tamura-Nei distance algorithm.

of a virus and the subsequent diversification of the virus population from the founding strain. The DENV-3, subtype III isolates from Nicaragua, Panama, and Costa Rica are closest to the Latin American group's originating node, with the more recent isolates found farther from that node, reflecting the viral population's ongoing evolution after the point source introduction.

The internal branch from the Latin American group shares a common node with the isolates from East Africa. The common hypothetical ancestor for Latin America and East Africa then shares a common node with the Sri Lankan group B virus isolates. Both on the phylogenetic tree and in pair-wise comparisons (Table 5), the Latin American group was more closely related to the isolates from East Africa than to the group B Sri Lankan isolates. Furthermore, the East African isolates pre-date the earliest Latin American isolates by 9 years, while the less closely related Sri Lankan group B isolates are nearly contemporaneous with the Latin American isolates. Therefore, the point source DENV-3 introduction into Latin America is most likely to have its origins in East Africa and not the Indian subcontinent (Figure 4).

Little is known about dengue activity in Africa, particularly DENV-3 (32). DENV-3 was first detected on the African continent in 1984 to 1985 during an outbreak in Mozambique (32). Later studies of U.S. troops in Africa and the Persian Gulf suggested that DENV-3 is endemic in those regions but largely undetected (33). Our results show that all East African DENV-3 isolates belong to subtype III. The fact that DENV-3 was only first isolated from East Africa in 1985, whereas the viruses were present in the Indian subcontinent at least as far back as 1966 (R.S. Lanciotti, pers. comm.), suggests that DENV-3, subtype III was introduced from the Indian subcontinent into East Africa in or before 1984 (Figure 4). This introduction led

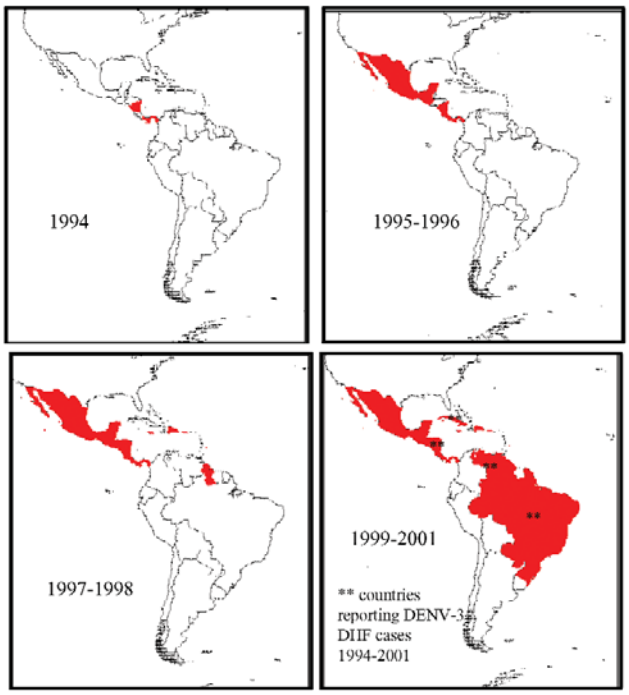

Figure 3. Map of the spread of dengue virus 3 (DENV-3), subtype III through Latin America and the Caribbean. The introduction of DENV-3, subtype III was first reported in November 1994 in Nicaragua and Panama. This virus strain has been isolated, identified, and reported in at least 16 other countries in the region. *Represents countries with dengue hemorrhagic fever (DHF) caused by DENV-3. These countries are Nicaragua in 1994 and 1998, Brazil and Venezuela in 2001 (Pan American Health Organization, unpub. data).

to the establishment of a stable East African group of DENV-3, subtype III because all the isolates from Mozambique, Kenya, and Somalia isolated from 1985 to 1993 form a distinct clade within subtype III (Figure 3).

The DENV-3, subtype III viruses introduced into Latin America are most closely related to subtype III viruses in East Africa (Figure 3). Although we can only speculate about the exact mode of transport of DENV-3 into Latin America, we propose that Panama, with its canal that attracts goods as well as civilians and military personnel from other parts of the world, may have been the point of introduction of subtype III into the Americas. Similarly, the introduction of DENV-2 in 1981 into Cuba may be

Table 5. Summary of within- and between-group nucleotide mean similarity for the dengue virus 3, subtype III virus isolates shown in Figure 2[a]

Subgroup	Within-group similarity	Between group similarity		
		Subgroup A	East Africa	Subgroup B
Subgroup A	99.4%			
East Africa	99.5%	98.2%		
Subgroup B	98.8%	97.9%	98.7%	
Latin America	99.5%	98.0%	99.0%	98.5%

[a]Mean similarities were calculated with the Tamura-Nei distance algorithm.

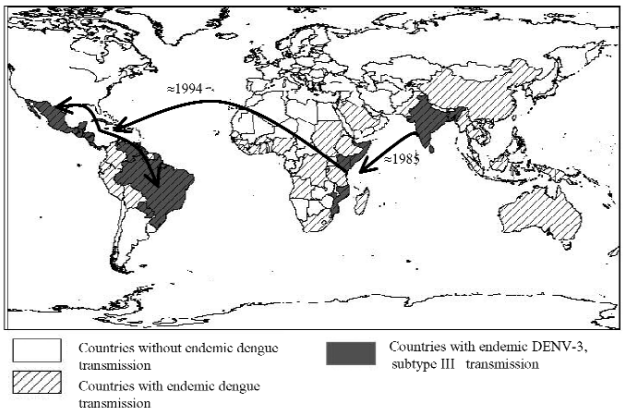

Figure 4. Global spread of dengue virus 3 (DENV-3), subtype III, which has been continuously circulating in the Indian subcontinent from the 1960s to the present. The virus was first isolated from East Africa in 1985 in Mozambique and subsequently from Kenya (1991) and Somalia (1993) (32,33). DENV-3 subtype III was first detected in the American continent in 1994 (Nicaragua and Panama) and the virus has subsequently spread through most of Latin America (13,14, 16,29,30). The arrows depict the most likely directions of spread based on the phylogenetic relationships between the viruses (see text for details). The map also displays countries in which dengue is known to occur.

attributable to Cuban military personnel traveling between Southeast Asia and Cuba (24,34).

Epidemiologic and clinical studies on dengue in Indonesia in the 1970s pointed to strain differences between DENV-3 viruses contributing to transmission and disease severity (35,36). Despite their overall similarity at the nucleotide level, the DENV-3, subtype III isolates examined in this study have been associated with severe or mild disease outbreaks (Figure 3). Sri Lankan group A viruses were isolated during a time of little to no DHF, while group B viruses were isolated after the emergence of DHF in Sri Lanka. The emergence of DHF in Sri Lanka was not accompanied by a change in dengue transmission or the abundance of any particular serotype (21). Implicating DENV-3 directly as the cause of DHF in Sri Lanka has been difficult because few virus isolates are available from DHF patients in Sri Lanka. However, during dengue surveillance studies in 1997, only DENV-3 was isolated from hospitalized dengue cases, whereas DENV-1, DENV-2, and DENV-3 were isolated from patients visiting outpatient clinics (22). These observations suggest that DENV-3 is responsible for severe dengue disease in Sri Lanka. Further studies are required to better establish the relative contribution of DENV-3 to severe disease in Sri Lanka.

The current studies support a viral genetic basis for severe and mild disease outbreaks. We found that the population of DENV-3 viruses associated with DHF in Sri Lanka did not appear to be direct descendants of the group A viruses that were circulating before DHF emerged in that country. The Sri Lankan 1989–1997 isolates are more closely related to the isolates from East Africa and the isolates from the Americas than they are to the isolates from 1981 to 1989 in Sri Lanka (Figure 3). All three groups of subtype III viruses (Sri Lankan group B, East African group, and Latin American group) associated with DHF are more closely related to each other than they are to the pre–DHF group A viruses from Sri Lanka (Figure 3). Thus, all the viruses within subtype III are closely related (mean 98.4% identity at the nucleotide level), yet they form distinct phylogenetic groups associated with mild or severe disease.

The Sri Lankan group B viruses may be associated with severe disease unlike group A viruses because the group B viruses are inherently more virulent. Alternatively, the ability of preexisting dengue antibody to neutralize group A viruses and enhance group B viruses may account for the observed associations with severe and mild disease. In a recent study, antibodies against American DENV-1 viruses neutralized the Native American DENV-2 genotype better than the Southeast Asian DENV-2 genotype that is currently circulating in the Americas and causing DHF (37). This study lends support to the idea that Asian DENV-2 may produce a more severe disease not because of inherent virulence properties but because persons with previous primary DENV-1 infections may enhance infection with this genotype and neutralize infections with the Native American DENV-2 genotype. Similarly, DENV-2 and -3 are the common serotypes in Sri Lanka, and persons with previous primary DENV-2 infections could neutralize the DENV-3 group A viruses better than the group B viruses. This difference may explain the unexpected emergence of DHF associated with group B. Further comparative studies with group A and B viruses are needed to understand their association with mild and severe disease, respectively.

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Molecular Epidemiology of O139 *Vibrio cholerae*: Mutation, Lateral Gene Transfer, and Founder Flush

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Vibrio cholerae in O-group 139 was first isolated in 1992 and by 1993 had been found throughout the Indian subcontinent. This epidemic expansion probably resulted from a single source after a lateral gene transfer (LGT) event that changed the serotype of an epidemic *V. cholerae* O1 El Tor strain to O139. However, some studies found substantial genetic diversity, perhaps caused by multiple origins. To further explore the relatedness of O139 strains, we analyzed nine sequenced loci from 96 isolates from patients at the Infectious Diseases Hospital, Calcutta, from 1992 to 2000. We found 64 novel alleles distributed among 51 sequence types. LGT events produced three times the number of nucleotide changes compared to mutation. In contrast to the traditional concept of epidemic spread of a homogeneous clone, the establishment of variant alleles generated by LGT during the rapid expansion of a clonal bacterial population may be a paradigm in infections and epidemics.

An epidemic of cholera began in Madras, India, in 1992 and within a year had spread across the Indian subcontinent, with cases numbering in the millions (1,2). *Vibrio cholerae* isolates from this epidemic had a previously unidentified serotype, subsequently designated as O139 Bengal (1,2). This new serotype appears to have resulted when a lateral gene transfer (LGT) event occurred that replaced the 22 kb of the *wbf* region (encoding the O1 antigen) of a seventh pandemic *V. cholerae* O1 El Tor strain with a 37-kb region encoding the O139 polysaccharide (3–5). The epidemic spread rapidly through all age groups, as persons with previous exposure to *V. cholerae* O1 were not immune to O139 infection. Since 1992, O139 strains have established endemicity in this geographic region and account for a variable percentage of cholera cases every year (6).

Genetic variation observed in O139 isolates has been attributed to many causes. Variation in restriction fragment length polymorphism (RFLP) analysis of rDNA genes (7) and in *recA* sequence (8) has been interpreted as evidence for multiple origins. Genetic variability in RFLP of the CTX element (6) has been attributed to phage-mediated recombination. Variation in antimicrobial susceptibility (9) has been attributed to plasmid exchange in response to selective pressure from drug use. The variation in pulsed-field gel electrophoresis (PFGE) analysis of genomic restriction fragments (6,10) has been attributed to point mutations. Multilocus sequence typing (MLST), which has been used in the evaluation of a number of other bacterial species (11–14), provides an alternative method for measuring genetic relatedness and has provided data for identifying both point mutations and LGT events (14). MLST has improved discriminatory power over PFGE in some cases, e.g., *Enterococcus* (15) and *Salmonella* (16); however, in the case of *Escherichia coli* O157, it does not because of an absence of sequence variation in the clonally derived isolates (17). A small MLST study of O139 isolates of *V. cholerae* did not identify any LGT events (18).

To understand the evolutionary dynamics of *V. cholerae* O139, we sequenced segments from nine loci, including seven that may be classified as traditional housekeeping genes, one that carries the genes for cholera toxin, and another that is next to the insertion sequence within the O139 *wbf* region (3–5). Thus, the last two loci might be expected to show LGT, because they are associated with known mobile elements, but the other seven loci would not be expected to show LGT. However, we found putative LGT alleles at all nine loci in the 96 clonally related O139 isolates.

Materials and Methods

We evaluated nine loci—*dnaE, lap, recA, pgm, gyrB, cat, chi, rstR,* and *gmd*—from 96 *V. cholerae* O139 isolated from patients seen at the Infectious Diseases Hospital, Calcutta, from 1992 to 2000 (see Appendix, online only). DNA was prepared from overnight cultures by using PrepMan Ultra (Applied Biosystems Inc., Foster City, CA) at the University of Maryland School of Medicine. Each

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locus was amplified by using polymerase chain reaction (PCR) with primers (Table 1) selected from a conserved region of the locus, as determined by aligning sequences from GenBank. Our primers selectively amplified the original O139 *rstR* gene found in all isolates and not the additional one found in some recently inserted CTX elements (19). The presence of amplified products was confirmed on agarose gels. Purification of the products was performed by using Millipore filters. The purified PCR products were sequenced in both directions by using the same primers used for amplification and Big Dye cycle sequencing kit (ABI) in accordance with manufacturer's instructions. The fluorescently labeled products were separated and detected by using either an ABI 377 or 3700 Automatic Sequencer (ABI). The trace files were read by using Phred (20,21) and Phrap (22). Low-quality sequence at the ends was trimmed, and the contigs from each individual isolate were aligned by using Clustal X (23). Variable nucleotides were identified manually. Isolates with identical alleles were identified from a distance matrix obtained from PAUP (24). The alleles have been assigned GenBank accession numbers AY297845 to AY297921.

The expected number of alleles that were a result of point mutations was calculated. All point mutations were assumed to occur independently; thus, the expected number of alleles with ≥2 nucleotides (nt) can be calculated, and the excess number of observed alleles was attributed to conspecific LGT of homologous genes. If one assumes that p is the probability of seeing a single mutation in an allele, the chance of seeing two mutations on the same allele is p^2; the probability of seeing three or more mutations is p^3. Probability can be calculated from the data by dividing the number of alleles with a single nucleotide difference, 34, by 785, the number of alleles in which observing a point mutation is possible (the 6-bp deletion; the recombinant *gmd, recA* alleles; and all duplicate novel alleles were excluded). Thus, p equals 0.043, p^2 equals 0.0018, and p^3 equals 8×10^{-5}. When these probabilities are multiplied by the total number of alleles, 785, the expected number of alleles containing two independent point mutations is 1.45, and the expected number containing three or more is 0.06 (Figure 1).

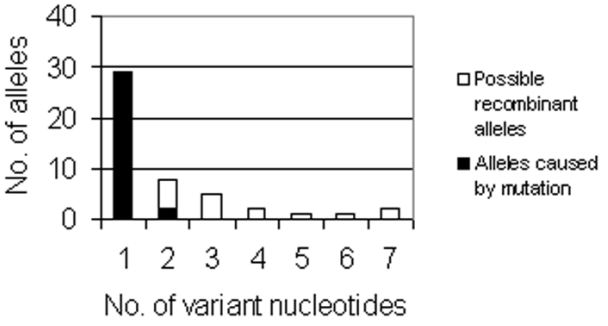

Figure 1. Bar graph of the number of novel alleles (y-axis) with a specific number of nucleotide differences from the ancestral allele. Two alleles with 24-bp and 113-bp differences are excluded from the graph.

Results

Each of the loci examined had a variable number of observed alleles: 9 for *dnaE*, 20 for *lap*, 11 for *rstR*, 11 for *gmd*, 2 for *recA,* 8 for *pgm*, 4 for *gyrB*, 7 for *cat,* and 5 for *chi*. The most variable, *lap* with 20 alleles, was expected because it is a highly variable locus when analyzed with multilocus enzyme electrophoresis (25). The most common allele was present in 91% of isolates (n=87) for *dnaE*, 77% (n=86) for *lap*, 79% (n=90) for *rstR*, 82% (n=87) for *gmd*, 99% (n=96) for *recA,* 90% (n=94) for *pgm*, 97% (n=92) for *gyrB*, 93% (n=88) for *cat,* and 94% (n=89) for *chi*. Thus, the pattern for each locus consists of a common or ancestral allele and a series of rare alleles, as expected for the expansion of a clone.

Of the 64 less frequent alleles, some result from LGT and others from mutation. The three alleles with the largest changes are unlikely to be due to point mutations. First, a *gmd* allele that differed by 113 of the 360 bp sequenced, when compared with sequences in GenBank using BLAST (available from: URL: www.ncbi.nlm.nih.gov/BLAST/) showed greater similarity to *gmd* from *E. coli* (AF061251) than *gmd* from *V. cholerae,* consistent with LGT of a homologous gene into the *V. cholerae* genome. Second, an alternative *recA* allele that differs by 24 nt is likely to be the result of LGT of a homologous gene. Although substantial, the number of nucleotide differences is not large enough for the allele to be clustered with sequences from

Table 1. Primers used for multilocus sequence typing

Locus	Primer 1	Primer 2
dnaE	CGRATMACCGCTTTCGCCG	GAKATGTGTGAGCTGTTTGC
lap	GAAGAGGTCGGTTTGCGAGG	GTTTGAATGGTGAGCGGTTTGCT
rstR	CGTGTTAGAGCACAC	GAGTGAATCGTCGTG
gmd	CCTTATGCKGTGGCRAA	CTWGGATCACCTAACA
recA	GAAACCATTTCGACCGGTTC	CCGTTATAGCTGTACCAAGCGCCC
pgm	CCKTCSCAYAACCCGCC	TCRACRAACCATTTGAADCC
gyrB	GAAGGBGGTATTCAAGC	GAGTCACCCTCCACWATGTA
cat	ATGGCTTATGAATCGATGGG	TCCCATTGCCATGCACC
chi	CAYGAYCCRTGGGCWGC	ACRTCTTCAATCTTGTC

V. mimicus, the closest sibling species to *V. cholerae* (8), a finding that suggests that recombination occurred within *V. cholerae.* Third, a *lap* allele had a 6-bp deletion and a single nucleotide difference that may be the result of a double-strand break repair.

We calculated that at least 26 putative conspecific LGT events occurred in the 96 isolates studied. Figure 1 shows the number of nucleotide differences between each novel allele and the ancestral allele. If all point mutations are assumed to occur independently, the expected number of alleles with two or more variable nucleotides can be calculated and the excess number of observed alleles attributed to conspecific LGT of homologous genes. The expected number of alleles containing two independent point mutations is 1.45, and the expected number containing three or more is 0.06. Since 11 alleles were observed with 2 nt differences, 9 more than expected, and 16 were observed with ≥3 differences, 16 more than expected, all of these alleles probably did not occur through mutation; more likely, these alleles are the result of LGT. Thus, we would estimate that 26 alleles (9 + 16 + *recA* allele above) are putatively due to conspecific LGT of homologous genes.

The putative conspecific LGT alleles, although fewer in number (26 alleles) than the assumed number of mutation-derived alleles (34 alleles), provide most of the nucleotide differences between alleles. The 120 nt changes introduced by conspecific LGT events are approximately three times the 38 (34 single mutations + 4 2x2 double mutations) introduced by mutation. This calculation is conservative: The 26 conspecific LGT events may represent an underestimate of the number because some of the alleles differing by ≤1 nt may have resulted from LGT.

The analysis of all nine loci from each isolate was based on the sequence type (ST). Each isolate was defined by a 9-digit number composed of the assigned allele number at each of the nine loci in the following order: *dnaE*, *lap*, *rstR*, *gmd, recA, pgm, gyrB, cat,* and *chi*. The most common allele was arbitrarily assigned as number 1. Thus, the ST of all the most common alleles is ST 1,1,1,1,1,1,1,1,1. Missing data were assigned the most common allele. This assumption is conservative, minimizes the observed amount of variation, and is consistent with the preponderance of common alleles found at each locus.

Fifty-one unique STs were found in the 96 isolates tested, reflecting relatively extensive genetic diversity. The overall average of 0.53 unique STs per isolate examined is similar to that seen in every year including 1992 (Table 2). Six STs occur more than once. As expected, the ancestral ST:1,1,1,1,1,1,1,1,1, found in 40 isolates, occurred in all years. Among the others, ST:1,1,2,1,1,1,1,1,1 was found three times, once each in 1995, 1996, and 1997. ST:1,2,1,1,1,1,1,1,1 and ST:1,1,7,1,1,1,1,1,1 were found twice in 1992 and 1994, respectively. ST 1,1,1,6,1,1,1,1,1 was found once in 1998 and again in 1999. ST:1,1,1,1,1,4,1,1,1 was found in 1995 and 1998. Since the number of STs is large (51 types), and number of samples in a collection period is small (8–13 samples; Table 2), STs seen in multiple years must not only persist but also represent a substantial portion of the epidemic O139 *V. cholerae* population.

Five of the novel STs are related to other novel STs by allelic change at another second or third locus. One sequence type evolved into three related types found in subsequent years (Figure 2a). The starred *gmd* allele is one related to the *E. coli* sequence, and its presence in two distinct related STs in two different years demonstrates its establishment in the population. That the pattern seen in Figure 2b of ancestral alleles *rstR* 1 and *chi* 1 and two variant alleles, *rstR* 7 and *chi* 5, was found in all combinations is indicative of an LGT event. Figure 2c-e shows three additional groups of related sequences. In Figure 2a, b, and d, the ST with the larger number of novel alleles occurred in later years. In contrast, in Figure 2c and e, the ST with the larger number of novel alleles occurred in the earlier years. The lack of an overall temporal relationship may result from the small sample size (8–13 isolates) in any year.

One isolate, CRC5, is unusual because it has no sequenced alleles of the ancestral type. Nevertheless, the alleles from this isolate are closely related to those of the ancestral type. Each CRC5 allele differs from the ancestral allele by 7 nt for *dnaE*, 3 nt for *lap*, 4 nt for *rstR*, 24 nt for *recA*, 6 nt for *pgm*, 10 nt for *gyrB*, and 4nt for *chi*. A comprehensive survey of the genetic distances for these loci could determine the average distance between alleles for each of these loci. The data would provide insight into whether this isolate represents a second derivation of the O139 clinical type from an environmental strain or if it is a genetic outlier within the clonally related, but diversified, O139 epidemic type.

Discussion

The emergence and pandemic spread of *V. cholerae* O139 Bengal represented a chance to examine evolution of a bacterial strain in the midst of a clonal expansion. Our results are consistent with clonal expansion and subsequent divergence as described by Spratt and Maiden (26). Putative recombinant alleles were found at all nine loci among the 96 clonally related O139 isolates. One *gmd* allele from *V. cholerae* was most similar to a *gmd* allele from *E. coli*. The number of base-pair differences among other alleles was higher than expected on the basis of a simple computation for the accumulation of independent mutations. This finding suggests that many of these events were due to LGT. When we applied our criteria to the

Table 2. Number of isolates tested and distinct sequence types, by year

No.	1992	1993	1994	1995	1996	1997	1998	1999	2000	Total
Isolates examined	9	9	12	10	13	11	12	12	8	96
Novel sequence types	3	6	6	7	8	6	7	4	7	51
Novel sequence types per isolate examined	0.33	0.66	0.5	0.7	0.62	0.55	0.58	0.33	0.88	0.53

novel alleles identified in a previous study (18), 11 of the 13 would be considered to have resulted from LGT, since the number of nucleotide differences to the ancestral allele varied from 4 to 19. Thus, for *V. cholerae*, like *Neisseria*, *Streptococcus*, and other bacterial species (11–14), conspecific recombination of homologous genes appears to be common and responsible for most of the alleles with multiple nucleotide differences and the majority of the nucleotide differences. The genetic variability at the nine loci alters our understanding of evolution in bacteria, showing that recombination in *V. cholerae* occurs frequently and most nucleotide changes occur by means of a recombination that can alter any gene.

The proportion of recombinants from conspecific recombination, 3.5% (28/785) is greater than that from transgeneric recombination (0.01% from the acquisition of *E. coli gmd* by one isolate). One potential implication of a greater rate of conspecific recombination may be that, over time, it will maintain the species identity of each individual bacterium, despite the constant bombardment of homologous genes from other genera. Although at first glance the frequency of the novel sequence types appears to conflict between our study and an earlier study (18), the observations may be reconciled on the basis of both the observed frequencies and the timing of the observations. Both studies identified a common ancestral allele in from 77% to 99% of isolates in our study and a series of rare alleles with 1–19 variant alleles for each locus. These studies reported 10% novel sequence types in 29 isolates that were collected from "the first epidemic period," from 1992 to 1993 (18). Our data from 1992 showed 33% novel sequence types from a sample of nine. These data are not statistically different (chi-square test=2.4, p=0.12). However, the researchers' estimate of frequency (18) is more likely to be correct because of the larger sample size. The dates of collection may also be important because our collection of isolates from 1993 began in March, when the number of O139 cases at the Infectious Diseases Hospital rose from <10 to >80 per month, corresponding to a rapid population expansion or flush. Thus, we can predict that we would see substantial variation in our sample.

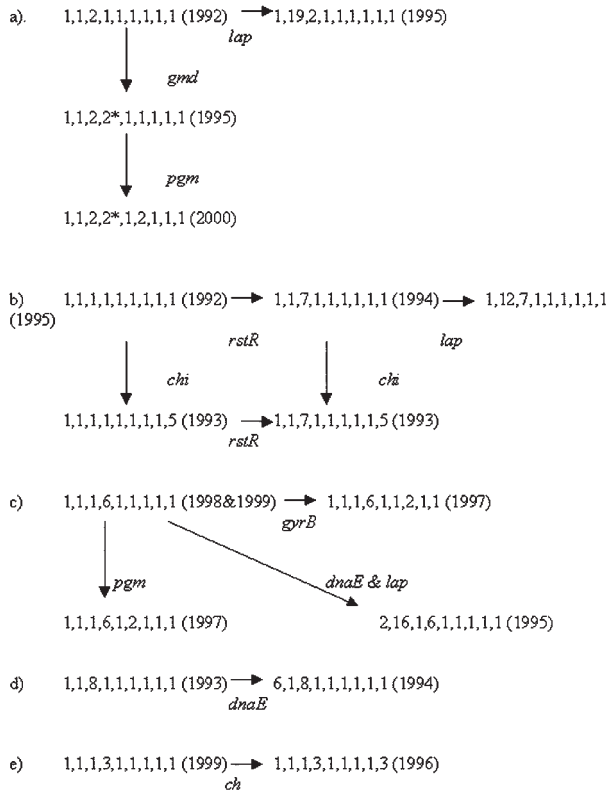

Figure 2. Five groups of related sequence types of *Vibrio cholerae* O139.

The genetic diversity was greater in the *V. cholerae* O139 isolates than in other clinically associated clones. In *V. parahaemolyticus* O3:K6, a pandemic strain, 94% of strains were identical at four loci (N. Chowdhury et al., unpub. data). In *E. coli* O157, all 77 isolates were identical at seven loci in spite of variation between isolates on PFGE (17). Although *V. parahaemolyticus* and *E. coli* are widespread pathogens, they differ from *V. cholerae* O139 because their population size has expanded much more slowly.

Among O139 isolates, the substantial genetic diversity found in the first year of the epidemic may reflect a "founder flush" phenomenon. During times of population expansion, i.e., a flush, any novel genotype with similar or even slightly deleterious fitness compared to the founder genotype will produce sufficient offspring to become established in the population (27). A founder flush appears to have occurred in the establishment of *Helicobacter pylori* in a single person (28). Although other previous descriptions of this phenomenon have been limited to insects, specifically butterflies (29) and drosophilids (30), we believe that the founder flush phenomenon may become the paradigm for epidemic bacterial expansion in individual patients and populations. This founder flush phenomenon, in turn, has implications for our interpretation of "clonality" among epidemic isolates and for our

understanding of factors that contribute to the emergence of new pathogenic strains.

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Dr. Garg completed this work as part of her Ph.D. dissertation at University of Calcutta. She is a postdoctoral fellow at the University of Maryland School of Medicine, pursuing studies of thrombospondin in human microvascular endothelial cells.

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Amoeba-Resisting Bacteria and Ventilator-Associated Pneumonia

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To evaluate the role of amoeba-associated bacteria as agents of ventilator-associated pneumonia (VAP), we tested the water from an intensive care unit (ICU) every week for 6 months for such bacteria isolates; serum samples and bronchoalveolar lavage samples (BAL) were also obtained from 30 ICU patients. BAL samples were examined for amoeba-associated bacteria DNA by suicide-polymerase chain reaction, and serum samples were tested against ICU amoeba-associated bacteria. A total of 310 amoeba-associated bacteria from 10 species were isolated. Twelve of 30 serum samples seroconverted to one amoeba-associated bacterium isolated in the ICU, mainly *Legionella anisa* and *Bosea massiliensis*, the most common isolates from water (p=0.021). Amoeba-associated bacteria DNA was detected in BAL samples from two patients whose samples later seroconverted. Seroconversion was significantly associated with VAP and systemic inflammatory response syndrome, especially in patients for whom no etiologic agent was found by usual microbiologic investigations. Amoeba-associated bacteria might be a cause of VAP in ICUs, especially when microbiologic investigations are negative.

Hospital-acquired pneumonia occurs in 0.5% to 1% of admitted patients admitted, representing 10% to 15% of all nosocomial infections; pneumonia is the most common cause of nosocomial infection in intensive-care units (ICUs) (1). This pneumonia is associated with high death rates. As the etiologic agent of pneumonia remains unknown in 20% to 50% of cases (2), identifying new lung pathogens is a major public health goal. Aquatic bacteria such as *Legionella* spp., *Pseudomonas* spp., *Stenotrophomonas* spp., *Burkholderia* spp., or *Acinetobacter* spp. may colonize in hospital water supplies and have previously been shown to be causally associated with cases of nosocomial infections (3). Free-living amoebae have been shown to be a reservoir of pathogens such as *Legionella* sp., *Burkholderia picketti,* and *Cryptococcus neoformans* (4–7). The most studied amoebae-associated bacterium is *Legionella pneumophila,* the agent of Legionnaires' disease (8), which frequently results from exposure to contaminated aerosols. Additional amoeba-associated bacteria might be implicated in community-acquired pneumonia, including *Legionella*-like amoebal pathogens (9) and members of the genus *Parachlamydia* (10). As part of research into the diversity of bacterial agents associated with amoebae in hospital water supplies, we have identified a new α-Proteobacteria belonging to the *Bradyrhizobiaceae* family (11–13). We demonstrated that patients with nosocomial pneumonia hospitalized in the vicinity of the contaminated water in a public hospital of our city have elevated antibody titers to these bacteria (14). In this study, we performed the same kind of analysis but focused our work on a single ICU during a 6-month period. Amoeba-associated bacteria were periodically evaluated in all ICU water taps. To evaluate contact of patients hospitalized in this ICU and amoeba-associated bacteria in the water, serum and bronchoalveolar lavages (BAL) samples were periodically sampled. Serum samples were tested in an immunofluorescence assay against the isolated bacteria to detect seroconversions, and DNA of these bacteria were detected in BAL samples by suicide-polymerase chain reaction (PCR) (15,16), a PCR technique without positive controls that incorporates "disposable" primers to avoid false-positive results. The second part of this work was to evaluate if exposure to the amoeba-associated bacteria in the ICU could be associated with disease. Thus, we specifically studied some clinical markers of infection, including fever, systemic inflammatory response syndrome (SIRS), and pneumonia for patients admitted to the ICU. As a definition of pneumonia based only on clinical and roentgenographic criteria has been criticized for low specificity (17–19), we used strict criteria in the definition. These criteria were applied to cases in which bacterial documentation was negative to determine if disease observed in patients hospitalized in an ICU may be attributed to amoeba-associated bacteria.

Materials and Methods

All patients admitted to the ICU during a 26-week period who needed intubation and mechanical ventilation

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were included. Patients were evaluated at admission by the Acute Physiology and Chronic Health Evaluation II score (20). At admission and every week thereafter, temperature, leukocytes and platelet counts, hepatic enzymes, and presence of SIRS and ventilator-associated pneumonia (VAP) were recorded. Serum samples were obtained from patients at admission, every 7 days afterwards, and at discharge. BAL was obtained at admission immediately after intubation by using protected mini-bronchoalveolar lavage (Combicath, Plastimed, Le-Plessy-Bouchard, France), then performed when a lung infiltrate suggestive of pneumonia appeared, and repeated every week until pneumonia resolved. BALs are part of the routine diagnosis and follow-up of pneumonia in the ICU and were not performed specifically for the study. Informed consent was obtained from the patient's family, according to French legislation. Data about bacteria isolated from blood cultures, lung secretions, and urine by conventional procedures were recorded. When isolated from urine and lung secretions, bacteria were only considered as pathogenic when concentration in the specimen was $\geq 10^5$ and $\geq 10^6$ CFU/mL, respectively (21).

Definitions of VAP and SIRS were based on previously published criteria (22–25), but to increase specificity, we limited our study to severe cases of VAP by adding strict criteria (Table 1). Taps and ice machine water were sampled every other week, as previously reported (11). The procedure for isolating bacteria from water and lung secretions by using cocultivation with *Acanthamoeba polyphaga* followed by subculture onto BCYE agar plates has been detailed elsewhere (11,26). Bacteria were identified by using 16S rRNA gene sequence comparisons as previously described (27). *Legionella* species were identified by using *mip* gene amplification and sequencing (28).

Twenty bacterial antigens were tested by microimmunofluorescence as previously reported (14,29). Bacterial species isolated from the ICU water during the studied period and 10 other species previously isolated in the same conditions from other sites (*Bosea eneae, B. vestrisii, B. thiooxydans, Mesorhizobium amorphae, Azorhizobium caulinodans, Afipia felis, A. felis* genospecies A, *A. clevelandensis, A. birgiae,* and *A. massiliae* [11–14]) were tested. The serologic tests were performed on the first serum samples from all patients admitted to the ICU; these patients were available for a second sample (30 patients). The control group comprised 10 patients in the same ICU. These patients had shorter stays and had samples taken at admission but was not available (100 patients) and 114 patients with other diseases, including Q fever (10 samples), trench fever (5 samples), tularemia (8 samples), Mediterranean spotted fever (10 samples), epidemic typhus (5 samples), syphilis (10 samples), cat scratch disease (5 samples), pneumonia caused by *Chlamydia pneumoniae* (5 samples), *C. psittaci* (5 samples), *Mycoplasma pneumoniae* (10 samples), *L. pneumophila* (10 samples), hepatitis C virus (5 samples), infections caused by cytomegalovirus (5 samples), Epstein Barr virus (11 samples), and HIV (10 samples). Serum samples were diluted at 1:25, 1:50, and 1:100 for immunoglobulin (Ig) G and IgM determination. The cutoff titer for a positive detection was determined as the lowest titer for which all first serum samples and control serum samples were negative. Then, all patients' serum samples were tested and serial twofold dilutions from 1:50 to 1:1600 were made on these samples with titers at least equal to the cutoff titer. Tests by Western blot were performed as previously described (29) for any patient with a seroconversion.

DNA was extracted from BAL samples by using QIAMP Tissue kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. PCR detection was attempted on all BAL samples for bacteria against which at least one seroconversion was generated. DNA extraction from serum samples was performed in a laboratory other than the one in which the isolates were identified to avoid vertical contamination from previous amplifications, and no positive control was used to avoid horizontal contamination from the same experiment (16). We used a nested PCR that incorporated two primer pairs used only once (Table 2) followed by sequencing and comparison to the targeted sequence, as previously described for suicide-PCR (15,16). All samples were tested the same day in the same assay. Sample testing was blinded, and positive amplicons were sequenced. Negative controls consisted of BAL samples from 10 patients of the same ICU obtained at admission, BAL samples from 200 patients with nosocomial pneumonia hospitalized in other medical centers of the city, water samples, and a suspension of *A. polyphaga.* At least one negative control was used for every two serum samples.

Table 1. Definition criteria for ventilator-associated pneumonia (VAP) and systemic inflammatory response syndrome (SIRS)

VAP	SIRS	Unexplained VAP	Unexplained SIRS
New and persistent roentgenographic lung infiltrate and new onset of: a) Increase in white blood cells >10 g/L b) Fever or hypothermia ($\geq$38°C or $\leq$36°C) c) Purulent sputum d) Duration of at least 2 weeks e) PaO_2/FiO_2 ratio <100	At least two of: a) T° >38°C or <36°C b) Heart rate >90/min c) Respiratory rate >20/min or $PaCO_2$ <32 mmHg d) Leukocytes >12 or <4 g/L or immature (band) forms	Lack of recovery of bacteria from: a) Lung secretions b) Blood cultures	Lack of recovery of bacteria from: a) Lung secretions b) Blood cultures c) Urine

Table 2. Polymerase chain reaction primers used for amplification and sequencing of bacterial DNA within human samples

Primers	*Bosea*-related strain	*Legionella anisa*	*Afipia broomeae*	*L. quinlivanii*
External forward	5′-TGCGAGTGTA GAGGTGAAATT-3′	5′-TATTGGTGC TGATTTAGGAA-3′	5′-TCTTTTGTGCG GGAAGATAATG-3′	5′-TTGTTGATGTTT GTTTTGAGACC-3′
External reverse	5′CGCTCGTTG CGGGACTTAA-3′	5′-GCTAAGTCTGAA GGTACA-3′	5′-TAAACTTTCCAA CGGCTGGCAT-3′	5′-TTCAACACTTCT TTCATCTGATC-3′
Internal forward	5′-GAGGTGAAA TTCGTAGATATT-3′	5′-GCCCAATTG ATTTTGACAG-3′	5′-GCTAACTTCGT GCCAGCAG-3′	5′-TCCAAGAATAA AAGGGGATTG-3′
Internal reverse	5′-GAGCTGACG ACAGCCAT-3′	5′-GCATTAATTGT AATGCTTCA-3′	5′-GTTTGCTCC CCACGCTTTC-3′	5′-CCATACCAT CCTGTAAGCCTT-3′

Comparisons of demographic, clinical, and laboratory data between patients with evidence of amoeba-associated bacteria contact (seroconversion or positive detection in BAL samples) were performed by using chi square and Mann-Whitney tests, respectively. The tested variables were age; sex; an underlying disease; severity score; intubation duration; hospitalization duration; SIRS; death; increase of hepatic enzymes, platelet count, and leukocytes; VAP; and fever. We also compared demographic, clinical, and laboratory data between patients with and without unexplained VAP, fever, and SIRS. Multivariate analysis adjusted for age, sex, prolonged intubation, and an underlying disease was performed to confirm observed associations. STATA software (v. 7.0, Stata Corporation, College Station, TX) was used for analysis.

Results

Ten species (310 isolates) were identified from 864 water samples (Table 3). *B. massiliensis* and *L. anisa* were the two most commonly isolated species (62.3% of the 310 isolates). In the ICU admission rooms , isolation of *L. anisa* ranged from 75% to 100% of the tested taps during the first 20 weeks; whereas all were negative during the last 6 weeks, after taps were changed. No amoeba-associated bacteria were isolated from BAL.

Ninety serum samples from the 30 patients and the 214 control serum samples were tested by an immunofluorescence assay for IgG and IgM on the 20 amoeba-associated bacteria antigens (12,160 tests). The 30 first serum samples and the samples from blood donors did not have an IgG titer of ≥1:50 or IgM titer >1:25. Cutoff titers for positive serologic tests with 100% specificity are shown in Table 4. Twelve (40%) patients seroconverted from 10 to 35 days after admission to at least one antigen; 10 showed IgM antibodies (Table 5). Five patients seroconverted to *L. anisa,* six to *B. massiliensis,* including one to both, one to *L. quinlivanii,* and one to *A. broomeae*. Western blots confirmed the seroconversions, with the appearance of several reacting bands on convalescent-phase serum samples (Figure). Patients also seroconverted to amoeba-associated bacteria not detected in the water in this study: two to *A. clevelandensis* (patient 2, IgG=1:100 and IgM=1:100; patient 8, IgG=1:50 and IgM=1:200;) and two to *A. felis* (patient 2, IgG=1:100 and IgM=1:100; patient 9, IgG=1:1600 and IgM=1:25). Seroconversions were significantly more frequent against amoeba-associated bacteria obtained in this ICU than against amoeba-associated bacteria isolated in previous studies: 13 of 300 tests versus 4 of 300 tests and 12 of 30 patients versus 4 of 30 patients (p=0.046 and p=0.039, respectively). Patients also seroconverted more frequently to the most commonly isolated bacteria, *L. anisa* and *B. massiliensis* (>50 isolates, p=0.021).

Table 6 shows the clinical characteristics of patients with serologic evidence of exposure to amoeba-associated bacteria isolated in the ICU. Analysis for risk and potential confounding factors did not indicate differences between patients with or without seroconversion. However, seroconversion was statistically associated with VAP (p=0.026), unexplained VAP (p=0.034), SIRS (p=0.024), and unexplained SIRS (p=0.045). Multivariable logistic regression demonstrated that seroconversion was independently associated with VAP even after we adjusted for intubation duration, hospitalization duration, number of serum samples, and underlying disease (p=0.014 to 0.030).

The DNA of *L. anisa* and *B. massiliensis* were each detected once in the 66 BAL samples from 30 patients. For two samples, seroconversion to the identified bacteria, *L. anisa* and *B. massiliensis,* respectively, was observed 4 and 2 weeks after the PCR was positive in BAL. None of the 210 control patients was positive for these bacteria in BAL samples compared to 2 of 30 patients in the ICU (p<0.01).

Table 3. Identification of the 310 bacterial strains isolated by using amoebal co-culture procedure

Species	No. of isolates
Legionella anisa	126
Bosea massiliensis	67
Rasbo bacterium	45
Bradyrhizobium liaoningense	29
L. quinlivanii	12
L. pneumophila	11
L. rubrilucens	7
L. worsleiensis	6
B. japonicum	5
Afipia broomeae	2

Table 4. Definition of cutoff titers for positive serologic tests and 100% specificity according to antigen tested by using the 224 control serum samples[a]

Antigen	IgG	IgM
Legionella anisa	≥1:50	≥1:25
Bosea massiliensis	≥1:50	≥1:25
Rasbo bacterium	≥1:100	≥1:25
Bradyrhizobium liaoningense	≥1:50	≥1:25
L. quinlivanii	≥1:100	≥1:25
L. pneumophila	≥1:50	≥1:25
L. rubrilucens	≥1:200	≥1:25
L. worsleiensis	≥1:200	≥1:25
B. japonicum	≥1:100	≥1:25
Afipia broomeae	≥1:200	≥1:25
Bosea eneae	≥1:800	≥1:25
B. vestrisii	≥1:100	≥1:25
B. thiooxydans	≥1:50	≥1:25
Mesorhizobium amorphae	≥1:100	≥1:25
Azorhizobium caulinodans	≥1:100	≥1:25
Afipia felis	≥1:100	≥1:25
A. felis genospecies A	≥1:100	≥1:25
A. clevelandensis	≥1:100	≥1:25
A. birgiae	≥1:50	≥1:25
A. massiliae	≥1:50	≥1:25

[a]Ig, immunoglobulin.

The death rate was 33.3%; disease was mainly associated with fever (96.6%), VAP (56.6%), and SIRS (76.6%). No microbial etiologic agent was found in 18 (62%) of 29 patients with fever, 10 (43%) of 23 patients with SIRS, and in 8 (47%) of 17 patients with VAP. VAP was significantly associated with duration of hospitalization (median hospitalization days/interquartile range of 23/18–41 with VAP versus 14/10–21 without VAP, p=0.04), SIRS (16/17 with VAP versus 7/13 without VAP, p=0.025) and seroconversion to amoeba-associated bacteria (10/17 with VAP versus 2/13 without VAP, p=0.026). No other statistical difference was observed between patients with VAP and without VAP in terms of demographic, clinical, and paraclinical data, risks, and potential confounding factors. Most patients received cephalothin for the first 3 days of hospitalization as an antibiotic prophylaxis. The most commonly used antibiotics in patients with VAP were third-generation cephalosporins. Patients with VAP received antibiotics more frequently and for more than 1 week (16/17 with VAP versus 7/13 without VAP, p=0.025).

Discussion

We confirmed that several amoeba-associated bacteria are common in the water in ICU; we recovered 310 amoeba-associated bacteria isolates from 10 species from the water of the ICU. As exposure to a microorganism is a prerequisite to infectious disease, we first evaluated contact of patients hospitalized in the ICU with amoeba-associated bacteria in water. We found that 12 (40%) of 30 of patients seroconverted to amoeba-associated bacteria and that these seroconversions were significantly more common against local isolates than to amoeba-associated bacteria from other ICUs. Moreover, antibody response parallels that of water contamination with *B. massiliensis* and *L. anisa,* which cause 83% of seroconversions identified in 62% of isolates (p=0.021). The cutoff titers, chosen to have 100% specificity, and the detection of several reactive antigens in the Western blots (Figure) suggest that these seroconversions represent specific serologic reactions. Detection of antibodies reacting to amoeba antigens would also be important because infections could occur through inhalation (perhaps after colonization) of infected amoebae acting as a "Trojan horse" (6). In future studies, isolation of amoebae in the aquatic environment as in the BAL samples of patients will be performed for further use as antigens.

Patient exposure to amoeba-associated bacteria from the ICU was also evident in results of amoeba-associated bacteria DNA detection in BAL samples from 2 of 30 patients as compared with 0 of 210 control patients (p<0.01). Moreover, for these patients, bacterial DNA was detected in the BAL sample before the seroconversion to the same bacteria 2 to 4 weeks after admission. This rate is compatible with an acute infection occurring during hospitalization rather than colonization of the respiratory tracts of patients. Contamination is unlikely when using a suicide-PCR procedure (15,16). Isolation of these agents was probably hampered by the antibiotic prophylaxis instituted at admission in this ICU for trauma patients and likely explains the lack of amoeba-associated bacteria isolation in BAL samples.

Among the 30 patients, VAP occurred frequently (56.6%) and was associated with hospitalization duration, as previously reported (30,31). Of the patients with VAP, 58.8% seroconverted to amoeba-associated bacteria, as compared to 15.4% of the remaining 13 patients (p=0.026). In patients with seroconversion, SIRS was also more prevalent (p=0.024). The percentages of unexplained VAP and unexplained SIRS were four times more common in amoeba-associated bacteria seroconverters than in non-seroconverters and remained statistically significant in spite of the small population studied (Table 6). Thus, amoeba-associated bacteria may be a common cause of unexplained VAP and SIRS.

Finally, we identified a cryptic outbreak in the ICU caused by *L. anisa,* a pathogen commonly encountered in the environment (32,33), only implicated in a few epidemics of Pontiac fever (34,35) and four cases of legionellosis (26,36–38). Five (16.7%) of 30 patients were infected with this bacterium, considered a relatively rare pathogen. Serologic tests are not currently used for *L. anisa*, and no urinary antigen test is available. Therefore,

Table 5. Antibody titers of 12 serum samples with seroconversion to at least one of the bacteria isolated in the intensive care unit

		Bosea massiliensis		*Legionella anisa*		*L. quinlivanii*		*Afipia broomeae*	
Case	Wk of sampling	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
1[a]	1			<1:50	<1:25				
	4			**1:50**	**1:100**				
	7			**1:800**	**1:50**				
2[a]	1	<1:50	<1:25						
	3	**1:400**	**1:100**						
7	1			<1:50	<1:25				
	3			<1:50	**1:200**				
8	1					<1:50	<1:25		
	3					**1:200**	**1:800**		
9	1	<1:50	<1:25	<1:50	<1:25				
	3	**1:400**	**1:50**	**1:50**	**1:400**				
	5	**1:50**	**1:25**	**1:800**	**1:50**				
11	1	<1:50	<1:25						
	5	**1:50**	**1:50**						
12	1			<1:50	<1:25				
	3			**1:400**	**1:50**				
13	1							<1:50	<1:25
	3							1:50	<1:25
	5							1:100	<1:25
	7							**1:200**	<1:25
19	1			<1:50	<1:25				
	3			**1:400**	<1:25				
22	1	<1:50	<1:25						
	3	**1:200**	**1:50**						
23	1	<1:50	<1:25						
	5	**1:400**	**1:25**						
28	1	<1:50	<1:25						
	3	**1:400**	**1:25**						
	5	**1:200**	**1:25**						

[a]Patients with positive PCR results in bronchoalveolar lavage samples (patient 1, *L. anisa*; Patient 2, *B. massiliensis*); Ig, immunoglobulin. All patients were sampled at admission (wk 1); titers in bold are those greater than or equal to defined cutoff titers (Table 4).

diagnosis based only on isolation may explain why *L. anisa* is so rarely reported. However, its absence in the BAL samples from the 200 patients from other ICUs shows that local epidemiology plays a major role. This study confirms that *L. anisa* is common in the environment (32,33).

Members of the *Bosea* genus are gram-negative, oxidase-positive, catalase-positive rods belonging to the α-2 subgroup of Proteobacteria. All are motile. They grow well on BCYE agar from 25°C to 37°C but do not grow or grow weakly on Columbia agar with 5% sheep blood. Colonies are smooth, mucoid, round, and cream colored and are urease positive and α-hemolytic on Columbia agar with 5% sheep blood and 0.2% yeast extract. *B. massilensis* are negative in assays for arginine dihydrolase activity, esculin and gelatin hydrolysis, β-galactosidase activity, maltose assimilation, and acid production by fermentation or oxidation of substrates tested in API 50 CH (Biomérieux, Marcy l'étoile, France), especially D-glucose, D-fructose, D-mannose, and sucrose. The species of the *Bosea* genus have high MICs to penicillin and amoxicillin and low MICs to doxycycline (13). In co-culture with *A. polyphaga, B. vestrisii, B. eneae,* and *B. massiliensis* are phagocyted and form progressively large vacuoles that lead to amoebal lysis; however, they have never been reported as pathogenic agents before. As *B. massiliensis* has not been tentatively isolated elsewhere, whether our findings reflect a local phenomenon or whether the bacterium is widely encountered is not known. We think that our data support the role of *B. massiliensis* in severe VAP, but confirmation is needed to definitely establish a role.

Our study indicates that most patients with VAP received β-lactam agents, mainly amoxicillin-clavulanic acid and third-generation cephalosporin. These antibiotics may have inhibited bacterial culture, which explains why no amoeba-associated bacteria were isolated from BAL samples. *B. massiliensis* has low MICs (≤0.5 mg/L) to ceftriaxone, doxycycline, rifampin, and erythromycin (13). However, β-lactam agents are also active in vitro on *Legionella* spp., but animal models and clinical studies have demonstrated their inefficacy in the treatment of legionellosis (39).

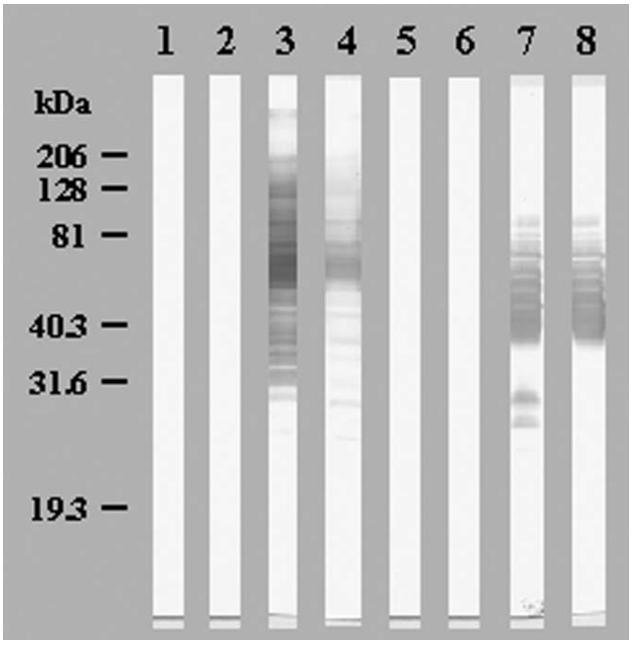

Figure. Western blot showing seroconversions in immunoglobulin (Ig) G (Lanes 1, 3, 5, 7) and IgM (Lanes 2, 4, 6, 8) of patient 2 against *Bosea massiliensis* (Lanes 1 to 4) and patient 9 against *Legionella anisa* (Lanes 5 to 8). Lanes 1, 2, 5, 6: acute-phase sera; Lanes 3, 4, 7, 8: convalescent-phase sera.

The results of our study confirm that the bacteriologic tests of hospital water supplies is largely ignored. Our work demonstrates that patients are exposed specifically to the most common water amoeba–associated bacteria in their environment, as evidenced by seroconversion against these bacteria and DNA of these bacteria in BAL samples. The route of infection, even if caused by aerosols generated in the ICU, remains unclear. Patients of this ICU sometime receive water through nasogastric tubes but only bottled, sterile water. However, we cannot exclude mistakes caused by not following recommended procedures. Patients for whom exposure to these bacteria is supported by seroconversion or DNA detection in BAL samples have unexplained VAP and SIRS more commonly. We speculate that amoeba-associated bacteria in the environment of intubated patients may concurrently cause unexplained infections and cryptic outbreaks. Research of new etiologic agents of pneumonia in ICUs should be based on environmental study of each ICU since ecologic findings of amoeba-associated bacteria in water points in hospital vary.

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Table 6. Clinical characteristics of patients with or without seroconversion to one of the amoeba-associated bacteria[a]

Clinical characteristics	Seroconversion (N=12)	No seroconversion (N=18)	p value
Demographic data			
Median age in y (IQR)	35 (25–43)	24 (21–52)	0.85
Male (%)	10 (83.3)	15 (83.3)	1
Risk and potential confounding factors			
Underlying disease (%)	2 (16.6)	3 (16.6)	1
Circulation injury (%)	9 (75)	14 (77.8)	1
Median APACHE II[a] score (IQR)	21 (14–4)	23 (16–34)	0.12
Intubation in ICU (%)	8 (66.6)	7 (38.9)	0.26
Median hospitalization days (IQR)	25 (19–41)	17 (10–23)	0.094
Median intubation duration in days (IQR)	11 (7–20)	11 (7–20)	0.8
Median number of serum samples (IQR)	3 (3–5)	3 (2–4)	0.12
Clinical data			
VAP (%)	10 (83.3)	7 (38.9)	**0.026**
Unexplained VAP (%)	6 (50)	2 (11.1)	**0.034**
Fever $\geq$ 38.5 °C (%)	12 (100)	17 (84.4)	1
Unexplained fever (%)	7 (58.3)	11 (39.3)	0.27
SIRS (%)	12 (100)	11 (61.1)	**0.024**
Unexplained SIRS (%)	7 (58.3)	3 (14.3)	**0.045**
Death (%)	2 (16.7)	8 (44.4)	0.23
Paraclinical data			
Leukocytes > 12 g/L (%)	12 (100)	14 (77.8)	0.13
Platelets > 500 g/L (%)	5 (41.7)	6 (33.3)	0.7
PCR detection of ARB in BAL samples(%)	2 (17)	0	0.15

[a]IQR, interquartile range; VAP, ventilator-associated pneumonia; SIRS, Systemic Inflammatory Response Syndrome; APACHE II, Acute Physiology and Chronic Health Evaluation II; PCR, polymerase chain reaction; ARB, angiotensin receptor blockers; BAL, bronchoalveolar lavage samples; bold p values are those that are significant (<0.05).

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Antimicrobial Resistance Markers of Class 1 and Class 2 Integron-bearing *Escherichia coli* from Irrigation Water and Sediments

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Municipal and agricultural pollution affects the Rio Grande, a river that separates the United States from Mexico. Three hundred and twenty-two *Escherichia coli* isolates were examined for multiple antibiotic resistance phenotypes and the prevalence of class 1 and class 2 integron sequences. Thirty-two (10%) of the isolates were resistant to multiple antibiotics. Four (13%) of these isolates contained class 1–specific integron sequences; one isolate contained class 2 integron–specific sequences. Sequencing showed that the class 1 integron–bearing strain contained two distinct gene cassettes, *sat-1* and *aadA*. Although three of the four class 1 integron–bearing strains harbored the *aadA* sequence, none of the strains was phenotypically resistant to streptomycin. These results suggest that integron-bearing *E. coli* strains can be present in contaminated irrigation canals and that these isolates may not express these resistance markers.

Integron gene sequences contribute to the spread of antimicrobial resistance alleles by lateral gene transfer of gene cassettes in a variety of enteric bacteria, including *Campylobacter* spp., *Escherichia coli,* and *Salmonella enterica* serotype Typhimurium (1–4). The gastrointestinal environment is suspected of serving as a reservoir for integron-bearing strains; when antimicrobial exposure occurs, gene transfer events—which spread cassettes between commensal organisms that are expelled into the environment (2)—would also occur.

The Rio Grande, the river separating the United States from Mexico along the Texas-Mexico region, serves as a source for irrigation water in Texas and Mexico. Previous studies in our laboratory and others have shown that the transboundary region is subject to extensive microbial and chemical contamination. This contamination has been associated with agricultural, municipal, and industrial wastes originating from both sides of the border (5,6). Leaking septic tanks and wastewater effluent discharges result in fecal contamination levels as high as 2,000 CFU/mL of fecal coliforms (7,8).

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Because of the strategic importance of the Rio Grande for U.S. agriculture and the potential transmission of antimicrobial resistance determinants by means of food crops, we investigated the prevalence and characteristics of class 1 and class 2 integron–bearing *E. coli* strains. These strains were previously isolated from a study investigating fecal contaminants in irrigation water and associated sediments at specific locations along the river (9).

Methods

Three hundred and twenty-two *E. coli* isolates were previously isolated from irrigation water and associated sediments at the El Paso, Presidio, and Weslaco regions of the river (9). After being confirmed as *E. coli* by MUG (4-methyl umbelliferyl-β-D-glucoronide)–based fluorescence, these isolates were screened for antimicrobial susceptibility by using the agar dilution method (10,11). The isolates were tested against ampicillin, tetracycline, ceftriaxone, cephalothin, gentamicin, kanamycin, streptomycin, chloramphenicol, ciprofloxacin, and trimethoprim/sulfamethoxazole. The antibiotics were tested at concentrations established by the National Antimicrobial Resistance System (12).

Isolates that were multidrug resistant (resistant to two or more antimicrobial agents) were grown overnight in 5 mL of Mueller-Hinton broth (Accumedia, Baltimore, MD) with the appropriate concentration of antimicrobial compound. A 1-mL aliquot of the culture was centrifuged at 10,000 rpm for 2 min. The cell pellet was resuspended in 500 µL of sterile water and boiled for 10 min. The resulting DNA suspension was used as template DNA in polymerase chain reaction (PCR) amplification for the class 1 and class 2 integrase gene and variable regions using the primer sequences shown in the Table (13–15).

The PCR reactions used 10 µL of template DNA, 5 µM of primers, 25 mM MgCl, 10 mM deoxynucleotide triphosphate, and 23 ng bovine serum albumin. Nuclease-free water (Ambion, Austin, TX) was added to achieve a volume of 50 µL. A "hot start" method was used, and 1.25 U of *Taq* DNA polymerase (Sigma, St. Louis, MO) was

Table. Oligonucleotide primer sequences used for amplification of class 1 and class 2 integrase and variable regions

Primer	Primer sequence	Target	Reference
integ-1	5′-GGCATCCAAGCAAG-3′	5′-Class 1 integron variable region	Levesque et al. 1995 (13)
integ-2	5′-AAGCAGACTTGACCTGA-3′	3′-Class 1 integron variable region	Levesque et al. 1995 (13)
hep51	5′-GATGCCATCGCAAGTACGAG-3′	5′-Class 2 integron variable region	White et al. 2001 (14)
hep74	5′-CGGGATCCCGGACGGCATGCACGATTTGTA-3′	3′-Class 2 integron variable region	White et al. 2001 (14)
intI1.F	5′-GGGTCAAGGATCTGGATTTCG-3′	5′-*intI1* gene	Mazel et al. 2000 (15)
intI1.R	5′-ACATGGGTGTAAATCATCGTC-3′	3′-*intI1* gene	Mazel et.al. 2000 (15)
intI2.F	5′-CACGGATATGCGACAAAAAGGT-3′	5′-*intI2* gene	Mazel et al. 2000 (15)
intI2.R	5′-GTAGCAAACGAGTGACGAAATG-3′	5′-*intI2* gene	Mazel et al. 2000 (15)

added after initial template denaturation. The PCR cycle was as follows: initial denaturation for 12 min at 94°C, hot start pause at 80°C followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 5 min at first cycle. An additional 5 s was progressively added to each cycle to reach a final of 7 min, 55 s. PCR products were analyzed on 1% agarose gel.

Amplification products were extracted from the gels with the QIAGEN QIAquick gel extraction kit (Valencia, CA). The amplified products were sequenced at a commercial facility (MWG Biotech Inc., High Point, NC) with the class 1 and class 2 integron variable region primers (*integ* and *hep*) (Table). Contiguous sequences were created from single sequence reads by using the CAP3 sequence assembly program (16). Contiguous sequences were analyzed by using the GenBank database of the National Center for Biotechnology Information and the BLASTX search engine (17). Putative gene relationships and sequence data were analyzed by using a multiple sequence alignment created by using Clustal W version 1.82 (18).

Results

Of the 322 *E. coli* isolates from sediment and irrigation water samples analyzed for antimicrobial resistance, 104 (32%) isolates showed resistance to at least one of the antimicrobial compounds (Figure 1). Approximately 10% (32/322) of all the isolates showed a multidrug resistance phenotype. Eighteen percent of the isolates were resistant to cephalothin; however, only 5 (2%) of 322 were resistant to ceftriaxone, which also belongs to the cephalosporin family. Resistance to ampicillin was prevalent in approximately 35 (11%) of the isolates. Resistance to tetracycline (9%), kanamycin (2%), gentamicin (0.3%), and streptomycin (4%) was also observed. Resistance to the fluoroquinolone ciprofloxacin was seen in one isolate. Three (<1%) of the 322 isolates were resistant to sulfonamide sulfamethoxazole. On the basis of analysis of variance, antimicrobial resistance and the sampling location were correlated. Isolates from the El Paso sampling region had significantly higher ($p<0.05$) antimicrobial resistance as compared with the Presidio and Weslaco sampling regions (data not shown).

The 32 isolates identified as multiple antimicrobial resistant were assayed by PCR amplification for class 1 and class 2 integrase genes. Four isolates (approximately 13%) had the class 1 integrase gene *intI1* (Figure 2A), and one isolate had the class 2 integrase gene *intI2* (Figure 2B). Isolates identified as having the class 1 or class 2 integrase genes were further characterized through PCR amplification of the class 1 and class 2 variable regions. Of the four amplified class 1 integron variable regions, three isolates (isolate 16, isolate 19, and isolate 21) were approximately 1 kb in size, but the fourth isolate (isolate I-6) harbored a 2-kb fragment (Figure 3A). The 1-kb amplification products were observed in isolates from the El Paso area. Nucleotide sequencing showed that all of the 1-kb sequences contained a conserved configuration of a 780-bp gene cassette identified as the *aadA* gene (Figure 4). The 2-kb amplification product was seen in an isolate from the Presidio sampling region. Nucleotide sequencing showed that the variable region contained a 498-bp gene cassette, identified as the *dhfrXII* gene, which encodes trimethoprim resistance. The gene cassette did not exhibit perfect homology with the *dhfrXII* gene (Figure 4). Within the identified 498-bp gene cassette, a 323-bp stretch showed 97% sequence homology; in addition, 59-bp and 56-bp fragments showed 88% and 89% homology, respectively. "Islands" of sequence within the variable region showed no sequence homology to any known genes.

When the 32 multiply antimicrobial-resistant isolates were screened for class 2 integrons, only 1 isolate was pos-

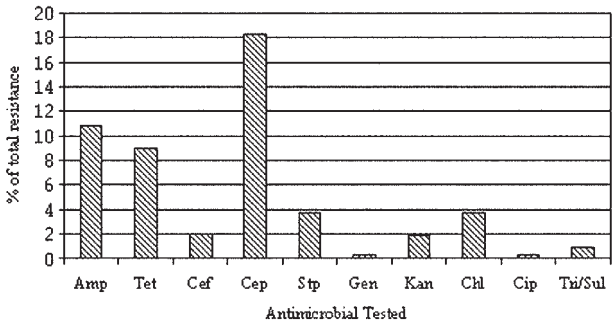

Figure 1. Frequency of antimicrobial resistance observed in 322 *Escherichia coli* isolates from irrigation water along the Texas-Mexico border.

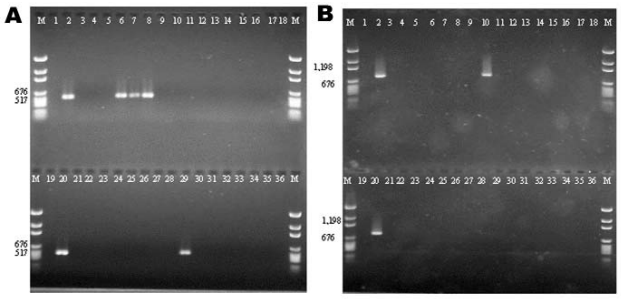

Figure 2. Agarose gel electrophoresis of integrase gene polymerase chain reaction (PCR) amplification products. A: PCR products of class 1 integrase gene intI1. Lane M; molecular marker; lanes 1 and 19: no template (negative) control; lanes 2 and 20: positive control (In2); lanes 3–36: multiple drug–resistant isolates. B: PCR products of class 2 integrase gene intI2. Lane M: molecular marker; lanes 1 and 19: no template control (negative) control; lanes 2 and 20: positive control (Tn7); lanes 3–36: multidrug-resistant isolates.

itive (Figure 2B). This particular isolate (isolate 29) was obtained in the Presidio region and had a 2,600-bp variable region (Figure 3B). Nucleotide sequencing identified two distinct gene cassettes, namely, the *sat-1* and *aadA* genes, which code for streptothricin acetyl transferase, and aminoglycosede adenyltransferase, respectively (Figure 5).

Discussion

Antimicrobial resistance in human pathogens has become a major public health issue. Resistant organisms have been isolated from a number of natural and man-made environments (6,19,20). In natural environments, resistant organisms can be indigenous or introduced through natural or anthropogenic causes (21,22). Integron gene sequences have been identified as a primary source of resistance genes and are suspected to serve as reservoirs of antimicrobial resistance genes within microbial populations (1,2,23,24). Previous studies along the Texas-Mexico border have shown that fecal contamination of the Rio Grande does occur (7,25). The isolation of 322 *E. coli* isolates from irrigation water and associated sediments further confirms that fecal wastes are affecting this body of water. Previous studies have reported that municipal and animal wastes regularly harbor multidrug-resistant *E. coli* strains (6,26,27). In this study, 18% of the isolates were resistant to cephalothin. These results are similar to those from a recent survey of U.S. rivers, which found cefotaxime (a third-generation, cephalosporin-resistant, gram-negative bacterium) to range from 16% to 96% across 22 rivers (19). The higher frequency of isolation of resistant strains from the El Paso region compared with the other, less urbanized sampling locations is not surprising since the effluent from a number of wastewater treatment plants enters the river at that region (W. McElroy, unpub. data; 28). Previous studies with sludge and septic tank wastes showed relatively high levels of antimicrobial resistance in *E. coli* (6). The precise sources of the *E. coli* isolates used in this study could not be identified because of technical limitations in source tracking (29).

Class 1 and class 2 integron gene sequences were found within these *E. coli* isolates. Together, they accounted for 5 (16%) of 32 multidrug-resistant isolates characterized in this study. This prevalence was higher than that reported by Rosser et al. (30), who showed that 3.6% of gram-negative bacteria in an estuarine environment contained the class 1 integron. Three of the four class 1 integron-bearing *E. coli* in this study contained the nucleotide sequence of the spectinomycin-streptomycin resistance gene *aadA1* (31). Resistance to streptomycin was not observed in these isolates, but resistance to the closely related kanamycin was seen. These results are similar to those reported by Zhao et al. (3), who identified that the *aadA* gene transferred to a strain of *Hafnia alvei* but did not report resistance to streptomycin or spectinomycin. These researchers attributed their findings to the inefficient expression of the inserted gene cassette by the integron promoter. Previous studies have also shown that the antimicrobial resistance phenotype can be modulated once these strains are exposed to specific environmental conditions (32).

The *aadA* gene cassette is not novel in class 1 integrons. Earlier work by Zhao et al. (3) and Bass et al. (24) has shown that the *aadA* gene is highly conserved among Shiga toxin–producing and avian clinical *E. coli* isolates, respectively. The only class 2 integron-bearing strain isolated in this study also contained the *aadA* gene in addition to the *sat-1* gene, which codes for resistance to kanamycin, a finding in agreement with the phenotypic expression.

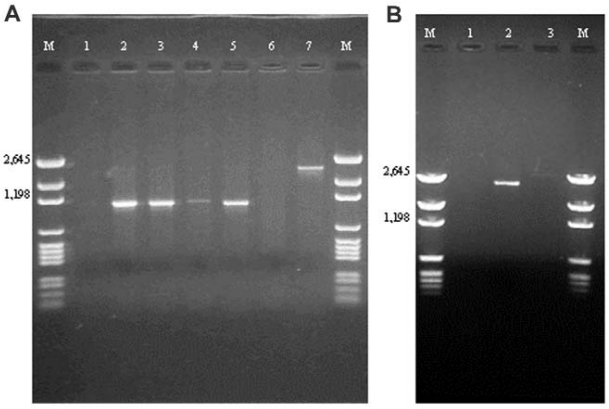

Figure 3. A: Polymerase chain reaction (PCR) amplification products with primers targeted against the class 1–specific conserved sequences. Lane 1: no template control; lane 2: positive control (In2); lane 3: *Escherichia coli* isolate 16; lane 4: *E . coli* isolate 19; lane 5: *E. coli* isolate 21; lane 6: blank; lane 7: *E. coli* isolate I-6. B: PCR amplification products with primers targeted against the class 2–specific conserved sequences. Lane 1: no template control; lane 2: positive control (In2); lane 3: *E. coli* isolate 29.

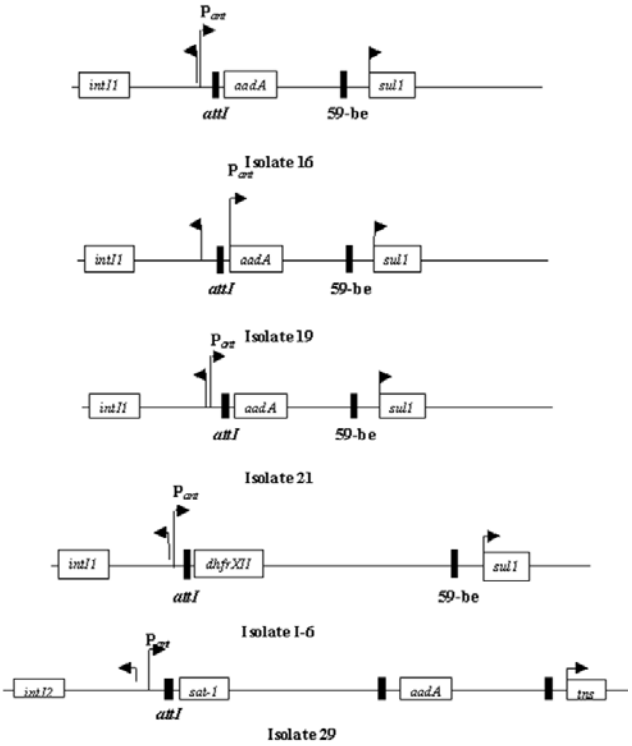

Figure 4. Schematic representations of the four class 1 integrons and one class 2 integron sequenced from multiple antibiotic–resistant *Escherichia coli* isolates.

The *sat-1* gene, which codes for the streptothricin acetyl transferase, was not detected in any other *E. coli* isolate. The presence of the *sat-1* gene cassette, in combination with the *aadA* gene, suggests that this class 2 integron is likely a derivative of the class 2 integron found on transposon Tn*7* (33,34).

The *aadA* gene was conserved among the class 1 and class 2 integrons, which suggests a possible selective mechanism for this cassette in enteric bacteria from natural waters. The 2-kb integron-specific variable region–containing strain, which was isolated from the Presidio area, harbored the dihydrofolate reductase gene (*dhfrXII*) instead of the *aadA* gene (35).

Overall, these results suggest that the irrigation canals and sediments associated with the Rio Grande are contaminated by bacteria of fecal origin that contain antimicrobial resistance genes. Of 322 *E. coli* isolates, 32 (approximately 10%) were resistant to multiple antimicrobial drugs. Five of these 32 *E. coli* isolates harbored class 1 and class 2 integron sequences. This study did not investigate the possibility that other integron-bearing nonfecal bacteria were present. The occurrence of integron-bearing *E. coli* in irrigation water is important since these organisms are known fecal contaminants, and the potential for lateral gene transfer exists. The results also indicate that integron-bearing strains may not always express the antimicrobial phenotype; thus, phenotype-based isolation of resistant organisms can underestimate the levels of resistant organisms. Studies are needed to identify whether integron-mediated antimicrobial resistance transfer does indeed occur within the irrigation canal sediments and on vegetable surfaces, when they are irrigated with contaminated irrigation water.

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Mr. Roe conducted this study while an M.S. student in the Food Safety and Environmental Microbiology Laboratory in the Poultry Science Department at Texas A&M University. His research interests are in environmental microbiology and food safety.

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Hantavirus Prevalence in the IX Region of Chile

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An epidemiologic and seroprevalence survey was conducted (n=830) to assess the proportion of persons exposed to hantavirus in IX Region Chile, which accounts for 25% of reported cases of hantavirus cardiopulmonary syndrome. This region has three geographic areas with different disease incidences and a high proportion of aboriginals. Serum samples were tested for immunoglobulin (Ig) G antibodies by enzyme-linked immunosorbent assay against Sin Nombre virus N antigen by strip immunoblot assay against Sin Nombre, Puumala, Río Mamoré, and Seoul N antigens. Samples from six patients were positive for IgG antibodies reactive with Andes virus; all patients lived in the Andes Mountains. Foresting was also associated with seropositivity; but not sex, age, race, rodent exposure, or farming activities. Exposure to hantavirus varies in different communities of IX Region. Absence of history of pneumonia or hospital admission in persons with specific IgG antibodies suggests that infection is clinically inapparent.

Hantaviruses, RNA-containing viruses, compose a genus within the family *Bunyaviridae*. The natural reservoirs of the pathogenic New World hantaviruses are rodents of the family *Muridae*, subfamily *Sigmodontinae*, in which a chronic and asymptomatic infection develops (1). Hantavirus is a zoonosis transmitted from rodents to humans by inhaling contaminated aerosols from feces, urine, and saliva of infected mice (2).

Human infection with hantaviruses have been associated with two diseases. One is the hemorrhagic fever with a renal syndrome (HFRS) caused by Hantaan, Puumala, Seoul, and Dobrava/Belgrade viruses, first recognized during the Korean War in 1950 to 1953. HFRS occurs mainly in Asia and Europe; death rates range from 0.1% to 15% (3,4). The other disease, a severe respiratory illness known as hantavirus cardiopulmonary syndrome (HCPS), occurs in the Americas and has a death rate of 40% (1,5–7). We prefer the term hantavirus cardiopulmonary syndrome to an alternative term, hantavirus pulmonary syndrome, because most deaths associated with HCPS are related to cardiac failure rather than pulmonary failure, and this aspect of the syndrome remains underappreciated by practitioners and others.

HCPS has been identified in several countries in North and South America and is caused by different hantaviruses: Sin Nombre in North America, Juquitiba virus in Brazil (8), Laguna Negra virus in Paraguay and Bolivia (9,10), Andes in Argentina and Chile (11,12), Choclo virus in Panama (13), and several subspecies or viral genotypes of Andes virus in Argentina (e.g., Oran, Lechiguanas, et al.) (14–16).

In 1995, Andes virus was first identified in the Argentinean Patagonia and was recognized in central and southern regions of Chile. The reservoir is the long-tailed pygmy rice rat (*Oligoryzomys longicaudatus*), a species that occurs primarily in temperate forest (17).

Since HCPS emerged in Chile, 287 cases have been confirmed as of March 2003, causing a substantial impact on the public health system; the death rate for HCPS in Chile has exceeded 40%. In addition to the 287 cases of HCPS, 17 cases of mild infection with no cardiopulmonary involvement have been reported, demonstrating that hantavirus infection may have variability in its expression. Serologic studies have established both clinically asymptomatic infections and symptomatic infections not recognized at the time as hantavirus infection (18–23).

The phenomenon of clinically nonapparent infections varies in different areas and populations of the Americas. In the United States, the proportion of infection versus disease is thought to be close to 1% (i.e., disease develops in most of the infected patients, and human population seroprevalence varies between 0.2% to 1.7%) (4,24,25). Seroprevalence studies in South America, however, have shown that some populations have had much more frequent exposure to hantaviruses in the absence of known clinical manifestations, as seen in some populations native to Paraguay and Northern Argentina (40% and 17%, respectively) (26), the general population in Aysen, Southern Chile (2.0% and 13.1% in urban and rural areas,

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respectively) (27), São Paulo and Bahia, Brazil (1.23 % and 13.1%, respectively) (28,29), and recently in Panama (30%) (13). The populations with the highest seroprevalence were indigenous persons rather than those of European ancestry.

Two hypotheses have been proposed to explain these phenomena. The first one presumes that strains in South America are of lesser pathogenicity, though in many cases nonpathogenic viruses have yet to be detected in local rodent populations. The second hypothesis involves the existence of at least two variables: the nature of the exposure and genetic constitution of the host population. The high seroprevalence seen in Paraguay (26) could be caused by a higher resistance in the aboriginal population and greater exposure to the virus in some regions. The prevalence and high case-fatality ratios seen in North America might be the result of a lower exposure, a lesser genetic resistance to disease, or both.

More than 20 strains of hantaviruses are known worldwide; not all of them are associated with human disease. The observations discussed in this article suggest the need to study whether hantaviruses of lesser pathogenicity and other clinical entities until now unrecognized may occur in areas where the seroprevalence is high and disease incidence is low (11,14–16,18). For example, hantaviruses previously thought to be endemic only in Europe and Asia have also been recognized in the North American continent (30–32).

To study the seroprevalence of hantavirus in Chile, we studied the general population in the IX Region for the reasons that follow. This area is ranked third among those affected by HCPS, accounting for 25% of the known cases in Chile. Three different areas can be distinguished on the basis of geographic features in relation to the two mountain ranges that traverse the region (the Pacific Coast and the coastal range [Coastal], the Central Valley [Central], and the pre-Andean Region [Andean]). The Mapuche, the main native ethnic population of Chile, account for 26.3% of the total regional population. Members of the tribe can readily be identified from surnames, which are derived from both parents. Therefore, we were able to study the seroprevalence of hantavirus in the same region but in areas having different incidence rates of the disease and evaluate the asymptomatic infection in the Mapuche aboriginal population.

Methods

Study Design and Area

We conducted a cross-sectional epidemiologic and serologic survey to determine the prevalence of IgG antibodies against hantavirus N antigen in the adults living in nine communities of the IX Region of Chile. The IX Region of Chile is located in the southern part of the country, between parallel 37° and 40° South and the meridians 70° and 74° East (Figure). The region has a surface area of 31,842.3 km^2 and 781,242 inhabitants in 31 communities; of the total population, 38.71% is rural. The characteristics of these populations can be distinguished according to geographic features. The Mountainside Andes range (Andean) is rural area with small towns; the population there is primarily sustained with farming, woodcutting, and tourist services. The population composes 13.8% of the region's total population, yet accounts for 82% of HCPS cases. The Central Valley intermediate depression (Central) is a predominantly urban area in which the main sources of income are industry, farming, and ranching. Temuco, the capital and main urban center, is located in this area. The Central Valley contains 67.3% of the total population and accounts for 5.9% of HCPS cases. Coastal inhabitants account for 19% of the region's population; the community sustains itself with farming and forestry activities, fishing, and tourism. Twelve percent of HCPS cases originate in this area.

Sample Design

The sample included 847 persons, enough to give us accurate point seroprevalence given estimated seroprevalence rates of 7%, 3%, and 5% for the Andean, Central, and Coastal areas, respectively, as derived from previous studies in the country (27) and the distribution of HCPS cases in the region. The sample was designed to be representative of each community according to information available from the National Statistics Agency about population sex, ethnicity, age distribution, and rural-urban proportion. Persons were contacted in their homes with a predetermined plan that included the selection of the housing blocks to be studied, the home at which to start enrollment, and how to proceed thereafter. Only one person was

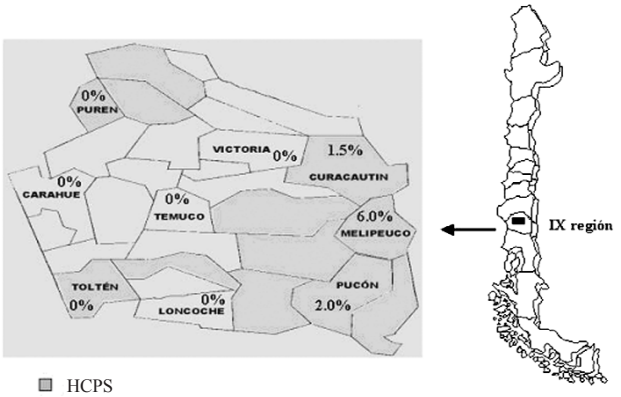

Figure. Geographic IX Region, Chile, and hantavirus seroprevalence in the tested communities of the IX Region. HCPS, Hantavirus cardiopulmonary syndrome.

enrolled in each household contacted. That person was informed of the study, signed an informed consent, and agreed to answer a questionnaire and donate a sample of blood. Only persons ≥15 years of age who were free of any current febrile respiratory illness were asked to enroll. The epidemiologic questionnaire, previously validated in the community, requested information about sex, age, race, residency, labor activity, rodent exposure at home or work, cardiorespiratory disease history, hospital admissions, and direct contact with HCPS patients.

Nine communities were included, three per geographic area. Curacautín, Melipeuco, and Pucón (Figure) were chosen as representative of the Andean area (n=277); Victoria, Temuco, and Loncoche from the Central area (n=279); and Purén, Carahue, and Toltén from the Coastal area (n=291). The Andean and Coastal areas were considered rural; the Central area was considered to be a composite of rural and urban. Persons were classified as Mapuches when they had at least one surname of Mapuche derivation. The study was approved by the Catholic University Ethics Committee.

Collection, Processing, and Analysis of Samples

Blood samples were collected, transported, and centrifuged the same day. Serum samples were kept frozen at –20°C and sent for analysis to the Laboratory of Virology at the Pontificia Universidad Católica de Chile, Santiago, Chile. An enzyme-linked immunosorbent assay (ELISA) was performed to detect hantavirus-specific immunoglobulin (Ig) G antibodies in all samples.

ELISA was performed as described (33,34) by using recombinant antigen for the Sin Nombre strain (produced in *Escherichia coli* and provided by Centers for Disease Control and Prevention [CDC]), which crossreacts with all American hantaviruses. Briefly, serial dilutions (1:100, 1:400, 1:1,600, and 1:6,400) of patient serum samples were incubated for 1 h at 37°C in antigen-coated, 96-well plates. Peroxidase-coupled anti-human IgG was used as secondary antibody and incubated 1 h at 37°C. After substrate reaction, plates were read at 414 nm.

The net absorbance values are results of the hemiplates absorbance subtraction with and without antigen. To be considered positive, the net absorbance values for a sample had to be ≥0.2 in the 1:100 and 1:400 dilutions, and the sum of all net absorbance values had to be ≥0.95. Positive serum samples were tested twice and verified to be positive at CDC.

A confirmatory strip immunoblot assay (SIA) was performed an all positive samples (B.H.). Four recombinant N antigens from Sin Nombre, Puumala, Rio Mamoré, and Seoul hantaviruses were fixed onto a nitrocellulose membrane by vacuum. Serum samples (1:200) were incubated with the strips and alkaline phosphatase-coupled anti-human IgG added. Reactivity was estimated visually, and each band given an intensity value on a four-point scale, as previously described (35,36). We regarded a sample as positive for antibodies when reactive to both Sin Nombre and Rio Mamoré on the basis of the presumption that antibodies to Andes virus will always react to closely related Rio Mamoré and generally will crossreact with the other sigmodontine rodent-borne virus, Sin Nombre. A sample was regarded as confirmed seropositive when the serum was reactive in the ELISA and confirmed by the SIA. Fisher exact test was used for the analysis of independent variables. A p value <0.05 was considered significant.

Results

Sample Population Characteristics

A total of 829 persons were included in the study, with 271, 272, and 286 from the Andean, Central, and Coastal, respectively. The age range was 15–88 years of age (mean 39.4 years of age); 47.6% were men, and 18.3% were of Mapuche origin. In all, 73.3% of the sampled population was considered rural. Men worked in farming, forestry, or both in 61.8% of the cases; 62.2% of the women were housewives. A history of exposure to rodents, their excreta, or both at home was reported in 88.0% of enrolled persons; 54.6% reported exposure at work.

Serology

Six (0.72%) samples were hantavirus antibody-positive by both ELISA and SIA. The samples came from persons who lived in the Andean area, giving a seroprevalence of 2.15% (6/271) for this area, significantly higher than the other two regions studied (p=0.0001). The seropositive cases belonged to each of the three counties studied; however, relative frequencies varied: Curacautín, 2.6% (2/132); Melipeuco, 6.1% (2/33); and Pucón, 2.0% (2/100). All three counties have previously had HCPS cases reported (Figure). As shown in Table 1, four case-patients were male, and two were Mapuche. The case-patients ranged from 28 to 76 years of age, with a mean of 52.8 years of age. Seropositivity could not be associated with sex (p=0.43), race (p=0.30), or age (p=0.18). Five of the case-patients worked in farming or forestry, although only forestry had a significant association with seropositivity (p=0.018), meaning the risk for infection was 10 times higher (relative risk 9.72; confidence interval 1.15 to 82.44). Five of the case-patients said they had been exposed to rodents or their excreta either at home or work. This exposure, however, did not reach statistical significance (p=0.1 and p=1.0, respectively). The sixth case-patient was not exposed to rodents; however, he had been working in a large shed, the woods, and a sawmill; he also had been weeding. Finally, none of the six antibody-posi-

Table 1. Relationship between independent variables and seropositivity to hantaviruses, IX Region, Chile

Variables	Seropositive (total)	p value	RR (95% CI)[a]
Sex			
Male	4 (395)	0.43	
Female	2 (435)		
Age			
15–44 y of age	2 (550)	0.18	
>44 y of age	4 (280)		
Race			
Mapuche	2 (151)	0.30	
Other	4 (679)		
Area			
Andean	6 (271)	0.0001[a]	NA[b]
Central	0 (273)		
Coastal	0 (286)		
Risk activity or labor			
Agrarian[c]			
No activity	1 (271)		
Two or more	4 (341)	0.38	
Foresting[d]			
No activity	1 (279)		
With activity	5 (135)	0.018	9.72 (1.15 to 82.44)
Exposure to rodents			
Peridomestic	5 (731)	0.10	
Laboral	5 (453)	1.00	

[a]p ≤0.05; p values were determined by using 2-tailed Fisher exact test; RR, relative risk; CI, confidence interval.
[b]Not applicable.
[c]Clearing, shrubbery cutting, working in pastures or cellars.
[d]Foresting, working at a sawmill.

tive persons had previous contact with HCPS patients, history of pneumonia, or hospital admission.

Discussion

This study shows serologic evidence of past infection by hantavirus in the Chilean population. The hantavirus associated with symptomatic infections in Chile is Andes virus, identified in patient blood samples by reverse transcription-polymerase chain reaction (RT-PCR) and recently isolated from human blood (12,37).

Our results show the global seroprevalence to hantavirus in the adult general population in the IX Region to be 0.72% (ranging from 0 to 6%, depending on the community). Seroprevalence is significantly higher in the Andes rural area, consistent with the observed elevated incidence of HCPS disease in this area of Region IX between 1997 and 2000 (Table 2). This geographic distribution is probably related to the great tracts of native forest, where *Chusquea quila*, a bamboo-like shrub that protects and feeds the carrier rodent, is abundant. Moreover, the increasing development of the forestry industry plus an increase in the rodent population caused by the favorable climatic condition because of the El Niño effect have caused humans and mice to interact closely.

We did not find an association between seroprevalence and reported exposure to rodents, probably because this type of exposure is frequent in all groups in the region, occurring both at work and at home. The exposure to rodents or their excreta is necessary but apparently not sufficient to acquire the infection. Presumably an exposure to a specific reservoir mouse or virus carrier, human behavior, and biologic factors are important.

Forestry is associated with a higher risk for infection. This labor, whether in the woods or at sawmills, is performed almost exclusively by men; both men and women share farm work in most instances. This finding may explain why a higher proportion of HCPS develops in men (75% of reported cases). However, any apparent association between hantavirus exposure and a particular occupation does not necessarily implicate these occupations as a risk factor. In fact, occupation may be a marker for living, sleeping, or housing conditions that constitute the proximate risk factor for exposure. Indoor exposure to rodents is common in patients with HCPS (38).

On the basis of these results, we argue that infections by hantavirus follow a gradient of exposure to the virus in the

Table 2. Incidence of hantavirus cardiopulmonary syndrome (HCPS) and seroprevalence in Chile's IX Region according to geographic area

	Region					
	Global	Andean (A)	Central (B)[b]	Coastal	p value[a] (A vs. B)	RR (95% CI)[a]
Disease						
Cases	34	29	1	4	0.0001	29.6 (11.46 to 76.47)
Population	781,242	127,974	424,278	229,190		
Cumulative Incidence (1997-2001) ($x10^6$)[c]	4.35	22.66	0.23	1.74		
Infection						
Seropositive	6	6	0	0	0.00013	NA[d]
Sample	829	271	272	286		
Prevalence (%)	0.72	2.15	0	0		

[a]p values were determined using 2-tailed Fisher exact test; RR, relative risk; CI, confidence interval.
[b]B is Central and Coastal regions combined.
[c]Chile's Department of Health, March 2001.
[d]Not applicable.

Table 3. Incidence of hantavirus cardiopulmonary syndrome (HCPS) and seroprevalence in Chile's IX Region according to race

	Seroprevalence	Incidence	HCPS cases/population	
Race	Positive/total (%)	RR (95% CI)[a]	(rate x 100.000)	RR (95% CI)[a]
Mapuche	2/151 (1.3)	2.23 (0.41 to 12.06)	4/205,466 (1.9)	0.37 (0.13 to 1.06)
Other	4/678 (0.6)	–	30/575,776 (5.2)	

[a]Relative risk (95% confidence interval). p values were determined by using two-tailed Fisher exact test; RR, relative risk; CI, confidence interval.

IX Region. Our population has a different epidemiologic profile to those of the aborigines of Paraguay (40%) and North Argentina (17%) in South America, which have low seroprevalence, similar to that described for North American populations, as reported by Vincent (13) and Ferrer (26). This lower seroprevalence could be because of a greater pathogenicity and clinical severity in infections by the prototypical (southern) form of Andes virus, similar to Sin Nombre virus.

Less pathogenic hantaviruses may cause a greater amount of asymptomatic infections, as seen for HFRS in Europe and Asia (seroprevalence 7.9% to 10%, death rate, 0.1% to 15%) (39) and some American hantaviruses (Laguna Negra in Bolivia and Paraguay, Choclo in Panama, Oran and Lechiguanas in Argentina) for which seroprevalence is high and case-fatality ratios are <30% (9,13,15). This finding is in contrast to the findings of hantaviruses with severe clinical syndromes and high death rate (i.e., Sin Nombre and Andes viruses, both of which have been associated with few subclinical infections). The lack of subclinical infections can be caused by a variation in virulence or by different genotypes of the hosts, which give them greater resistance to infection and disease.

A slightly disproportionate fraction of seropositive samples (33%) were from Mapuche (18.3% of those sampled were Mapuche). Moreover, when both hantavirus seroprevalence and the HCPS incidence rates in this region are considered, more infection but less disease (not significant) is found in the Mapuche population (Table 3). Future epidemiologic studies should address this finding and use a larger sample to evaluate possible associations between racial origin and the incidence rates of infection and disease.

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Antimicrobial Susceptibility Breakpoints and First-Step *parC* Mutations in *Streptococcus pneumoniae*: Redefining Fluoroquinolone Resistance

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Clinical antimicrobial susceptibility breakpoints are used to predict the clinical outcome of antimicrobial treatment. In contrast, microbiologic breakpoints are used to identify isolates that may be categorized as susceptible when applying clinical breakpoints but harbor resistance mechanisms that result in their reduced susceptibility to the agent being tested. Currently, the National Committee for Clinical Laboratory Standards (NCCLS) guidelines utilize clinical breakpoints to characterize the activity of the fluoroquinolones against *Streptococcus pneumoniae*. To determine whether levofloxacin breakpoints can identify isolates that harbor recognized resistance mechanisms, we examined 115 *S. pneumoniae* isolates with a levofloxacin MIC of >2 μg/mL for first-step *parC* mutations. A total of 48 (59%) of 82 isolates with a levofloxacin MIC of 2 μg/mL, a level considered susceptible by NCCLS criteria, had a first-step mutation in *parC*. Whether surveillance programs that use levofloxacin data can effectively detect emerging resistance and whether fluoroquinolones can effectively treat infections caused by such isolates should be evaluated.

The emergence of *Streptococcus pneumoniae* resistance to β-lactam and macrolide antimicrobial agents has led to recommendations that fluoroquinolones with increased activity against *S. pneumoniae*, such as levofloxacin, moxifloxacin, and gatifloxacin, be used to treat patients at risk for infection caused by such multidrug-resistant strains (1–6). Fluoroquinolone resistance in *S. pneumoniae* is primarily due to mutations in the genes encoding the target topoisomerase enzymes, namely *parC*, which encodes the A subunit of DNA topoisomerase IV, and *gyrA*, which encodes the A subunit of DNA gyrase (7). Mutations in *parE* and *gyrB* have been reported, but to a lesser extent (8–10). Most pneumococcal isolates with reduced susceptibilities to fluoroquinolones have amino acid substitutions in either ParC alone or ParC and GyrA (11–14). Resistance can also be mediated by active efflux (15), although the role of efflux in contributing to resistance by the newer fluoroquinolones is unclear (16).

The MIC of an antimicrobial agent is a value that has been used to determine breakpoints that predict the probability of clinical success, detect resistant populations, or both (17). Clinical breakpoints are dependent on the antimicrobial activity and pharmacology of the drug; such breakpoints are ascertained with the goals of eradicating the infection and ultimately achieving clinical success with the antimicrobial agent. In contrast, microbiologic breakpoints are established to identify isolates that may be categorized as susceptible when applying clinical breakpoints but harbor resistance mechanisms that result in their reduced susceptibility to the agent being tested. These microbiologic breakpoints are therefore useful in monitoring the emergence of resistance. The current National Committee for Clinical Laboratory Standards (NCCLS) guidelines make no distinction between these two interpretations of MIC, with clinical breakpoints used to characterize most antimicrobial agents, including the fluoroquinolones.

Levofloxacin has been used as a surrogate marker to predict fluoroquinolone susceptibility in clinical laboratories and surveillance studies (18). To establish whether current levofloxacin breakpoints are also able to function as microbiologic breakpoints, we determined the percentage of *S. pneumoniae* isolates with first-step *parC* mutations that would go undetected by using the current NCCLS breakpoints for levofloxacin (19).

Materials and Methods

A total of 6,076 clinical isolates of *S. pneumoniae* were collected as part of a 1993–1998 surveillance program

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throughout Canada. All isolates were identified as *S. pneumoniae* by standard methods. The isolates were frozen at –70°C, thawed, subcultured onto blood agar, and incubated at 37°C in 5% CO_2 for 24 h twice before testing. In vitro susceptibility testing was performed by broth microdilution, according to NCCLS guidelines (20,21). Susceptibility interpretive criteria used were those published in the NCCLS M100-S12 document (19). The nonsusceptible category was defined as those isolates with MICs of fluoroquinolines in the intermediate and resistant category. The *parC* gene of 115 isolates with a levofloxacin MIC ≥2 μg/mL (82 = MIC 2 μg/mL; 8 = MIC 4 μg/mL; 10 = MIC 8 μg/mL; and 15 = MIC ≥16 μg/mL) was amplified by polymerase chain reaction (PCR), and the nucleotide sequence determined as previously described (9). All isolates (n=33) with a levofloxacin MIC of ≥4 μg/mL, and a random sample of 29 isolates with a levofloxacin MIC of 2 μg/mL were examined for *gyrA* mutations. For comparative purposes, the *parC* gene of 14 isolates with a ciprofloxacin MIC of 2 μg/mL, regardless of their levofloxacin MIC, was amplified and sequenced. Although numerous single mutational events occur in *parC*, the focus of this investigation was on amino acid substitutions for Ser-79 or Asp-83, because previous studies have consistently demonstrated that mutations at either of these positions are associated with decreased susceptibility (9,14).

Crude cell lysates were used as DNA templates for PCR. After overnight growth on Columbia nutrient agar and supplemented with 5% sheep blood, a loopful of growth was suspended in 100 μL of lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.3], 1 mM EDTA, 1% Triton X-100) and boiled for 10 min. Ten microliters of the supernatant was used as the template in a 50-μL reaction volume. The quinolone-resistance–determining regions of *parC* and *gyrA* were amplified by PCR. Primers used were based on published sequences (7,8), and amplification products were purified with either the QIAquick PCR purification kit (Qiagen Inc., Mississauga, Ontario, Canada) or the Concert Rapid PCR purification kit (Life Technologies, Burlington, Ontario, Canada).

DNA sequencing was performed by ABI prism Big Dye terminator cycle sequencing with the ABI 377 automated sequencer (PE Applied Biosystems, Mississauga, Ontario, Canada). Nucleotide and amino acid sequence comparisons were performed by the multiple-alignment sequence function of Vector NTI Suite software (InforMax Inc., Bethesda, MD). The GenBank accession numbers for the wild-type sequences used for comparison purposes were Z67739 for *parC* and *parE* (22), AB010387 for *gyrA*, and Z67740 for *gyrB* (23).

Isolates were examined for active efflux by agar dilution on Mueller-Hinton agar containing 5% sheep blood in the presence of ciprofloxacin with or without 10 mg/mL of reserpine (Sigma Chemical Co., St. Louis, MO) (24). Strains for which a fourfold or greater decrease in the MIC of ciprofloxacin existed in the presence of reserpine were considered in this study to be positive for reserpine-inhibited efflux. *S. pneumoniae* strain P121/1N27 and clinical isolate BSP 823 were used as quality control strains, the latter of which demonstrated a 16-fold decrease in the ciprofloxacin MIC in the presence of reserpine (9).

Results

Of the 115 *S. pneumoniae* isolates with a levofloxacin MIC ≥2 μg/mL, 78 (69%) had an amino acid substitution in ParC (Ser-79 or Asp-83) (Table 1). Mutations in *gyrA* were not found in any of the randomly selected isolates with a levofloxacin MIC of 2 μg/mL, but were present in three (38%) of eight isolates with a levofloxacin MIC of 4 μg/mL and in all isolates with a levofloxacin MIC ≥ 8 μg/mL (Table 2). The specific ParC amino acid substitutions of the isolates and their corresponding levofloxacin MICs are shown in Table 1. The most common substitution was Ser-79 to Phe, accounting for 60% of all observed amino acid substitutions. The prevalence of first-step ParC amino acid substitutions among all strains according to their levofloxacin and ciprofloxacin MICs is shown in Table 3. Using the current MIC interpretive standards for levofloxacin, 48 (59%) of 82 of isolates with a first-step mutation fall in the susceptibility category of levofloxacin

Table 1. ParC amino acid substitutions found in 115 *Streptococcus pneumoniae* isolates with levofloxacin MICs ≥2 μg/mL and corresponding levofloxacin MICs

ParC amino acid substitution	No. isolates inhibited by levofloxacin MIC (μg/mL) of					Total no. of strains
	2	4	8	16	≥32	
Ser79→Phe	28	4	3	9	3	47
Ser79→Tyr	7	1	3	2	1	14
Ser79→Ala	1	0	0	0	0	1
Asp83→Asn	7	0	3	0	0	10
Asp83→Gly	1	0	0	0	0	1
Asp83→Tyr	3	0	0a	0	0	3
Asp83→Val	1	0	0	0	0	1
Asp83→Ala	0	0	1	0	0	1
No. isolates/total with amino acid substitutions	48/82 (59%)	5/8[a] (63%)	10/10	11/11	4/4	78/115 (69%)

[a]One isolate with no ParC amino acid substitution found to have active efflux; two isolates had ParC amino acid substitutions at sites other than Ser79 or Asp83.

Table 2. Number of isolates with ParC and GyrA amino acid substitutions and their corresponding levofloxacin MICs

MIC (μg/mL)	No. strains with amino acid substitutions in ParC (%)	ParC and GyrA (%)
2	48/82 (59)	0/29[a] (0)
4	5/8 (63)	3/8 (38)
8	0/10 (0)	10/10 (100)
≥16	0/15 (0)	15/15 (100)

[a]29/82 isolates were randomly examined for GyrA mutations.

(MIC<4 μg/mL). In comparison, 4 (29%) of 14 randomly chosen isolates with a ciprofloxacin MIC of 2 μg/mL harbored a first-step mutation.

Thirty-three isolates were nonsusceptible to levofloxacin (MIC≥4 μg/mL); for 25, which harbored both ParC and GyrA amino acid substitutions, the levofloxacin MIC was ≥8 μg/mL (Table 3). For eight isolates, the levofloxacin MIC was 4 μg/mL; three (38%) of those isolates had a substitution in GyrA (Ser-81-Phe) as well as a substitution in ParC (Ser-79-Phe, Asp-78-Asn and Ala-115-Pro) (Table 4). In addition, two of the eight (25%) isolates had no substitution in GyrA, but were considered positive for reserpine-inhibited efflux, while three isolates had a Ser-79-Phe amino acid substitution in ParC. No mutations were found in either *parE* or *gyrB* in the isolates with a levofloxacin MIC of 4 μg/mL.

Table 3. The prevalence of ParC amino acid substitutions among all strains according to their levofloxacin and ciprofloxacin MICs

MIC (μg/mL)	No. strains with ParC amino acid substitution at 79 or 83	
	Ciprofloxacin (%)	Levofloxacin (%)
2	4/14 (29)	48/82 (59)
4	24/37 (65)	5/8 (63)
8	11/12 (92)	10/10 (100)
>8	22/22 (100)	15/15 (100)
Total	62/87 (71)	78/115 (69)

Discussion

Before the development of fluoroquinolones such as levofloxacin, ofloxacin was used to determine trends of pneumococcal fluoroquinolone resistance in the United States (25). By using this system, an increase of ofloxacin-nonsusceptible isolates from 2.6% in 1995 to 3.8% in 1997 was reported. However, the significance of such an increase was questioned, since ofloxacin-resistant strains could be observed with only a single topoisomerase mutation, whereas for fluoroquinolones such as levofloxacin, multiple mutations are required for a strain to be classified as resistant according to NCCLS breakpoints (25–27). As a consequence, ofloxacin was replaced by levofloxacin in 1998 as a marker for fluoroquinolone nonsusceptibility, and not surprisingly, given levofloxacin's increased activity against *S. pneumoniae*, fluoroquinolone resistance rates were only 0.2% in 1998 and 1999 (25).

Since effective surveillance depends upon the ability to detect the emergence of resistance, the prevalence of pneumococci that harbor resistance mechanisms to the fluoroquinolones may not be accurately represented if surveillance systems that rely on levofloxacin MIC data are used (25,28–34). We found that 59% of isolates with a levofloxacin MIC of 2 μg/mL, a level considered susceptible according to NCCLS criteria, had a first-step mutation in *parC*. Similarly, Davies et al. (12) found that of 14 strains for which levofloxacin MICs were 2 μg/mL, 10 (71%) had a *parC* mutation. Therefore, if the goal of surveillance is to detect emerging problems, then by extension, the detection of first-step mutations is also important and the use of current NCCLS breakpoints to estimate fluoroquinolone resistance is clearly inadequate. Apart from DNA sequencing, currently no accurate test can reliably identify isolates with first-step mutations (35). Although decreasing levofloxacin breakpoints has been proposed as a solution to this problem, we found that 8 (25%) of 32 strains for which the levofloxacin MIC was 1 μg/mL already had first-step mutations (data not shown). Similarly, the replacement of levofloxacin as a surveillance indicator by another fluoroquinolone has also been suggested. However, use of ciprofloxacin does not fare significantly better, with 4 (29%) of 14 isolates in the susceptible category (MIC of 4 μg/mL to define nonsusceptible isolates) harboring first-step mutations.

In addition to causing an underestimation of the emergence of fluoroquinolone resistance, the use of clinical breakpoints has therapeutic implications, as supported by recent reports of treatment failure when a fluoroquinolone was used to treat an infection caused by a strain of pneumococci with a first-step mutation (36,37). Clearly, a first-step mutation is necessary for the development of subsequent mutations, the latter of which result in MICs that fall within the nonsusceptible category. However, studies have shown that upon acquisition of a first-step mutation, the likelihood of developing a subsequent mutation is enhanced in comparison to the development of the first-

Table 4. Characterization of *Streptococcus pneumoniae* isolates with levofloxacin MIC 4 μg/mL[a]

Isolate no.	Amino acid substitution		Change in MIC with inhibition of efflux
	In ParC	In GyrA	
1	Ser79→Phe	None	8-fold
2	Ser79→Phe	None	No effect
3	Asp78→Asn	Ser81→Phe	No effect
4	Ala115→Pro	Ser81→Phe	No effect
5	Ser79→Phe	None	No effect
6	Ser79→Phe	None	No effect
7	None	None	4-fold
8	Ser79→Phe	Ser81→Phe	No effect

[a]*parE* and *gyrB* sequencing was performed on all isolates, but no mutations were found in the quinolone-resistance–determining region.

step mutation itself (38–40). Studies are required to determine whether isolates with one or more mutations in genes encoding ParC, GyrA, or both, can still be effectively treated with a fluoroquinolone when that fluoroquinolone is found to be susceptible by using current clinical breakpoints. Recognizing the presence of underlying mutations may be especially important when using these agents to treat patients with large biomass infections such as pneumococcal pneumonia.

Lastly, the acquisition of a second-step mutation appears more likely than not to raise the MIC to ≥8 μg/mL and not to 4 μg/mL as would be expected. Isolates with a levofloxacin MIC of 4 μg/mL represented 0.1% of the total number of isolates in our study, which is notable, considering that a levofloxacin MIC of 4 μg/mL is currently used to define nonsusceptibility. Furthermore, these isolates were for the most part either genotypically or phenotypically distinct from other isolates characterized (Table 4); two had efflux mechanisms, one singly and the other concurrent with a ParC amino acid substitution, and two had unusual substitutions in ParC (Asp78→Asn and Ala115→Pro). The importance of this latter finding remains to be determined.

In summary, levofloxacin susceptibility testing that uses current MIC clinical breakpoints does not identify most *S. pneumoniae* isolates with only first-step *parC* mutations. This finding may not only have implications for the ability of surveillance programs to detect emerging resistance, but therapeutic implications as well.

Dr. Lim is a physician trained in internal medicine and currently a fellow in infectious diseases and medical microbiology at the University of Toronto. Her research interests include infections in the immunocrompromised host and transplant recipients.

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Mutations in Putative Mutator Genes of *Mycobacterium tuberculosis* Strains of the W-Beijing Family

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Alterations in genes involved in the repair of DNA mutations (*mut* genes) result in an increased mutation frequency and better adaptability of the bacterium to stressful conditions. W-Beijing genotype strains displayed unique missense alterations in three putative *mut* genes, including two of the *mutT* type (Rv3908 and *mutT*2) and *ogt*. These polymorphisms were found to be characteristic and unique to W-Beijing phylogenetic lineage. Analysis of the *mut* genes in 55 representative W-Beijing isolates suggests a sequential acquisition of the mutations, elucidating a plausible pathway of the molecular evolution of this clonal family. The acquisition of *mut* genes may explain in part the ability of the isolates of W-Beijing type to rapidly adapt to their environment.

Tuberculosis (TB) and AIDS cause more deaths in adults worldwide than any other infectious disease. Globally, the number of TB cases is growing at a rate of 2% per year. Resistance, especially multidrug-resistance (MDR), is an increasing problem (1) and a growing hazard to human health. Many outbreaks of MDR-TB, defined as resistance to at least rifampicin and isoniazid, have been reported, with poor response to therapy and very high disease and death rates. Some TB outbreaks have involved patients with HIV co-infection (2,3). Although in several instances, MDR outbreaks associated with a particular genotype, such as the W strain, have been identified (4,5), drug-susceptible variants of the W strain account for most of this group of isolates characterized to date.

In 1995, the largest proportion of the *Mycobacterium tuberculosis* strains from Beijing, China, shared a high degree of similarity in IS*6110* restriction fragment length polymorphism (RFLP) patterns and identical spoligo patterns (6). Subsequent molecular analyses have indicated that the W and Beijing isolates constitute a single group of strains designated as the W-Beijing genotype (Figure 1). The global distribution and success of *M. tuberculosis* isolates of the W-Beijing genotype have led to the hypothesis that these strains may have selective advantages over other *M. tuberculosis* strains. In addition to the W-MDR strain identified in New York City, and areas in Cuba, Estonia, Vietnam, and Russia, the W-Beijing genotype has been significantly associated with drug resistance (7 and unpub. data). Several studies have suggested that the W-Beijing genotype strains are disseminating throughout the world (7). In Vietnam, the proportion of W-Beijing strains was 71% in patients <25 years of age and 41% for those >25 years of age (8). Furthermore, W-Beijing strains have been implicated in several TB epidemics globally, including ones in New York, Texas, California, South Carolina, and New Jersey in the United States (9) and South Africa, Russia, and Spain (10). A recent study showed that 82% of MDR strains isolated in a prison in Azerbaijan, Eastern Europe, are of the W-Beijing genotype (11).

Ongoing research is focused on identifying the factors responsible for the worldwide spread of the W-Beijing strains and their ability to adapt and enhance their pathogenicity or virulence. Identifying a possible mechanism for increased adaptation of these bacteria to the human immunologic host defense system or human interventions such as anti-TB treatment is of the utmost importance. Such mechanisms may indicate how the bacterium adapts to the host, a prerequisite for an enhanced accumulation of genomic mutations associated with resistance. In *M. tuberculosis,* resistance to antibiotics occurs because of genomic mutations in certain genes, such as the *katG* gene for isoniazid (INH) resistance and the *rpoB* gene for rifampicin resistance (12). In contrast to several other pathogens with MDR phenotypes, plasmid or transposon-mediated mechanisms of resistance have not been reported in *M. tuberculosis* (13–15). Since resistance to bacteriostatic in *M.*

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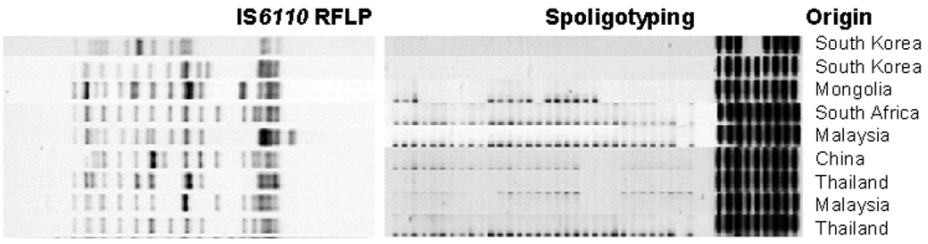

Figure 1. Characteristic patterns of *Mycobacterium tuberculosis* Beijing genotype strains. RFLP, restriction fragment length polymorphism.

tuberculosis is exclusively due to genomic mutations, the bacterium would benefit from an increased mutation rate.

Recent studies provided evidence for a role of mutator phenotypes in the emergence of MDR clinical *Pseudomonas* isolates (16). Such phenotypes not only enable the bacteria to acquire resistance to antibiotics more easily but also facilitate their adaptation to a new niche. Bacteria can escape immune surveillance by modulating bacterial resistance to host defense mechanisms (16–18). This finding prompted us to investigate whether a similar situation exists in *M. tuberculosis*. We have undertaken a comprehensive comparative sequence analysis of selected target genes to evaluate and study the presence of mutations in putative genes expected to play a role in the mutation frequency in such strains.

Mutated phenotypes commonly result from defects in DNA repair (19). An in silico analysis suggested that most mismatch repair systems (e.g., *mutS, mutL,* or *mutH*) were missing in the *M. tuberculosis* genome (20). However, the frequency of spontaneous mutations in *M. tuberculosis* (in vitro cultures) is similar to that found in other bacteria-carrying mismatch repair systems (21), which suggests that other DNA repair mechanisms must be present. Hypothetical open reading frames (ORF), similar to genes known to be responsible for the avoidance or repair of DNA lesions resulting from the alkylation or oxidation of nucleotides, are present in the genome of *M. tuberculosis*. We searched for variations in these genes in 139 clinical isolates to detect possible mutations that could allow an enhanced adaptability to the host and increased resistance to anti-TB drugs.

Methods

We searched for *mut* genes variation in 139 *M. tuberculosis* complex strains originating from 35 different countries. Ninety-four of these strains were selected because they were representative strains characterized with 13 different genetic markers in previous studies (6,22).

This set comprised 125 *M. tuberculosis* strains, 1 *M. africanum*, 8 *M. bovis*, 3 *M. bovis* BCG, and 2 *M. microti*. Fifty-five strains had a W-Beijing genotype; 12 had an MDR phenotype. Strains representing different branches of the W-Beijing genotype were studied. Eight MDR *M. tuberculosis* strains with a genotype other than Beijing were included. Five *M. tuberculosis* strains of the W-Beijing genotype and three strains of unrelated genotype were obtained from the national program for surveillance of MDR tuberculosis in Spain. Four *M. tuberculosis* W-Beijing genotype strains isolated in the Netherlands and one from Vietnam were included because they showed spoligo patterns with fewer than nine spacers. Five other W-Beijing genotype strains showed hybridization to an additional spacer, as demonstrated by using the extended set of spacers, two of which lacked hybridization to spacer 37. Strain W4 is part of a drug-susceptible outbreak in New Jersey (4); W147 is a drug-resistant isolate widely spread in Russia (23). Eleven strains were representative of ancestral W-Beijing strains, which diverged early in the evolution of the W-Beijing phylogenetic lineage. Finally, 29 strains of another frequently observed genotype, the Haarlem genotype (6), were investigated.

The collection consisted of 55 W-Beijing genotype isolates, 29 Haarlem genotype isolates, 8 strains of the African genotype, 1 *M. bovis* strain, and 46 representatives of other genotypes. Principal genetic grouping (PGG), according to the polymorphism in *katG* and *gyrA*, was known (24) for most of the isolates in this study. Seventy-four strains belong to PGG 1, 54 to PGG 2, and 3 to PGG 3. All isolates were subjected to at least IS*6110* RFLP typing and spoligotyping (6). Drug susceptibility was determined for 41 of 139 strains (Table 1 and 2). Several putative *mut* genes were annotated as such in the released genome sequence of *M. tuberculosis* (25). In addition, using the BLAST program (26), we identified Rv3908 as an ORF carrying a *mutT* domain (27) and have since named it *mutT4*.

Primers were designed to amplify putative *mut* genes: *mutY* (5'-CCGGCGACGAATCGCTCGTT-3', 5'-AGCTGGGACAGTCGTCGCGG-3'), *mutM* (5'-CTGGTTCGATGGTGATGACC-5', 5'-GTGCGCTCGACCCACAG-3'), *mutT2* (5'-TCCGGATGATGATTTACCTCC-3', 5'-TCCGCCGGGTCGGGGAC-3'), *mutT1* (5'-ATCGTCGGCGTGCCGTG-3', 5'-GTCAGCGTCCTGCCCGG-3'), *mutT4* (5'-TCGAAGGTGGGCAA- ATCGTG-3' 5'-TGGGGTTCGCTGGAAGTGG–3'), *ogt* (5'-CAGCGCTCGCTGGCGCC-3', 5'-GACTCAG CCGCTCGCGA-3'), and *mut T3* (5'-GTCACGTCTGTTAGGACCTC-3', 5'-CGCGCAACGGCTGCCGG-3'). Similar primers were

designed to amplify the *rpoB* gene (5'-TACGGTCGGC-GAGCTGATCC-3', 5'- TACGG- CGTTTCGATGAACC-3').

DNA sequencing was performed directly on the amplified fragments by using the dideoxy chain-termination method with the Big Dye Terminator Cycle sequencing Kit (Perkin Elmer Applied Biosystems, Courtaboeuf, France) on a GeneAmp polymerase chain reaction (PCR) system 9600 (Perkin Elmer) and run on a DNA analysis system model 373 or 3100 (Applied Biosystems). Sequences of *mutY*, *mutT2*, *mutT4*, *rpoB, mutT1, mutT3,* and *ogt* of the *M. tuberculosis* strains H37Rv, CDC1551, and MT210 were obtained from published sequences or at the TIGR Web site (available from: URL: http://www.tigr.org/).

```
                  #     #       #    #
MutT              GGKIEMGETPEQAVVRELQEEVG
MutT2             GGKVAAGETERAALARELAEELG
MutT3             GGARDSHETPEQTAVRESSEEAG
MutT4             KGHIELGETAEQTAIREVAEETG
MutT1             KGKVDPGETAPVGAVREILEETG
                   *     *       **   **
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Figure 2. MutT proteins' sequences alignment. *Mycobacterium tuberculosis* Rv2985(MutT1), Rv1160(MutT2), Rv0413(MutT3), and Rv3908(MutT4) were selected from the *M. tuberculosis* genome because of their annotation or after a BLAST analysis. These sequences were compared to *Escherichia coli mutT* by using alignments available from: URL: http://www.biochem.uthsc-sa.edu/~barnes/mutt.html. The detected region of similarity is shown here. # absolutely conserved residues; * residues that are strongly conserved and that define the mutT or nudix motif.

Results

We searched for allele variation in putative genes coding for DNA repair enzymes: *mutT* (which hydrolyzes 8-oxo-deoxyguanosine triphosphate) (28), *ogt* (which removes methyl groups from O6-methylguanine in DNA) (29), *mutM* (formamidopyrimidine-DNA glycosylate) (30), and *mutY* (specific adenine glycosylate) (31) in 12 MDR *M. tuberculosis* strains. Subsequently, we genotyped for the observed single nucleotide polymorphism (SNP) variation in 124 strains, members of the *M. tuberculosis* complex, and in the three published sequences of *M. tuberculosis* strains. In total, the sampling comprises 55 W-Beijing genotype *M. tuberculosis* strains, including 11 ancestral W-Beijing isolates (unpub. data), 84 *M. tuberculosis* strains of other genotypes, and 1 *M. bovis* strain.

Several putative *mut* genes were annotated as such in the released genome sequence of *M. tuberculosis*. A BLAST search using the *E. coli mutT* sequences as template identified, in addition to *mutT1*, *mutT2*, *mutT3*, the hypothetical ORF Rv3908, which we have designated as *mutT4*. The best matches with *E. coli mutT* gene were observed for *mutT2* and *mutT4*. Figure 2 depicts sequence alignment of the conserved region of the different genes of the *M. tuberculosis* genome showing similarity with *mutT* of *E. coli*. The search for sequences similar to *ogt, mutM,* and *mutY* identified a single ORF in each case. Primers were designed for PCR amplification of all the genes mentioned above.

We first determined the sequences of the different genes mentioned above in 12 MDR *M. tuberculosis* strains (ZA20, ZA65, ZA67, ZA68, ZA69, ZA11, ZA16, ZA12, ZA13, ZAA14, ZA17, and ZA19), including 5 W-Beijing strains (ZA20, ZA65, ZA67, ZA78, and ZA69). For the *mutY*, *mutM*, *mutT1*, and *mutT3* putative genes, PCR amplification was obtained in all strains tested, but sequence analysis did not indicate any nucleotide changes at these loci except for the same silent SNP in *mutT3* in strains with a Haarlem genotype. We confirmed these findings by sequencing *mutT1, mutT3*, *mutM,* and *mutY* in a collection of 26 MDR strains from North Africa. No variation was observed in *mutT1* or *mutM*. Only one strain had a major variation in *mutY*. All Haarlem strains carried one characteristic silent mutation in *mutT3* and one characteristic mutation in *ogt* (Ser 15 replaced by Thr). These defining SNPs were also observed for all Haarlem strains of this study. No other variations were observed in *mutT1, mutT3, mutM,* or *mutY*. However, comparative sequence analysis of H37Rv, CDC1551, and the five MDR–W-Beijing isolates indicated polymorphisms in *mutT2*, *mutT4,* and *ogt*. These mutations in *mutT4*, *mutT2,* and *ogt* were also found in the W-Beijing strain 210 (TIGR) but not in MDR strains other than those belonging to the W-Beijing genotype. We therefore decided to extend this investigation and look for mutations in these three genes in a collection of *M. tuberculosis* complex isolates, including well-defined branches of the W-Beijing phylogenetic lineage (Table 1 and 2).

In 43 of 55 strains with a W-Beijing genotype, either susceptible to bacteriostat or MDR, we found a mutation in *mutT4*. Codon 48 (CGG) of the annotated ORF had been changed to GGG, resulting in the amino acid substitution of Arg by Gly (Table 1 and 2). All 11 W-Beijing isolates known to be closely related to the ancestral W-Beijing strain (AM, HI, N16, DU2, DV, LB2, KY, IK, 122(C11), 113, and 107(LB)) were found to have the wild-type genotype as all other 84 isolates with a genotype other than W-Beijing.

Thirty-nine of 43 W-Beijing strains with the mutation in *mutT4* carried an additional mutation in *mutT2* and in *ogt*. The *mutT2* mutation constitutes a change in codon 58 (GGA to CGA), resulting in an amino acid substitution of Gly by Arg. The active site of the *E. coli* MutT enzyme comprises amino acids 53, 56, 57, and 98. Therefore, a mutation Gly to Arg at position 58 may have a important effect on enzyme activity and lead to a mutator phenotype.

All 39 W-Beijing isolates carrying the *mutT2* polymorphism at codon 58 also displayed a concurrent silent muta-

Table 1. Characteristics of *Mycobacterium tuberculosis* complex strains originating from 35 different countries

Strains	Genotype	No. isolates	Country of isolation	Group	*mutT2*	*mutT4*	*ogt*
ZA20/65	W-Beijing	2	Spain	1	wt	Arg CGG 48 GGG Gly	Arg CGC 37 Leu CTC
ZA67-69	W-Beijing	3	Spain	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
ZA11/16	Haarlem	2	Spain	2	wt	wt	Thr ACC 15 Ser AGC
ZA12-14/17	other	4	Spain	nd	wt	wt	wt
ZA19	*M. bovis*	1	Spain		wt	wt	wt
ZA15	other	1	Spain	nd	wt	wt	wt
ZA60-62	W-Beijing	3	Spain	1	wt	Arg CGG 48 GGG Gly	Arg CGC 37 Leu CTC
CDC1551		1	USA	2	wt	wt	wt
H37Rv		1	USA	3	wt	wt	wt
MT210	W-Beijing	1	USA	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
20	W-Beijing	1	Mongolia	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
30	W-Beijing	1	South Africa	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
34	W-Beijing	1	Malaysia	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
43	W-Beijing	1	China	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
44	W-Beijing	1	Thailand	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
45	W-Beijing	1	Malaysia	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
91/102-6	W-Beijing	6	Vietnam	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
110/116/119/124-5/140-2	W-Beijing	8	the Netherlands	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
133	W-Beijing	1	South Africa	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
W4/10/126/129	W-Beijing	4	USA	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
W99	W-Beijing	1	Singapore	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
W147	W-Beijing	1	Russia	1	Gly GGA 58 CGA Arg	Arg CGG 48 GGG Gly	Gly GGG 12 Gly GGA
94	W-Beijing	1	Vietnam	1	wt	Arg CGG 48 GGG Gly	Arg CGC 37 Leu CTC
111	W-Beijing	1	South Korea	1	wt	Arg CGG 48 GGG Gly	Arg CGC 37 Leu CTC
115	W-Beijing	1	the Netherlands	1	wt	Arg CGG 48 GGG Gly	Arg CGC 37 Leu CTC
5107(HG1)	W-Beijing	1	USA	1	wt	Arg CGG 48 GGG Gly	Arg CGC 37 Leu CTC
114, 139	W-Beijing	1	the Netherlands	1	wt	Arg CGG 48 GGG Gly	wt
166(HD6)	W-Beijing	1	USA	1	wt	Arg CGG 48 GGG Gly	wt
165(001)	W-Beijing	1	USA	1	wt	wt	Arg CGC 37 Leu CTC
107(LB)	W-Beijing	1	USA	1	wt	wt	wt
113	W-Beijing	1	the Netherlands	1	wt	wt	wt
122(CI1)	W-Beijing	1	USA	1	wt	wt	wt
IK/KY/LB2/DV/DU2/HI	W-Beijing	6	Russia	1	wt	wt	wt
N16	W-Beijing	1	USA	1	wt	wt	wt
AM	W-Beijing	1	USA	1	wt	wt	wt

[a]wt, wild-type alleles (identical to H37Rv strain); nd, not determined

tion in codon 12 (Gly GGG to GGA Gly) of the *ogt* gene. Of four possible codons encoding for glycine, GGG and GGA had the lowest relative synonymous codon usage (RSCU) in genes with high expression levels (0.20 and 0.17, respectively, compared to 1.32 and 2.31 for GGU and GGC). For genes with low expression levels, the RSCU values are 0.92, 0.37, 0.65, and 2.06 for GGG, GGA, GGU, and GGC, respectively (32).

The five W-Beijing isolates with a mutation in *mutT4* and a wild-type *mutT2* gene did not contain the *ogt* silent mutation on codon 12 either. Instead, they all shared a dinucleotide substitution in codon 37 (ACC to CTC) of *ogt*, resulting in amino acid substitution of Arg to Leu. These five W-Beijing isolates of 43 with the *mutT4* mutations, without the *mutT2* (codon 58) or the *ogt* (codon 12) mutations, differed molecularly from all other W-Beijing isolates in their spoligotype pattern and accompanying deletion flanking the DR locus. Four of five were isolated from Dutch patients in the Netherlands; the fifth originated from a patient in Vietnam. The Vietnamese isolate (no. 94) shared >95% IS*6110* pattern similarity with the Dutch isolate 115 when standard RFLP analysis was used. Overall, the five isolates were closely related to each other according to IS*6110* profiling (>90% similarity). Spacer 37 in the DR locus of Dutch isolates 114 and 139 was absent, while sample 115 was missing spacers 37 and 38, and 111 had a deletion of spacers 38 and 39 but not spacer 37, suggesting that these isolates may belong to a different sublineage. A tentative phylogeny of the W-Beijing strains analyzed in this study is proposed in Figure 3. Seven of nine MDR W-

Table 2. Characteristics of *Mycobacterium tuberculosis* complex strains originating from 35 different countries

Strains	Genotype	No. isolates	Country of isolation	Group	*mutT2*	*mut T4*	*ogt*
AU	Haarlem	1	USA	2	wt	wt	Thr ACC 15 Ser AGC
3,5,22,32,39,48,50 ,52-3,55	Haarlem	10	Argentina	2	wt	wt	Thr ACC 15 Ser AGC
8	Haarlem	1	Vietnam	2	wt	wt	Thr ACC 15 Ser AGC
13/28	Haarlem	2	Sri Lanka	2	wt	wt	Thr ACC 15 Ser AGC
51	Haarlem	1	the Netherlands	2	wt	wt	Thr ACC 15 Ser AGC
57/59	Haarlem	2	Czech republic	2	wt	wt	wt
84	Haarlem	1	Czech Republic	2	wt	wt	Thr ACC 15 Ser AGC
86/143/145	Haarlem	3	Bolivia	2	wt	wt	Thr ACC 15 Ser AGC
87	Haarlem	1	USA	2	wt	wt	Thr ACC 15 Ser AGC
99	Haarlem	1	Italy	2	wt	wt	Thr ACC 15 Ser AGC
123	Haarlem	1	Czech Republic	2	wt	wt	Thr ACC 15 Ser AGC
144/146-7	Haarlem	3	Bolivia	2	wt	wt	wt
Apr-35	Africa	2	Rwanda	2	wt	wt	wt
37	Africa	1	Uganda	2	wt	wt	wt
40/120	Africa	2	Burundi	2	wt	wt	wt
72	Africa	1	Central African Republic	2	wt	wt	wt
97	Africa	1	Uganda	2	wt	wt	wt
121	Africa	1	Central African Republic	2	wt	wt	wt
2	BCG	1	the Netherlands	1	wt	wt	wt
6/47/73/130	*M. bovis*	4	the Netherlands	1	wt	wt	wt
12	Other	1	Tunisia	3	wt	wt	wt
15/31	Other	2	Iran	2	wt	wt	wt
16	Other	1	Canada	2	wt	wt	wt
17	Other	1	Greenland	2	wt	wt	wt
18	Other	1	USA	2	wt	wt	wt
19/36/74	Other	2	India	1	wt	wt	wt
25/62	*M. microti*	2	UK	1	wt	wt	wt
26	Other	1	Zimbabwe	2	wt	wt	wt
27	Other	1	Ethiopia	2	wt	wt	wt
38/42	Other	2	Tahiti	2	wt	wt	wt
41/46	Other	2	Chile	2	wt	wt	wt
49	Other	1	Tanzania	1	wt	wt	wt
56	Other	1	Curacao	2	wt	wt	wt
64	Other	1	Honduras	2	wt	wt	wt
65/112	Other	2	the Netherlands	1	wt	wt	wt
71	BCG	1	Japan	1	wt	wt	wt
76/101/126	*M. bovis*	3	Argentina	1	wt	wt	wt
83	BCG	1	Russia	1	wt	wt	wt
89/95	Other	2	Spain	2	wt	wt	wt
96	Other	1	the Netherlands	3	wt	wt	wt
98	Other	1	Ecuador	2	wt	wt	wt
100	*M. africanum*	1	the Netherlands	1	wt	wt	wt
108	Other	1	China	2	wt	wt	wt
118	Other	1	Honduras	2	wt	wt	wt

[a]nd, not determined; wt, wild-type alleles (identical to H37Rv strain).

Beijing strains carried missense mutations in two *muT* genes (*mutT2* and *mutT4*), and two had a missense mutation in both *mutT4* and *ogt* (Table 1 and 2).

No mutations in *mutT4* or in *mutT2* were observed in any of the 84 *M. tuberculosis* complex strains, including 19 strains of PGG1, 54 strains of PGG2, and 2 strains of PGG3; the strains originated from 29 countries and were a genotype other than W-Beijing. A Thr15Ser mutation was observed in 24 of 29 strains of the Harlem family. No other change was observed in *ogt*.

Resistance to rifampicin in MDR strains was correlated with mutations in the *rpoB* gene. The three tested MDR W-Beijing strains isolated in Spain, with the mutations at the *mutT2* and *mutT4* loci, harbored a different mutation in the *rpoB* gene. These strains were isolated from patients who had emigrated from Eastern Europe to Spain (ZA67,

ZA68, and ZA69). Analysis of the IS*6110* RFLP of the respective isolates showed a difference of a single band. These findings suggest that the three strains may be related. The acquisition of the three different mutations in the *rpoB* gene leading to rifampicin resistance must have occurred after the acquisition of mutations in the putative nucleotide repair enzyme genes *mutT4* and *mutT2*.

Discussion

Our results show that *M. tuberculosis* strains of the W-Beijing genotype acquired missense mutations in DNA repair genes. These *M. tuberculosis* W-Beijing genotype strains are genetically highly conserved and widespread. DNA repair genes have been previously shown to be associated with mutator phenotypes in other microorganisms. The success of this group of strains may result in part from mutations in DNA repair enzymes, which might provided a true selective advantage for these bacteria to adapt and persist, including through the acquisition of resistance to anti-TB drugs. Mutations in the DNA repair genes might be the evolutionary answer of the TB bacillus to increase adaptation to hosts. This adaptation will lead to increasing trends in the TB epidemic in the coming decades. The World Health Organization considers MDR and resistance as a problem of local rather than of global importance (1). If the relative contribution of W-Beijing genotype strains to the current worldwide TB epidemic is increasing as suggested (7), this approach should be revised. In areas with an increasing problem with MDR-TB, such as Estonia and Russia, W-Beijing genotype strains are predominantly associated with MDR cases (33). In Germany, the relative proportion of W-Beijing strains among isolates from resistant cases has increased from 12% in 1995 to 35% in 2000 (unpub. data). The latter observation may indicate an increasing influence of W-Beijing strains on the worldwide TB epidemic.

We identify polymorphisms in *M. tuberculosis* in genes that might result in a mutator phenotype and therefore a plausibly better adaptation of the bacilli to a hostile environment (34). Forty-three of 55 W-Beijing isolates analyzed were found to have a unique mutation on the ORF Rv3908. This ORF contains a MutT domain and is denoted here as *mutT4*. Thirty-nine of 43 W-Beijing strains carried an additional and identical mutation in a second putative gene of the *mutT* family, *mutT2*, and an identical silent mutation in *ogt*.

The W-Beijing phylogenetic lineage probably acquired the mutation on codon 48 of the *mutT4* only once and before other mutations associated with the mutator genes we describe. This mutation clearly distinguishes ancestral W-Beijing isolates from contemporary W-Beijing strains. The 11 W-Beijing isolates that did not have the characteristic *mutT4* mutation on codon 48, consist of a collection

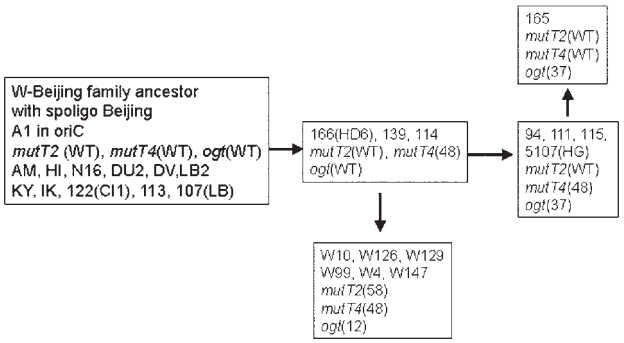

Figure 3. Schematic representation of a plausible pathway to explain the accumulation of mutations in *mut* genes.

of isolates known to be ancestral within this phylogenetic lineage, as determined by various other molecular techniques (unpub. data).

Nine of W-Beijing strains with the wild-type *mutT2* gene had a characteristic mutation on codon 37 of the *ogt* gene, which suggests that these isolates constitute a branch of the W-Beijing family that diverged after the acquisition of the *mutT4* mutation but before the development of the nucleotide substitution on *mutT2*. One strain carries the mutation 37 in *ogt* but no mutation in *mutT4*, a reversion that might have occurred after a transient mutator phenotype.

A mutation in *mutT2* was always associated with a mutation in *muT4*. A first mutation may have occurred in *mutT4* and thereafter a second mutation either in *mutT2* or *ogt* was acquired. As observed for other bacterial populations, mutator phenotypes may be transient in many cases to limit deleterious effects (35). Identifying these mutations may aid in the identification of *mut* genes in *M. tuberculosis*. These mutations associated with mutator genes provide a reliable tool for the identification of W-Beijing isolates and thus a useful marker for strains endowed with capacity to yield epidemics. The biologic consequences of these mutations and function of these DNA repair genes are currently been investigated in the laboratory.

Nine MDR strains with a W-Beijing genotype were among strains carrying two missense mutations in putative mutator genes. Phylogenetically unrelated *M. tuberculosis* MDR isolates had no mutations within the DNA repair genes investigated in this study. Our data support the idea that *M. tuberculosis* strains of the W-Beijing genotype may have adapted to hostile environments, including exposure to anti-TB drugs, because of a succession of alterations of DNA repair enzymes. Other genes involved in other DNA repair mechanisms or in the fidelity of DNA replication may also be involved and remain to be investigated.

The acquisition of mutator alleles was described as an adaptive response of bacteria to a succession of different

environments (18,35,36). After infecting a host, *M. tuberculosis* has to adapt to different environments such as alveolar macrophages and dendritic cells and subsequently to granuloma containing inactivated macrophages or to activated macrophages after induction of the acquired immune responses. In addition, the bacilli have to adapt to the caseous media with low oxygen concentration in the center of tubercles and to different types of tissues during dissemination of the disease. Such variable growth conditions might select for mutations in *M. tuberculosis* strains, as described in other bacterial populations exposed to different environmental challenges. Mutations and selection might occur with an increased frequency caused by the toxic radicals produced in phagocytic cells.

However, a mutator phenotype is often transient. Otherwise a continual accumulation of mutations would lead to deleterious effects and loss of fitness. No difference in the frequency of spontaneous mutations, resulting in a rifampin resistance phenotype, was observed for W-Beijing strains (37). We suggest that a transient mutator phenotype allowed a better adaptation of W-Beijing strains. Subsequent compensatory mutations occurred to reverse the mutator phenotype. An alternative hypothesis would be the existence of a higher mutation rate in specific conditions (i.e., in mutagenic radicals inside phagocytes). The accumulation of mutations leading to antibiotic resistance in W-Beijing strains may be a consequence of the appearance of strains with a better adaptation to the hosts. MDR strains would be easily selected when patients with strains that have adapted better received inadequate anti-TB regimens.

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Yellow Pygmy Rice Rat (*Oligoryzomys flavescens*) and Hantavirus Pulmonary Syndrome in Uruguay

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During 5,230 trapping nights, 672 small mammals were trapped in the areas where most hantavirus pulmonary syndrome (HPS) cases occur in Uruguay. Yellow pygmy rice rats (*Oligoryzomys flavescens*) were the only rodents that showed evidence of antibodies to hantavirus, with a seroprevalence of 2.6%. The rodents were trapped in all the explored environments, and most of the seropositive rodents were found in habitats frequented by humans. Nucleotide sequences were obtained from four HPS case-patients and four yellow pygmy rice rats of the M genome segment. Sequence comparison and phylogenetic analysis showed that rodent-borne viruses and viruses from three HPS case-patients form a well-supported clade and share a 96.4% identity with the previously characterized Central Plata hantavirus. These results suggest that yellow pygmy rice rat (*O. flavescens*) may be the host for Central Plata, a hantavirus associated with HPS in the southern area of Uruguay.

The family *Bunyaviridae* consists of five genera. Viruses in the *Hantavirus* genus are unique among them because all members (except Thottapalayam virus) are rodent-borne. Viruses in the other four genera are arthropod-borne. Hantavirus pulmonary syndrome (HPS) was first identified in the United States in 1993. The discovery of the outbreak was followed by the identification of Sin Nombre virus (SNV) as the primary etiologic agent of HPS (1). Since these findings, many countries in the Americas have identified cases and outbreaks of this syndrome, and several other related viruses (New World hantaviruses) have been recognized (2–9).

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New World hantaviruses are carried by different species of sigmodontine and arvicoline rodents (*Muridae*). Indeed, genetic diversity and geographic distribution of these viruses are related to the genetic diversity, geographic distribution, and phylogenetic history of their rodent hosts. In South America, studies of the correlation between rodent hosts and indigenous hantaviruses are complicated by the great diversity of sigmodontine rodents in this area. Also, the sympatric distributions between the different species of sigmodontine rodents in South America provide opportunities for spillover infections and host-switching events (10).

In Uruguay, the first evidence of the circulation of these viruses came from a study of serum specimens collected from blood donors between 1985 and 1987 that showed a seroprevalence of 1%, as measured by indirect fluorescent antibody (IFA) test using Hantaan and Seoul antigens (11). Since 1997, the Ministerio de Salud Pública, through the Departamento de Laboratorios, began the surveillance and diagnosis of HPS. In 2000 Padula et al. (12) reported partial sequences (G1 and G2 glycoprotein) derived from two HPS cases that occurred in Uruguay in 1997 and 1999. These viruses clustered within a previously reported lineage named Central Plata.

Knowledge about small mammal communities and habitat preferences is limited in Uruguay. However, some studies about systematic distribution, reproduction, and cytogenetic aspects have been published (13–17). Research regarding the distribution and habitat preferences of the *Muridae* family in Uruguay is currently being conducted (18–20).

The purpose of this study was to identify the carrier rodents of hantavirus in Uruguay and their potential association with HPS cases, to determine the prevalence of infection in different habitats, and to begin to genetically characterize the hantaviruses recovered from these rodents.

Material and Methods

HPS Case Identification

National surveillance for HPS was reinforced when a case definition was established by the Ministerio de Salud Pública in 1997. An HPS case was suspected in a previous healthy person with an acute febrile illness (temperature >38°C), associated with dyspnea, acute respiratory distress syndrome with pulmonary noncardiogenic edema, or interstitial bilateral infiltrates, hypotension or shock, elevated leukocyte count, and thrombocytopenia (21). A case of HPS was confirmed when, in addition to clinical illness, circulating specific hantavirus immunoglobulin (Ig) M was detected.

Human serum samples were tested for the presence of IgM and IgG antibodies with an enzyme-linked immunosorbent assay (ELISA) developed by MRL (Hantavirus ELISA IgM and Hantavirus ELISA IgG, MRL Diagnostics, Cypress, CA). The test was used to screen patients, and in every case, the results were confirmed by retesting the specimens by an in-house enzyme immunoassay with a recombinant nucleocapsid antigen specific to Andes virus, according to the procedure developed by Padula et al. (12).

Site Selection

Rodent sampling was conducted at the most likely sites of infection for known HPS case-patients and included the places where the person had lived or worked in the 6 weeks before onset of symptoms and nearby natural habitats. The trapping sites were classified as 1) domestic and peridomestic, including all sites in the immediate vicinity of houses, sheds, gardens, road borders, and fence lines, and 2) rural natural ecosystems and agro-ecosystems, including representative habitats of each area such as open fields, cultivated areas, wetlands, shrublands, brook borders, natural forests, and artificial woods (planted by humans) (Table 1).

The trapping expeditions were performed in the following areas: Puntas de Valdéz (34°32′S/56°36′W) and Piedritas (34º20′S/55°39′W) (one expedition each); Cerrillos (34°38′S/56°19′W), Melilla (34º44′S/56°16′W), and Sauce (34°35′S/56°08′W) (two expeditions each) (Figure 1). The geographic area covered by the trapping expeditions corresponded to areas where 16 HPS cases occurred in Montevideo and Canelones, two cases occurred in San José, and one case occurred in Florida. The other 19 cases were dispersed in the southern half of Uruguay, and for some of them, the probable site of infection was not clearly identified.

The landscape of Canelones, rural Montevideo, Florida, and San José shows cultivated areas, stubble areas, shrublands in the territories abandoned by rural people, range lands, natural and artificial woodlands, small wetlands, and small brooks. In recent years, many rural inhabitants have migrated to the cities; abandoned farmlands have thus been transformed into shrublands.

Small-Mammal Trapping and Processing

Small mammals were trapped in six expeditions in the above-mentioned areas from May 1997 until September 2001. Each trapping site was sampled with Sherman live-capture traps (model LFATDG 23 cm x 8 cm x 8.5 cm) (Sherman Traps Inc., Tallahassee, FL). The number of traps depended on the available area for trap placement at each trapping site. The traps were placed at 5-m intervals in line transects, along the different environments at the trapping site. The traps were set out in the late afternoon and checked in the early morning for the next two mornings. The animals were trapped and sampled according to established biosafety guidelines (22). Each animal was anesthetized, and blood was collected from the retroorbital sinus. The animals were humanely killed, and their size, mass, sex, and reproductive status were recorded. Samples of liver, kidney, lung, and brain were extracted and stored in liquid nitrogen for further

Table 1. Trapping efficiency/environment/species in the total of captures[a]

Env	TN	C	E%	*Md*	*Of*	*Od*	*St*	*Aa*	*No*	*Hb*	*Cl*	*Mm*	*Rr*	*Ca*
NW	288	27	9.4	-	1	7	10	6	2	-	1	-	-	-
RB	1,010	172	17.0	-	55	4	24	11	14	-	1	63	-	-
PD	1,420	44	3.1	1	19	1	7	-	2	-	-	11	-	3
WE	265	14	5.2	-	2	-	8	-	2	2	-	-	-	-
BB	680	57	8.4	1	11	1	23	4	10	-	-	7	-	-
AG	190	50	26.3	-	9	6	9	2	6	-	1	17	-	-
SH	1,198	268	22.4	1	93	2	117	11	13	-	-	30	-	1
AW	179	40	22.3	1	4	1	-	-	3	-	-	29	2	-
T	5,230	672	12.8	4	194	22	198	34	52	2	3	157	2	4
Sp%				0.6	28.9	3.3	29.5	5.1	7.7	0.3	0.4	23.4	0.3	0.6

[a]Env, environments; TN, trapping nights; C, captures; E, efficiency; NW, natural woods; RB, road borders; PD, peridomestic; WE, wetlands; BB, brook borders; AG, agroecosystems; SH, shrublands; AW, artificial woods; T, totals; Sp%, species %; *Md*, *Monodelphis dimidiate*; *Of*, *Oligoryzomys flavescens*; *Od, O. delticola; St*, *Scapteromys tumidus; Aa*, *Akodon azarae; No*, *Necromys obscurus; Hb, Holochilus brasiliensis*; *Cl*, *Calomys laucha*; *Mm, Mus musculus*; *Rr, Rattus rattus*; *Ca*, *Cavia aperea*.

Figure 1. Locations of capture sites and enzyme-linked immunosorbent assay–confirmed hantavirus pulmonary syndrome case-patients in Uruguay. Capture sites: triangle = Puntas de Valdéz, diamond = Piedritas, circle = Sauce, star = Cerrillos, square = Melilla.

processing. The individual animals were tentatively identified in the field by external characteristics, and the carcasses were kept in 10% formalin. Identification was confirmed by cranial measurements and dental examination at the laboratory. All specimens were deposited at the collection of the Sección Zoología Vertebrados, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay.

ELISA

Serologic testing of rodents was performed by IgG ELISA. Briefly, the IgG ELISAs were performed by coating polyvinyl chloride microtiter plates (Dynex Technologies, Chantilly, VA) overnight at 4°C with a Lechiguanas virus (LECV) antigen (inactivated, 3 M rad gamma-ray irradiation detergent-extracted lysate of Vero-E6 infected cells, with a 100% infection index controlled by indirect immunofluorescence). An uninfected Vero E6 cell culture antigen was used to determine the specificity of mouse antibodies. Unbound antigen was removed by washing three times with phosphate-buffered saline (PBS)-Tween 20, 0.1% (Sigma-Aldrich, St. Louis, MO). After blocking with PBS-Tween 20, 0.1%-dry milk 5% (37°C, 1 h), sera diluted fourfold, beginning with 1:100, were added to react with the antigen-coated wells. Bound antigen was measured by the use of a hyperimmune mouse ascitic fluid and by using goat anti–*Peromyscus leucopus* IgG (H+L) and goat-anti rat IgG (heavy- and light-chain–specific; Kirkegaard & Perry Laboratories, Gaithersburg, MD) conjugated to horseradish peroxidase. Optical densities (ODs) at 405 nm were recorded on a microplate spectrophotometer (Labsystems Multiskan EX; Thermo Labsystems, Finland, Vartaa, Finland), and the ODs of the uninfected antigen-coated well were subtracted from that of its corresponding viral antigen to yield the adjusted OD. A serum dilution was considered positive if OD was >0.2 U after adjustment. A serum titer ≥400 was considered positive.

Total RNA Extraction and RT-PCR

Total RNA was extracted from lung tissue of seropositive rodents and from blood clots from HPS case-patients. Approximately 100 mg of tissue was treated with 1 mL of TRIzol reagent (GIBCO BRL, Life Technologies, Rockville, MD), according to manufacturer's instructions. An M genome segment of the G2 glycoprotein encoding region was amplified by using reverse transcription-polymerase chain reaction (RT-PCR) and specific oligonucleotides as previously described by Levis et al. (4).

RT was carried out using MMLV reverse transcriptase (GIBCO BRL) and the oligonucleotide 3348(-) (5'CTGTCCAGATTTAGTGTTCCA 3'). cDNA was then precipitated with NaAc (ICN Biomedicals, Costa Mesa, CA) 3 M pH 5.6, ethanol (Merck Química Argentina, Buenos Aires, Argentina), and lineal polyacrylamide 2.5 µg/µL (ICN Biomedicals), and resuspended in 20 µL of double distilled water. Two microliters of first-strand cDNA was used in the PCR reaction. Two rounds of PCR were performed by using Taq DNA polymerase (GIBCO BRL). The first round was performed with oligonucleotides 3348 (-) and 2765 (+) (5'CTGTATGTGAGTACCAAG 3'), and the second round (heminested) was performed with 1 µL of first-round reaction and oligonucleotides 3221 (-) (5'TCAGAAGAGCAGTCAGTGTCATG 3') and 2765 (+), giving a 456-nucleotide (nt) fragment. PCR fragments were visualized on ethidium bromide 1.5% agarose gels.

Sequencing and Phylogenetic Analysis

The PCR fragments obtained from rodent and HPS case-patient samples were purified for further sequencing by using the Concert rapid gel extraction system (GIBCO BRL) or QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA). Nucleotide sequencing was conducted by using the oligonucleotide 2765(+) and an ABI 377 Genetic Analyser (PE Applied Biosystems, Inc., Foster City, CA).

Alignment of sequences was done by using CLUSTALX (1.5) (23). Phylogenetic analyses and sequence comparison were carried out with PAUP* 4.0b10 (24) and MEGA version 2.1 (25). Maximum parsimony

analysis was carried out by using the heuristic search option. Maximum parsimony trees were searched by applying the tree bisection reconnection branch-swapping algorithm. A consensus tree was obtained through 50% majority rule consensus. For the distance-based approach, MODELTEST 3.06 (26) was used to establish the most suitable model of DNA substitution that best fitted our dataset, and a phylogenetic tree was obtained by using the neighbor-joining algorithm. Bootstrap analysis (27) was performed to estimate topologic accuracy of the trees (500 replicates), and only values >70% were considered significant.

For comparison, existing sequence data from GenBank were used: hantavirus sequences from Argentina (GenBank accession nos. AF028023 to AF028027, AF028029 to AF028063), Central Plata genotype from Uruguay (GenBank accession nos. AY101184 and AY101185), and Sin Nombre virus (L37903, isolate NMR11); the last one was used as outgroup.

Results

HPS Cases

From April 1997 to August 2002, 38 cases of HPS were confirmed by ELISA, with a fatality rate of 21.0%. Twenty-four (63.2%) of these cases occurred in rural or suburban areas of Montevideo and Canelones, 6 (15.7%) in Colonia, 3 in Soriano (7.9%), 2 in San José (5.3%), 2 in Rocha (5.35%), and 1 in Florida (2.6%) (Figure 1). As of August 2002, HPS cases in Uruguay had occurred in the southern half of the country.

Distribution of Rodents by Species and Capture Site

During 5,230 trap-nights, 672 small mammals were collected (trap success = 12.8%). The trapped small mammals belonged to two families (*Muridae* and *Caviidae*) within the order Rodentia and one family (*Didelphidae*) in the order Didelphimorphia. The mammals belonged to 11 species, 75.1% of the captured animals corresponded to the Sigmodontinae subfamily, 23.7 % to the Murinae subfamily, 0.6% to the *Caviidae* family, and 0.6% to the *Didelphidae* family (Table 1).

Captures and percentage of trap success by habitats were as follows: natural woodlands, 27 (9.4%) of 288; road borders, 172 (17.0%) of 1,010, peridomestic areas, 44 (3.1%) of 1,420; wetlands,14 (5.2%) of 65; brook borders, 57 (8.4%) of 680; agroecosystems, 50 (26.3% of 190; shrublands, 268 (22.4%) of 1,198; and in artificial woodlands, 40 (22.3%) of 179 (Table 1). The most common captured small mammals were the following: swamp rats (*Scapteromys tumidus)*, 198 (29.5%); yellow pygmy rice rats (*Oligoryzomys flavescens),* 194 (28.9%); and house mice (*Mus musculus),* 157 (23.4%) (Table 1). No sigmodontine rodents were found inside the houses, where only house mice and black rats (*Rattus rattus)* were found. Yellow pygmy rice rats were found in areas of human disturbance such as peridomestic areas, agroecosystems, road borders, and shrublands. We found that the trapping success in these sites was higher than in natural areas. As shown in Table 1, yellow pygmy rice rats were found in all of the habitats where traps were set.

Screening for Hantavirus Infection of Rodents

Serum specimens collected from rodents were screened for IgG antibodies to LECV by ELISA. As mentioned above, 672 small mammals were trapped in six areas where HPS cases occurred between 1997 and 2001. Anti-LECV antibodies were detected in five rodents (*O. flavescens*) from four different locations. Absorbances with LECV antigen of positive samples screened at 1:400 dilution were at least fourfold the absorbance of the negative control. Further titration showed that three samples had titers >1:1,600, and one had a titer >1:6,400 (Table 2). The proportion of positive rodents in the different localities ranged from 2.1% to 2.9%. Piedritas was the only locality where no antibody-positive rodents were recorded (Table 3).

Total RNA Isolation, RT-PCR, and Sequence Analysis

Total viral RNA was extracted from the lungs of the five seropositive yellow pygmy rice rats and blood clots from four case-patients. RT of viral RNA and PCR amplification of a 456-nt fragment of the G2 glycoprotein–encoding region of the virus M genome segment (bases corresponding to LECV 2,805–3,215) and nucleotide sequences were obtained from four rodent samples and four human blood clots. Amplified DNA was not recovered from the rodent sample CE155, which had the lower antibody titer (Table 3). A 292-nt segment (LECV

Table 2. Hantavirus-seropositive rodents found in the different geographic areas where captures were performed

Sample	Rodent species	Geographic area	Habitat	Antibody titer (arbitrary units)
U89[a]	*Oligoryzomys flavescens*	Puntas de Valdéz (San José)	Road border	1,600
SA63[a]	*O. flavescens*	Sauce (Canelones)	Peridomestic	1,600
Ce20[a]	*O. flavescens*	Melilla (Montevideo)	Peridomestic	>6,400
Ce22[a]	*O. flavescens*	Melilla (Montevideo)	Shrublands	1,600
Ce155[a]	*O. flavescens*	Cerrillos (Canelones)	Shrublands	400

[a]Specimens deposited at the Specimen Collection of the Sección Zoología Vertebrados, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay with the following numbers: U89=ZVC-M2154, SA63=ZVC-M2155, Ce20=ZVC-M2156, Ce22=ZVC-M2157, and Ce155=ZVC-M2158.

Table 3. Small mammals trapped in the different sites, number and % of positives *Monodelphis dimidiate*

Area	*Md*	+/%	*Of*	+/%	*Od*	+/%	*St*	+/%	*Aa*	+/%	*No*	+/%	*Hb*	+/%	*Cl*	+/%	*Mm*	+/%	*Rr*	+/%	*Ca*	+/%	Total
PV	-	-/-	38	1/2.6	-	-/-	-	-/-	10	0/0	-	-/-	-	-/-	2	0/0	81	0/0	-	-/-	-	-/-	131
Ce	-	-/-	47	1/2.1	9	0/0	52	0/0	4	0/0	17	0/0	2	0/0	-	-/-	23	0/0	-	-/-	-	-/-	153
Me	-	-/-	67	2/2.9	8	0/0	37	0/0	-	-/-	17	0/0	-	-/-	1	0/0	10	0/0	-	-/-	3	0/0	297
Pi	-	-/-	4	0/0	-	-/-	8	0/0	14	0/0	-	-/-	-	-/-	-	-/-	2	0/0	-	-/-	-	-/-	28
Sa	3	0/0	42	1/2.4	1	0/0	92	0/0	5	0/0	18	0/0	-	-/-	-	-/-	12	0/0	-	-/-	-	-/-	173
Ca	1	0/0	-	-/-	-	-/-	9	0/0	1	0/0	-	-/-	-	-/-	-	-/-	29	0/0	2	0/0	1	0/0	43
Total	4	0/0	194	5/2.6	22	0/0	198	0/0	34	0/0	52	0/0	2	0/0	3	0/0	157	0/0	2	0/0	4	0/0	672

[a]*Md, Monodelphis dimidiate; Of, Oligoryzomys flavescens; Od, O. delticola; St, Scapteromys tumidus, Aa, Akodon azarae*, *No, Necromys obscurus; Hb, Holochilus brasiliensis*; *Cl, Calomys laucha; Mm, Mus musculus; Rr ,Rattus rattus; Ca, Cavia aperea;* PV, Puntas de Valdéz; Ce, Cerrillos; Me, Melilla; Pi, Piedritas, Sa, Sauce; Ca, Canelones. *O. flavescens, S. tumidus,* and *M. musculus* were the most frequently captured rodents.

2,815–3,106 G2 glycoprotein–encoding region) was used for further comparison and phylogenetic analysis.

Sequence Comparison and Phylogenetic Analysis

For phylogenetic comparisons, a 292-nt fragment of the M gene from lung RNA of four yellow pygmy rice rats (GenBank accession nos. AY204677 to AY204680), as well as clot RNA from four HPS case-patients (GenBank accession nos. AF283896 to AF283899) was used. These nucleotide sequences were compared with the equivalent region of published hantavirus sequences. Phylogenetic analysis indicated that two previously known hantavirus genotypes are circulating in Uruguay: Central Plata and LEC (Figure 2).

By both maximum parsimony and distance-based analysis, the four sequences recovered from Uruguayan yellow pygmy rice rats were closely related to each other and formed a monophyletic group with the hantavirus sequences derived from three HPS case-patients from Canelones and Montevideo and two HPS case-patients from the same geographic area, previously characterized as Central Plata. This clade was supported by high bootstrap values (Figure 2A,B). Comparison of these sequences at the nucleotide level showed 96.4% identity. The most closely related genotype was LEC, with 87.9% identity, followed by Bermejo (85.0%), Orán (83.1%), Andes (82.5%), and Hu39694 (82.1%). The less-related genotypes of the Argentinean hantaviruses were Maciel (79.3%) and Pergamino (78.6%). One viral sequence from an HPS case-patient in Soriano clustered into LEC genotype.

Conclusion

Most HPS cases were in rural and suburban areas of Montevideo and Canelones (24 of 38 cases) (Figure 1) in southern Uruguay. Rodent sampling was conducted at the most likely sites of infection for known HPS case-patients.

The trapping success rate was higher in the environments influenced by humans (agroecosystems, road borders, shrublands, artificial woods, peridomestic areas) than in the natural areas (natural woods, wetlands, brook borders): 574 (85.4%) of trapped individual animals were captured in environments influenced by humans, and 98 (14.6%) were captured in natural environments. Swamp rats, yellow pygmy rice rats, and house mice were the most frequently trapped species. Of 44 (3.1%) rodents trapped in peridomestic environments, 19 (43.2%) were yellow pygmy rice rats (Table 1). Five seropositive yellow pygmy rice rats were captured in modified environments: one was captured along a road border, two were captured in peridomestic environments, and two were captured in shrublands, at ≤150 m from homes. Three of five seropositive rodents were therefore trapped in environments frequented by humans (road borders and peridomestic environments). These findings could indicate an increased risk for infection for human inhabitants.

Yellow pygmy rice rats were the only rodents that showed evidence of antibodies to hantavirus, with a prevalence of 2.6%. Researchers have found that hantavirus seroprevalence in rodents may vary widely, according to the season, geographic area, altitude, and rodent species analyzed (29–33). We found that the percentage of seropositive rodents (2.6%) is the same as encountered in the central zone of Argentina (2.6%) (32), although the habitats are not similar to the southern area of Uruguay. In Uruguay, we found that only yellow pygmy rice rats were antibody positive, while in central Argentina seropositive yellow pygmy rice rats, Azara's field mice (*Akodon azarae)*, dark mice (*Necromys benefactus*), and small water rats (*Holochilus brasiliensis*) were found (2–4). In the different locations in Uruguay, seroprevalence was similar, ranging from 2.1% to 2.9%. In Piedritas, where no positive rodents were found, only four yellow pygmy rice rats were trapped. All seropositive rodents in Uruguay were adult males, which is consistent with horizontal transmission and in accordance with the findings of several authors (30–32).

The phylogenetic analysis on a 292-nt region of the M segment showed that these rodent sequences clustered together with those from five Uruguayan HPS case-patients from the same geographic area (Canelones and Montevideo); these data suggest that the yellow pygmy rice rat can be considered as the putative reservoir host for Central Plata hantavirus in this region of Uruguay. This study also showed the circulation of LEC genotype in the western location of Soriano, 250 km from Montevideo, separated from the Argentinean central HPS-endemic area by the Uruguay River. This virus shared a 99% identity at the nucleotide level with LEC genotype.

Phylogenetic analysis shows that the genotype Central Plata recovered from rodents and HPS case-patient from

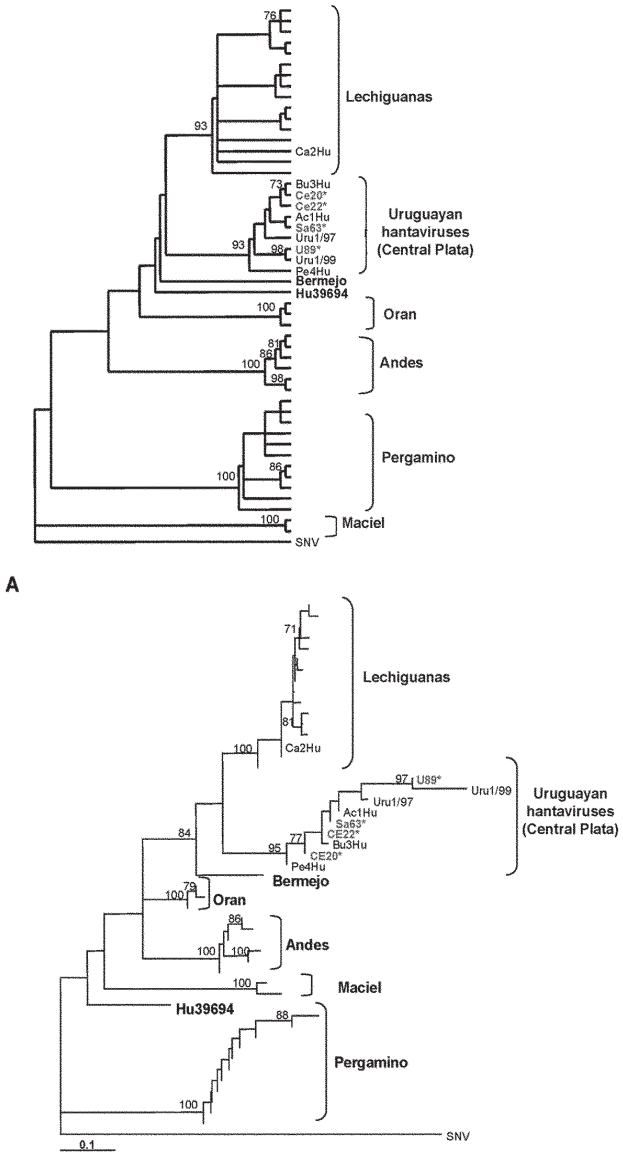

Figure 2. A: Maximum parsimony phylogenetic tree B: Distance-based phylogenetic tree. The tree was built under the Tamura-Nei model of DNA substitution with estimation of the shape parameter of the gamma distribution (28). This model and the associated parameters resulted from testing our dataset with the program MODELTEST 3.06 (26). Both trees include Argentinean and Uruguayan hantaviruses from hantavirus pulmonary syndrome (HPS) case-patients and rodents. Hantavirus sequences from HPS case-patients in Uruguay: Ac1Hu, Ca2Hu, Bu3Hu, Pe4Hu, Uru1/97, Uru1/99. Hantavirus sequences from yellow pygmy rice rats: U89, Sa63, Ce20, Ce22. Sin Nombre virus was used as outgroup.*Specimens deposited at the Collection of the Sección Zoología Vertebrados, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay, with the following numbers: U89=ZVC-M2154, SA63=ZVC-M2155, Ce20=ZVC-M2156, Ce22=ZVC-M2157, and Ce155=ZVC-M2158.

Canelones, San José, and Melilla is phylogenetically distinct from (although related to) the previously described LEC genotype, whose reservoir host in Argentina is also the yellow pygmy rice rat. Hantaviruses have been associated with subspecies of closely related rodents: Sin Nombre–like hantaviruses with mice from the genus *Peromyscus* (34) and Andes virus recovered in southwestern Argentina and Orán virus in northwestern Argentina, both recovered from long tailed pygmy rice rats (*O. longicaudatus*) (4). Recent studies have shown that these two rodent populations differ with respect to their mitochondrial DNA (10). This fact raises the question of whether rodents morphologically identified as *O. flavescens* in Uruguay are indeed a different subspecies of *O. flavescens* in Argentina. Further experiments will be needed to identify both the interspecific and intraspecific phylogenetic relationships of *O. flavescens* in these regions.

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Serologic Evidence of West Nile Virus Infection in Horses, Coahuila State, Mexico

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Serum samples were obtained from 24 horses in the State of Coahuila, Mexico, in December 2002. Antibodies to West Nile virus were detected by epitope-blocking enzyme-linked immunosorbent assay and confirmed by plaque reduction neutralization test in 15 (62.5%) horses. We report the first West Nile virus activity in northern Mexico.

West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) is a member of the Japanese encephalitis virus complex, which also includes Japanese encephalitis virus, Saint Louis encephalitis virus (SLEV), and Murray Valley encephalitis virus (1). These viruses are maintained in cycles between mosquitoes and birds (2). The principal vectors for WNV are *Culex* species mosquitoes, and many species of wild birds act as vertebrate hosts (3). Humans, horses, and other mammals usually serve as dead-end hosts. In humans and equines, WNV infection is usually asymptomatic or characterized by a mild febrile illness, although fatal meningoencephalitis or encephalitis may occur (3–5). WNV has a broad geographic distribution, recently including North America (4,5). The initial outbreak of WNV in North America was recognized in New York City in August 1999. Since then, WNV's geographic range has increased. WNV activity has now been reported in 44 states in the United States, the District of Columbia, and 5 of the 10 Canadian provinces (6,7).

In anticipation of the possible emergence of WNV into Mexico, we conducted equine infection surveillance in the northeastern states of Mexico. Coahuila State is bordered on the north by Texas. WNV activity has been detected in 204 (80%) of 254 Texas counties, including most counties that border Coahuila State (8). Therefore, Coahuila State was considered to be a likely point of incursion of WNV into Mexico from the United States.

Case Study

We present data from a small equine serosurvey conducted in Coahuila State in December 2002. A more extensive equine serosurvey is currently under way and will be described in detail elsewhere (B.J. Beaty, unpub. data). In the present study, blood samples were taken from 24 domestic horses at study sites located in Ciudad Acuña, Jiménez, and Saltillo (Figure). The Ciudad Acuña and Jiménez sites are approximately 40 km apart, and both are <15 km from the Texas border. Saltillo is located in the southeast region of Coahuila State and is approximately 220 km from Texas. All study sites are privately owned ranches.

The climatic conditions of the three study sites are similar and can be described as hot, dry, and arid. The average annual temperature ranges from 18°C to 22°C. The average rainfall is from 100 to 300 mm per year. The sites in Ciudad Acuña and Jiménez are approximately 300 m above sea level. The Saltillo site is situated at an elevation of approximately 1500 m.

A horse from the Ciudad Acuña ranch died October 17, 3 days after being observed with neurologic signs. The case was not reported immediately, and we were unable to obtain a tissue specimen postmortem. On December 19, with the assistance of a local veterinary practitioner, we sampled 14 horses at this site, 5 of which had developed neurologic disease in mid- to late October. Clinical symptoms included ataxia, weakness of limbs, trembling, and anxiety. All five horses survived. The horses with clinical signs were from 1 to 5 years of age; three were male, and two were female. Ages and sexes of horses without clinical symptoms were not documented. The veterinarian reported a great abundance of mosquitoes in the area. Another six horses were sampled in Jiménez and four in Saltillo, none of which had signs of illness. According to

Figure. Geographic location of West Nile virus study sites in Coahuila State, Mexico.

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the owners, none of the horses had ever been outside the State of Coahuila, and none of the horses had been vaccinated against WNV.

All serum samples were tested for antibodies to WNV by epitope-blocking enzyme-linked immunosorbent assay (ELISA). Blocking ELISAs were performed by using the WNV-specific monoclonal antibody (MAb) 3.1112G, as previously described (9). The ability of the Mexican horse serum samples to block the binding of the MAb to WNV antigen was compared to the blocking ability of horse serum without antibody to WNV (Vector Laboratories, Burlingame, CA). Data were expressed as relative percentages by using the formula of Hall et al., (10). Previously, we considered an inhibition value ≥30% to indicate the presence of viral antibodies (9). Recently, we have shown that ELISAs performed with MAb 3.1112G detect WNV antibodies in various vertebrate species, including horses (9,11).

Fourteen serum samples were positive in blocking ELISA that utilized MAb 3.1112G (Table). Serum from another horse (H-16) inhibited the binding of MAb by 25%, which is close to the diagnostic criterion. Previously, we observed that the nonspecific inhibition values for serum samples from noninfected control birds ranged from 0% to 24.3% (9). Therefore, if we used a less stringent threshold value of ≥25%, this serum could be considered positive for WNV antibodies.

To validate the above assays, we tested serum samples for neutralizing antibodies to WNV and SLEV by plaque reduction neutralization assay (PRNT). Testing for neutralizing antibody to SLEV was important because this virus is enzootic in the Americas and antibodies to WNV and SLEV often cross-react. Furthermore, horses are susceptible to SLEV infection, although clinical manifestations have not been reported (12). Viral isolates of WNV (strain NY99-35261-11) and SLEV (strain TBH-28) were obtained from the World Health Organization Center for Arbovirus Reference and Research, maintained at the Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Fort Collins, CO. PRNTs were performed by using Vero cells. Serum samples were tested by using a starting dilution of 1:20. Titers were expressed as the reciprocal of serum dilutions reducing the number of plaques that were ≥90% ($PRNT_{90}$).

Table. Summary of serologic data for horses in Coahuila State, Mexico[a]

Horse	Study site	Clinical illness	% inhibition by ELISA[b]	$PRNT_{90}$ titer[c]		PRNT diagnosis
				WNV	SLEV	
H-1	Ciudad Acuña	No	90	≥320	—[d]	WNV
H-2	Ciudad Acuña	No	5	—	—	Negative
H-3	Ciudad Acuña	No	0	—	—	Negative
H-4	Ciudad Acuña	No	0	—	—	Negative
H-5	Ciudad Acuña	No	90	≥320	40	WNV
H-6	Ciudad Acuña	No	93	≥320	—	WNV
H-7	Ciudad Acuña	No	93	≥320	—	WNV
H-8	Ciudad Acuña	Yes	93	≥320	—	WNV
H-9	Ciudad Acuña	Yes	86	≥320	—	WNV
H-10	Ciudad Acuña	Yes	90	≥320	20	WNV
H-11	Ciudad Acuña	No	89	≥320	—	WNV
H-12	Ciudad Acuña	No	92	≥320	20	WNV
H-13	Ciudad Acuña	Yes	91	≥320	—	WNV
H-14	Ciudad Acuña	Yes	78	≥320	20	WNV
H-15	Jiménez	No	82	≥320	—	WNV
H-16	Jiménez	No	25	40	—	WNV
H-17	Jiménez	No	7	—	—	Negative
H-18	Jiménez	No	93	≥320	20	WNV
H-19	Jiménez	No	0	20	20	Flavivirus
H-20	Jiménez	No	47	40	—	WNV
H-21	Saltillo	No	9	—	—	Negative
H-22	Saltillo	No	15	—	—	Negative
H-23	Saltillo	No	12	—	—	Negative
H-24	Saltillo	No	11	—	—	Negative

[a]ELISA, enzyme-linked immunosorbent assay; PRNT, plaque reduction neutralization test; WNV, West Nile virus; SLEV, Saint Louis encephalitis virus.

[b]Inhibition values ≥30% are considered significant.

[c]Neutralizing antibodies to WNV in selected horse serum samples were confirmed at Centers for Disease Control and Prevention-Division of Viral and Bacterial Infectious Diseases by PRNT.

[d]—, <20.

Conclusions

Overall, PRNT and ELISA data were in concordance. Fifteen (62.5%) horses were considered to be seropositive for WNV by PRNT because the antibody titers for WNV were greater than or equal to fourfold higher that the corresponding SLEV titer (Table). These 15 were the same serum samples that had inhibition values of ≥25% by ELISA. Of these, 11 horses were from Ciudad Acuña, and 4 were from Jiménez. Evidence for WNV infections was detected in 5 (100%) of 5 horses with clinical symptoms, and 10 (52.6%) of 19 horses without clinical symptoms. Therefore, the rate of asymptomatic seropositivity was high, with 10 (66.7%) of 15 WNV-infected horses showing no signs of illness. Similarly, 21 (58.3%) of 36 WNV-infected horses sampled during a serosurvey in New York in 1999 showed no clinical signs (13). However, the sample population (n=24) in the present serosurvey was notably small, and data from our large equine serosurvey will provide a more reliable estimate of the asymptomatic seropositivity rate.

We were unable to detect RNA in any horse serum by reverse-transcription polymerase chain reaction with WNV-specific primers (14). We plan to isolate and amplify WNV RNA sequences from tissue specimens obtained from seropositive horses, as well as from birds, in future studies.

We are currently conducting avian infection surveillance in the State of Coahuila and the neighboring states of Tamaulipas and Nuevo Leon. Preliminary evidence suggests that several birds from a region in Nuevo Leon State have antibodies to WNV (I. Fernandez-Salas, unpub. data). The birds were trapped in February 2003, 2 months after we obtained samples from the horses in Coahuila State. However, equine cases often precede the detection of seropositive birds. For example, an equine case was the first indication of WNV activity in 29% (660/2,289) of the United States counties to report virus activity in 2002 (6).

In summary, we have obtained serologic evidence for antibodies to WNV in horses in the State of Coahuila, Mexico. In the accompanying manuscript, we report the detection of antibodies to WNV in horses in the State of Yucatan (15). These two reports provide the first published evidence of WNV activity in horses in Mexico. Antibodies to WNV, or a closely related virus, were detected in a single bovine during a serosurvey in Chiapas, Mexico, in mid-2001 (16). WNV will probably become endemic in Mexico, which is a major concern to public health authorities in the Americas. Our findings demonstrate the importance for continued WNV surveillance in Mexico.

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Serologic Evidence of West Nile Virus Infection in Horses, Yucatan State, Mexico

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Serum samples were obtained from 252 horses in the State of Yucatan, Mexico, from July to October 2002. Antibodies to West Nile virus were detected by epitope-blocking enzyme-linked immunosorbent assays in three (1.2%) horses and confirmed by plaque reduction neutralization test. We report the first West Nile virus activity in the State of Yucatan.

West Nile virus (WNV) is a member of the Japanese encephalitis virus complex within the genus *Flavivirus*, family *Flaviviridae* (1). The virus is transmitted in natural cycles mainly between mosquitoes and birds, with humans and horses serving as incidental hosts (2). WNV was first isolated in 1937 from the blood of a febrile adult human in the West Nile District of Uganda (3). This virus has since been reported in Africa, the Middle East, Asia, southern Europe, Australia, and, more recently, North America (4,5). The initial outbreak of WNV in North America was recognized in New York City in August 1999, with deaths reported in humans, horses, and numerous species of birds. Since then, the geographic distribution of WNV in North America has greatly increased. WNV activity has now been reported in 44 states and the District of Columbia in the United States and in 5 of the 10 Canadian provinces (6,7).

In response to the incursion and rapid spread of WNV in North America, we established equine and avian infection surveillance in Yucatan State, Mexico, in March 2000. Yucatan State is a likely point of incursion of this virus into Latin America because this area is a principal landfall for many species of birds that migrate from the northeastern and midwestern United States (8).

To determine whether WNV had already reached this part of Mexico, we obtained blood samples from 252 domestic horses in 14 study sites from July to October 2002 (Table 1). The age distribution of the horses was 3 months to 25 years, and the mean age was 8.2 years. One hundred and fifty-one horses were male, and 101 were female. All study sites were on privately owned ranches, where the horses were primarily used to perform heavy labor and herd cattle. According to the owners, none of the horses had ever been outside the State of Yucatan. Furthermore, none of the horses had been vaccinated against WNV.

The climate and topography of the study sites are similar. The climate can be described as tropical. The average annual rainfall in each study site ranges from 600 to 1,100 mm, and the average annual temperature is 26°C. The average elevation is approximately 17 m.

All serum samples were screened for antibodies to flaviviruses by hemagglutination inhibition (HI) assays and epitope-blocking enzyme-linked immunosorbent assays (ELISAs) at the Universidad Autonoma de Yucatan in Merida. HI assays were performed by using Saint Louis encephalitis virus (SLEV) antigen as previously described (9). This antigen recognizes cross-reactive HI antibodies to WNV and to other flaviviruses. To preclude nonspecific HI reactions, samples were treated with kaolin, then adsorbed with goose erythrocytes, according to standard methods (9). Epitope-blocking ELISAs were performed by using the flavivirus group-reactive monoclonal antibody (MAb), 6B6C-1, or the WNV-specific MAb, 3.1112G as previously described (10). The ability of the Mexican horse serum samples to block the binding of MAbs to WNV antigen was compared to the blocking ability of horse serum without antibody to WNV (Vector Laboratories, Burlingame,

Table 1. Study sites and numbers of horses sampled per site, State of Yucatan, Mexico

Study site	Global Positioning System location	No. (%) of horses bled
Acanceh	20° 48' 46" N, 89° 27' 14" W	8 (3.2)
Caucel	21° 00' 53" N, 89° 42' 25" W	1 (0.4)
Hobonil	20° 00' 54" N, 89° 01' 15" W	26 (10.3)
Hunucma	21° 00' 55" N, 89° 52' 28" W	7 (2.8)
Mani	20° 23' 11" N, 89° 23' 37" W	1 (0.4)
Merida	20° 58' 04" N, 89° 37' 18" W	63 (25.0)
Molas	20° 48' 57" N, 89° 37' 55" W	5 (2.0)
Progreso	21° 17' 04" N, 89° 39' 48" W	31 (12.3)
Sierra Papacal	21° 07' 16" N, 89° 43' 41" W	14 (5.6)
Timucuy	20° 48' 34" N, 89° 30' 51" W	5 (2.0)
Tixkokob	21° 00' 08" N, 89° 23' 37" W	15 (6.0)
Tizimin	21° 08' 32" N, 88° 09' 03" W	49 (19.4)
Uman	20° 49' 38" N, 89° 41' 08" W	26 (10.3)
Xbec	21° 14' 54" N, 88° 49' 29" W	1 (0.4)
Total		252 (100)

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CA). Data were expressed as relative percentages, and inhibition values ≥30% were considered to indicate viral antibodies. Recent studies in this laboratory have shown that epitope-blocking ELISA provides a rapid and reliable serologic technique for the detection of WNV antibodies in various vertebrate species, including horses (10,11).

Six horses had evidence of flavivirus infection by HI assay or ELISA (Table 2). Serum samples from three of these horses (H-117, H-126, and H-252) were positive in the ELISA that used the WNV-specific MAb. H-117 (7-year-old stallion) and H-126 (2-year-old stallion) were both sampled at the Tizimin study site. Neither horse showed signs of illness at the time of serum collection or during the 7 months that followed. Furthermore, neither horse had a history of WNV-like illness. H-252 was a 3-year-old stallion from Caucel that exhibited neurologic and muscular symptoms at the time of sampling; it was euthanized several hours later. We were not able to obtain tissue specimens from this horse postmortem. Of the 252 horses sampled, the only other horse to exhibit signs of clinical illness was H-60, which had signs consistent with gastrointestinal illness.

Serum samples positive for flavivirus antibodies by HI assay or ELISA were tested by plaque reduction neutralization assay (PRNT) to identify the infecting virus. PRNTs were conducted in the BSL-3 facilities at Colorado State University. Serum sample results shown to be negative by HI assay and ELISA were not tested. PRNTs were done by using WNV (strain NY99-35261-11), SLEV (strain TBH-28), Ilhéus virus (ILHV, original strain), and Bussuquara virus (BSQV, strain BeAn-4073). Virus stocks were obtained from the World Health Organization Center for Arbovirus Reference and Research, maintained at the Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Fort Collins, CO. We tested serum samples for neutralizing antibodies to SLEV because the virus is enzootic in the Americas and because antibodies to WNV and SLEV often cross-react. Furthermore, horses are susceptible to natural SLEV infections, although clinical manifestations have not been reported (12). ILHV and BSQV are also present in the Americas, although neither virus is known to naturally infect horses (2). PRNTs were performed by using Vero cells. Serum samples were tested by using a starting dilution of 1:20. Titers were expressed as the reciprocal of serum dilutions yielding ≥90% reduction in the number of plaques ($PRNT_{90}$).

Neutralizing antibodies to WNV were detected in three horses (Table 3). The PRNT-positive horses were H-117, H-126, and H-252, which exhibited $PRNT_{90}$ antibody titers of 320, ≥2,560, and 160, respectively. The SLEV, ILHV, and BSQV antibody titers of the three horses were all <20. Therefore, we considered H-117, H-126, and H-252 to be seropositive for WNV because the $PRNT_{90}$ antibody titers for WNV were more than fourfold higher than the other flaviviruses tested. Overall, the PRNT and ELISA data were in concordance; all serum samples that contained neutralizing antibodies to WNV were positive in the assay that used MAb 3.1112G (Tables 2 and 3). However, H-252 was negative in the assay that used MAb 6B6C-1, although the percent inhibition value was close to the diagnostic criterion. The three other horses (H-60, H-134, and H-141) that were positive for flavivirus antibodies by HI assay or ELISA did not have neutralizing antibodies to WNV. H-60 had a low SLEV $PRNT_{90}$ titer, suggesting it had been infected with SLEV or a closely related virus. H-134 exhibited a HI titer of 10 but was negative by the other serologic tests, suggesting that the HI antigen had reacted nonspecifically. H-141 was positive by HI assay and ELISA but had no neutralizing antibodies to any flavivirus tested; thus, the identity of the infecting virus was not determined.

We obtained serologic evidence for antibodies to WNV in Yucatan State, Mexico. The mode of entry of this virus into Yucatan State is not known; however, the virus may have been brought in by birds migrating from the north.

Table 2. Summary of horses with HI assay or epitope-blocking ELISA antibodies to flaviviruses[a]

Horse	Sampling date	Study site	Age (y)	Sex	Clinical symptoms	Outcome	HI assay titer	% inhibition by blocking ELISA[b]	
								3.1112G[c]	6B6C-1[d]
H-60	July 2, 2002	Merida	8	Male	Gastrointestinal (recurrent colic)	Survived	10	0	59
H-117	July 5, 2002	Tizimin	7	Male	None	Survived	10	84	93
H-126	July 5, 2002	Tizimin	2	Male	None	Survived	40	87	93
H-134	July 5, 2002	Tizimin	3	Female	None	Survived	10	11	0
H-141	July 5, 2002	Tizimin	10	Male	None	Survived	80	5	47
H-252	Oct. 15, 2002	Caucel	3	Male	Neurologic and muscular symptoms	Euthanized	20	64	25

[a]HI, hemagglutination-inhibition; ELISA, enzyme-linked immunosorbent assay.

[b]Inhibition values ≥30% are considered significant.

[c]MAb 3.1112G is WNV-specific.

[d]MAb 6B6C-1 is flavivirus group–reactive.

Table 3. Neutralizing antibody titers to West Nile, Saint Louis encephalitis, Ilhéus, and Bussuquara viruses in serum samples from six horses[a]

Horse	$PRNT_{90}$ titer			
	WNV	SLEV	ILHV	BSQV
H-60	–[b]	20[c]	–[b]	–
H-117	320	–	–[b]	–
H-126	>2,560	–[b]	–	–
H-134	–	–	–	–
H-141	–	–	–	–
H-252	160	–	–	–

[a]WNV, West Nile virus; SLEV, Saint Louis encephalitis virus; ILHV, Ilhéus virus; BSQV, Bussuquara virus; PRNT, plaque reduction neutralization test; –, <20.
[b]$PRNT_{80}$ titer: 20.
[c]$PRNT_{80}$ titer: 40.

We have also detected antibodies to WNV in certain species of migratory birds, which supports this hypothesis. Data from the avian surveillance studies conducted in Yucatan State will be described separately (J.A. Farfán-Ale, unpub. data). We plan to isolate and amplify viral sequences from migratory and resident birds, as well as from specimens from other seropositive animals, to determine the origin of the WNV strain in Yucatan State. We also provide serologic evidence for WNV infection in horses in Coahuila State (13). These two reports provide the first published evidence of WNV activity in horses in Mexico. Neutralizing antibodies to WNV have also been detected in a bovine in Chiapas, Mexico, in mid-2001, indicating that the animal had been infected with WNV or a closely related virus (14). WNV may become endemic in this country, which demonstrates the importance for continued WNV surveillance in Mexico, and elsewhere in the south.

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Serologic Evidence of West Nile Virus Transmission, Jamaica, West Indies

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In spring 2002, an intensive avian serosurvey was initiated in Jamaica, Puerto Rico, and Mexico. We collected >1,600 specimens from resident and nonresident neotropical migratory birds before their northerly migrations. Plaque reduction neutralization test results indicated specific neutralizing antibodies to West Nile virus in 11 resident species from Jamaica.

West Nile virus (WNV) is maintained in nature between birds and *Culex* species mosquitoes (1,2). Unlike other viruses maintained in bird and mosquito transmission cycles (for example, St. Louis encephalitis, western equine encephalomyelitis, and eastern equine encephalomyelitis), the WNV strain responsible for the current epizootic in the Western Hemisphere is associated with a high number of avian deaths (3). Although crows and other corvids appear to be especially susceptible to disease (4), WNV has been documented in >190 bird species, including neotropical migratory species and exotic zoo specimens (5).

Birds have been implicated in spreading WNV during migratory events in Europe, Asia, Africa, and the Middle East (6–9). Because of the apparent ease of infecting a multitude of avian hosts, WNV can potentially be introduced during annual migratory events in the Western Hemisphere. Predicting the incursion of WNV into the tropics is complicated by our incomplete knowledge regarding geographic connectivity in populations of migratory birds between winter and summer. We do not know where most species of migratory birds from North America spend their winter, where wintering birds spend their summer, or the routes they use while in transit. Evidence from mark-and-recapture efforts suggests that birds from the northeastern United States tend to winter in the southeastern United States and Greater Antilles (e.g., Puerto Rico, Jamaica) whereas birds from the western United States migrate to Mexico and Central America (10–12). In response to the potential introduction of WNV in tropical America during the fall migrations, we established a network of monitoring sites on the overwintering grounds of neotropical migratory birds in Jamaica, Puerto Rico, and Mexico.

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The Study

The primary goal of the monitoring system was to obtain a large number of blood specimens from birds belonging to as many species as possible across the Caribbean. At seven study sites in Jamaica, Puerto Rico, and the Yucatan Peninsula of Mexico, we erected 15 mist nets (12-m) daily for 3 to 4 weeks during January through March 2002. We collected 1,619 avian blood specimens, which represented 98 species, 25 families, and eight orders. In Jamaica, 542 samples were collected from Westmoreland, Manchester, and St. Catherine Parishes; 649 samples were collected at Roosevelt Roads Naval Station in Puerto Rico; and 430 samples were collected from the states of Yucatan and Campeche in Mexico (Table 1). At the time of capture, all migratory birds were banded with an aluminum U.S. Fish and Wildlife Service band and 3–5 breast feathers were removed for isotope analysis. Resident birds had outer rectrices cut. All birds were evaluated for age and gender if possible, bled with microcapillary tubes from the brachial vein, and released. Blood was added immediately to BA-1 medium, consisting of M199 medium with Hank's salts, 1% bovine albumin, TRIS base (tris [hydroxymethyl] aminomethane), sodium bicarbonate, 20% fetal bovine serum (FBS), and antibiotics. The samples were placed in a cooler on ice packs until storage in a –20°C freezer. Samples were sent on dry ice or hand-carried on ice packs to the Arbovirus Laboratories, Wadsworth Center, New York State Department of Health for serologic analysis and virus isolation attempts.

Specimens were screened at 1:100 for antibodies against flaviviruses by using an indirect enzyme-linked immunosorbent assay (ELISA) (13). Samples with a P/N ratio >2.0 were tested further by a plaque reduction neutralization test (PRNT) for Ilhéus virus (ILHV), St. Louis encephalitis virus (SLEV), and WNV, as described (14). The particular virus strains used for the PRNTs were ILHV (original), SLEV 59268 Parton, and WNV (3100365), an isolate from a pool of *Culex* sp. mosquitoes collected in Staten Island, New York. The indirect ELISA was chosen to screen the samples in order to take advantage of its ability to detect antibodies against a wide range of flaviviruses. PRNT was used as a confirmatory assay to differentiate among recognized flaviviruses (15,16). Briefly, serial dilutions of test samples were mixed with an equal amount of virus suspension containing 200 PFU/0.1 mL and incubated at 37°C for 1 h. We then added 0.05 mL of each virus-

Table 1. Number of birds collected at seven field sites in the Caribbean

Field site	Migratory birds	Resident birds	Total	Flavivirus positives (%)[a]
Westmoreland Parish, Jamaica	156	232	388	18 (4.6)
Manchester Parish (site 1), Jamaica	2	21	23	1 (4.4)
Manchester Parish (site 2), Jamaica	24	83	107	4 (3.7)
St. Catherine Parish, Jamaica	12	12	24	4 (16.7)
Yucatan, Mexico	70	102	172	2 (1.2)
Campeche, Mexico	114	144	258	0 (0.0)
Mexico totals	184	246	430	2 (0.5)
Puerto Rico (one collection site) totals	391	256	647	5 (1.1)[b]
Jamaica totals	194	348	542	27 (5.0)
Totals	769	850	1,619	34 (2.4)[c]

[a]Based on screening enzyme-linked immunosorbent assay (ELISA) results.
[b]Results based on 441 samples suitable for ELISA (206 samples were blood clots only).
[c]Results based on 1,413 samples tested by ELISA.

diluted blood sample onto 1 well of a 12-well tissue culture plate containing confluent monolayers of African green monkey kidney cells (Vero). The plate was incubated for 1 h at 37°C, after which an agarose overlay was added and incubation was continued. When virus plaques became visible, we added a second overlay containing neutral red and counted plaques. The antibody titer reported is the dilution of serum that inhibited 90% of the test virus inoculum. For virus isolation attempts using confluent Vero cell monolayers, 0.1 mL of each serum specimen was added onto one well of a six-well tissue culture plate, incubated for 1 h, rinsed with phosphate-buffered saline, and then the cells were refed with minimum essential medium containing 2% FBS. Cells were monitored once a day for 5 days for cytopathic effect. Cell cultures showing any abnormal cell morphology were then blind passaged after 5 days.

ELISA results indicated 34 of 1,413 serum specimens tested from the three study sites contained immunoglobulin G antibody against a flavivirus. Of the 34 reactive samples representing 20 bird species, 27 were collected in Jamaica (26 residents, 1 migrant), 5 in Puerto Rico (3 residents, 2 migrants), and 2 in Mexico (1 resident, 1 migrant). PRNTs on the Jamaican bird samples indicated 18 WNV infections, 3 SLEV infections, 5 undetermined flavivirus infections (positive results for two or more viruses without a fourfold difference in antibody titer); one additional reactive serum sample was negative for the three viruses tested. Results on the serum samples collected in Puerto Rico indicated one WNV infection in a migratory bird and one SLEV infection in a resident bird; three additional reactive serum samples were negative for all three viruses tested. In Mexico, we found evidence of WNV infection in one migrant bird and SLEV infection in one resident bird (Table 2). Virus isolation attempts were negative for all 1,603 specimens tested (16 samples destroyed). Negative isolation results were not entirely unexpected, considering that birds are viremic for a short period of time (17) and maintaining a proper cold chain (i.e., temperatures) to preserve virus is difficult when working in the tropics.

Conclusions

We detected neutralizing antibodies to WNV in resident birds from two parishes in Jamaica. This detection marks the earliest evidence of WNV introduction into the neotropics; WNV antibodies have been demonstrated in birds and horses in Mexico (late 2002, spring 2003) (18,19) and detected in resident birds from the Dominican Republic (spring 2003) (20). This evidence of WNV in the neotropics may be an important development in the spread of the virus. The tropics provide all the necessary components (i.e., high temperatures, dense avian populations, and large numbers of *Culex* sp. mosquitoes) to maintain an enzootic focus. Furthermore, the climates of Mexico and the Caribbean are suitable for year-round transmission of the virus. No dead birds have been reported in Jamaica, but surveillance activity there is less intensive than in the United States; the study sites, being rural in nature, are not conducive to observing dead birds. Another contributing factor to the lack of reports of dead birds may be the rapid decomposition of dead birds as a result of the heat, humidity, and detritivore foragers, such as ants.

Arbovirus activity, particularly of flaviviruses, is well documented in the Caribbean and Mexico. Dengue and yellow fever viruses are recurring public health threats in these areas. SLEV is endemic to Mexico and has been isolated from mosquitoes and one Northern mockingbird (*Mimus polyglottos*) nestling in Jamaica (21). This disease is still active in the region, and its known range may have expanded into Puerto Rico, considering the one seropositive Caribbean elaenia (*Elaenia martinica*) sampled during this study, although the antibody may have resulted from infection with yet another flavivirus. Neutralizing antibodies to WNV in migratory birds collected in Mexico and Puerto Rico, coupled with the apparent absence of antibody to WNV in the resident bird population, indicate that

Table 2. Ninety percent plaque reduction neutralization titers to West Nile virus, St. Louis encephalitis virus, and Ilhéus virus in enzyme-linked immunosorbent assay reactive bird specimens[a]

Field site	Species	ILHV	SLEV	WNV	Interpretation
Westmoreland Parish, Jamaica	White-chinned thrush (*Turdus aurantius*)[b]	40	80	640	WNV
	White-chinned thrush *(T. aurantius*)[b]	<40	<40	320	WNV
	Jamaican elaenia (*Myiopagis cotta*)[b]	<40	160	640	WNV
	Bananaquit (*Coereba flaveola*)[b]	<40	80	160	Flavivirus
	Loggerhead kingbird (*Tyrannus caudifasciatus*)[b]	<40	<40	320	WNV
	Bananaquit (*C. flaveola*)[b]	<40	<40	160	WNV
	White-chinned thrush (*Turdus aurantius*)[b]	<40	40	≥1,280	WNV
	Bananaquit *(C. flaveola*)[b]	<40	<40	80	WNV
	Northern mockingbird (*Mimus polyglottos*)[b]	<40	<40	320	WNV
	Caribbean dove (*Leptotila jamaicensis*)[b]	<40	≥1,280	80	SLEV
	Jamaican elaenia (*Myiopagis cotta*)[b]	<40	<40	160	WNV
	Bananaquit (*C. flaveola*)[b]	<40	40	80	Flavivirus
	White-chinned thrush (*Turdus aurantius*)[b]	<40	40	640	WNV
	Jamaican elaenia (*Myiopagis cotta*)[b]	<40	80	<40	SLEV
	Common ground-dove (*Columbina passerina*)[b]	<40	<40	160	WNV
	Caribbean dove (*Leptotila jamaicensis*)[b]	<40	<40	<40	Negative
	Black-faced grassquit (*Tiaris bicolor*)[b]	<40	<40	320	WNV
	Jamaican vireo (*Vireo modestus*)[b]	<40	<40	80	WNV
Manchester Parish (site 1), Jamaica	Caribbean dove (*Leptotila jamaicensis*)[b]	<40	<40	80	WNV
Manchester Parish (site 2), Jamaica	Black-faced grassquit (*Tiaris bicolor*)[b]	<40	<40	160	WNV
	Jamaican oriole (*Icterus leucopteryx*)[b]	<40	160	≥1,280	WNV
	White-eyed thrush (*Turdus jamaicensis*)[b]	<40	<40	320	WNV
	Orangequit (*Euneornis campestris*)[b]	<40	<40	40	Flavivirus
St. Catherine Parish, Jamaica	Greater Antillean grackle (*Quiscalus niger*)[b]	40	160	160	Flavivirus
	Loggerhead kingbird (*Tyrannus caudifasciatus*)[b]	80	320	320	Flavivirus
	Golden warbler (*Dendroica petechia* sp.)[c]	<40	80	<40	SLEV
	Northern waterthrush (*Seiurus noveboracensis*)[d]	<40	40	320	WNV
Yucatan State, Mexico	Caribbean dove (*Leptotila jamaicensis*)[b]	<40	≥1,280	80	SLEV
	Yellow warbler (*Dendroica petechia*)[d]	<40	40	320	WNV
Roosevelt Roads Naval Station, Puerto Rico	Prairie warbler (*Dendroica discolor*)[d]	<40	<40	<40	Negative
	Golden warbler (*Dendroica petechia* sp.)[c]	<40	<40	<40	Negative
	Pearly-eyed thrasher (*Margarops fuscatus*)[b]	<40	<40	<40	Negative
	Caribbean elaenia (*Elaenia martinica*)[b]	<40	80	<40	SLEV
	Black and white warbler (*Mniotilta varia*)[d]	<40	40	≥1,280	WNV

[a]ILHV, Ilhéus virus; SLEV, St. Louis encephalitis virus; WNV, West Nile virus.
[b]Resident bird.
[c]Resident yellow warbler subspecies.
[d]Migrant bird.

infection likely occurred in an enzootic area of the United States, but this observation shows that individual birds from at least three species of neotropical migratory birds have survived WNV infection and may serve as hosts for spreading the virus. The results from this study suggest that WNV now appears to be established in Jamaica, on the basis of the neutralizing antibodies to WNV found in the resident bird population.

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Sulfa Resistance and Dihydropteroate Synthase Mutants in Recurrent *Pneumocystis carinii* Pneumonia

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Failure of sulfa or sulfone prophylaxis is associated with mutations in *Pneumocystis carinii* gene coding for dihydropteroate synthase (DHPS). The DHPS genotype was analyzed in AIDS patients who had two separate episodes of *P. carinii* pneumonia. The results suggest that DHPS mutations can be selected de novo within patients by the pressure of a sulfa or sulfone drug.

Co-trimoxazole, the antifolate drug combination of trimethoprim and sulfamethoxazole, is the drug of choice for the prophylaxis and treatment of *Pneumocystis carinii* pneumonia (PCP), a life-threatening disease in immunosuppressed patients. Trimethoprim is an inhibitor of dihydrofolate reductase, whereas sulfamethoxazole inhibits dihydropteroate synthase (DHPS). The antipneumocystis activity is believed to be due mainly to sulfamethoxazole (1). Dapsone is a sulfone drug, also frequently used, that targets DHPS. Widespread use of sulfa and sulfone drugs to prevent and treat PCP in recent years has correlated with an increase of the prevalence of mutations in the gene coding for DHPS (2,3). The most frequent DHPS mutations occur at nucleotide positions 165 and 171, which lead to an amino acid change at positions 55 (Thr to Ala) and 57 (Pro to Ser). These mutations are located in the sulfa-binding site and may appear as either a single or double mutation in the same isolate. Similar mutations in other microbial pathogens confer sulfa resistance (4,5). In *P. carinii,* DHPS mutations are associated with failure of sulfa or sulfone prophylaxis (1,6) and decreased survival of the patient at 3 months after PCP (2). However, patients harboring *P. carinii* types with DHPS mutations are most often successfully treated with high-dose co-trimoxazole (6). Because a standardized culture system for *P. carinii* does not exist, the level of sulfa resistance conferred by these mutations cannot be determined with in vitro susceptibility tests. A key issue is whether the recent emergence of DHPS mutations is a result of *P. carinii* transmission between patients or arises from selection within patients by the pressure of a sulfa or sulfone drug, two possibilities that are not mutually exclusive. To investigate the latter possibility, we analyzed patients who had had two separate episodes of PCP.

The Study

P. carinii DNA was extracted from bronchoalveolar lavage specimens by using the Qiamp Blood Kit (QIAGEN GmbH, Hilden, Germany). Bronchoalveolar lavage specimens from 13 patients with recurrent PCP episodes were collected from four European hospitals (Lyon, France; Copenhagen, Denmark; Lausanne, Switzerland; and La Chaux-de-Fonds, Switzerland). To determine the prevalence of the different *P. carinii* molecular types, we analyzed bronchoalveolar lavage specimens from 310 PCP patients from two Swiss hospitals (Lausanne, 111 patients; Zurich, 64 patients) and Lyon's hospital (135 patients). Specific information on demographic and clinical characteristics, chemoprophylaxis, and treatment regimens were obtained from the medical charts. *P. carinii* infecting humans (now named *P. jiroveci* [7]) was typed by using the multilocus method developed in our laboratory as previously described (8–10). In this method, four variable regions of the *P. carinii* genome are amplified by polymerase chain reaction (PCR), followed by the detection of polymorphisms using single-strand conformation polymorphism (SSCP). A *P. carinii* type is defined by a combination of four alleles corresponding to the four genomic regions. If a specimen harbored two alleles of one or more of the four genomic regions, the patient was considered to be co-infected with two or more *P. carinii* types (9). This typing system has been validated and the stability of its markers assessed; its index of discriminatory power has been estimated to be 0.93 (10). The full length of the DHPS gene was amplified by PCR as described previously (1). PCR products (765 bp) were cloned, and both strands were sequenced (5 clones per sample). The five clones had identical sequences for all samples, except for those from patients 3 and 4, which contained a mixture of DHPS sequences.

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[1]Aimable Nahimana contributed to the design of the study, analyzed the samples by polymerase chain reaction and DNA sequencing, and wrote the draft of the manuscript. Meja Rabodonirina and Jannik Helweg-Larsen reviewed medical charts and provided bronchoalveolar lavage specimens. All authors contributed to the analysis of data and writing of the paper. Philippe M. Hauser initiated and supervised all aspects of the study.

Thirteen patients with two separate PCP episodes were analyzed (Table). All patients had recovered between episodes. The intervals between the episodes ranged from 4 to 25 months. All patients had AIDS and all, except patients 8 and 9, were men, with a median age of 35 (range 23–51) and median CD4 cell count of 9.5 cells/μL (range 0–98). Some patients were co-infected with two different *P. carinii* types, as shown by PCR-SSCP multilocus typing method (patients 4, 5, 8, 11, and 13) or DHPS genotyping (patients 3 and 4). In seven (54%) patients (patients 1–7), the same PCR-SSCP type was observed in both episodes of PCP; six (46%) patients (patients 8–13) had different types in the first and second episodes. This rate of genotype switch is similar to that reported in previous studies, in which such a change was observed in approximately half of recurrent episodes (11–14). The importance of a genotype switch remains uncertain. Indeed, the switch might be due to a de novo infection or to the reactivation of a genotype not detected in the first episode because of the compartmentalization of different co-infecting *P. carinii* types in the lungs (15).

A second episode of PCP could result either from reactivation of organisms that caused the first episode or from de novo infection with a new *P. carinii* type acquired from an exogenous source. In seven patients (patients 1–7), reactivation was strongly suggested by the detection of identical SSCP types in both episodes of PCP. An alternative explanation could be de novo infection in the second episode by the same *P. carinii* PCR-SSCP type as that which caused the first episode. However, the prevalence of the types observed in the seven "reactivation" cases was low in Lyon and Switzerland during the study period (types no. 2, 5, and 7 represented 7%, 6%, and 10%, respectively, of Lyon's isolates; type 6 represented only 3.5% of the Swiss isolates [Figure]). Thus, reinfection with these specific types was unlikely. All Danish patients (1, 3, and 6) were infected with type 6. Although no prevalence data for SSCP genotypes in Denmark are available, no indication of possible contact between these patients, over-

Table. *Pneumocystis carinii* DHPS and PCR-SSCP genotyping in AIDS patients with recurrent pneumonia

Patient no.	City[b]	Age	Date of episode 1/ date of episode 2/ interval (mo)	CD4/mm^3	Prophylaxis at PCP episode[c]	Treatment	Outcome of treatment	*P. carinii* PCR-SSCP type	DHPS genotype[d]
1	Co	29	7/16/1993	9	D	CO → P[e]	Success	6	WT
			6/8/1994 (11)	0	P	CO	Success	6	M1
2	Ly	36	1/31/1994	58	D	A	Success	7	M2
			5/18/1995 (16)	16	CO	A	Success	7	M3
3	Co	51	8/19/1994	0	No	CO → C/P[e]	Success	6	WT/M1
			12/23/1994 (4)	0	P	T	Success	6	M1
4	Ly	32	11/23/1994	75	No	CO	Success	2, 5	WT/M3
			3/23/1995 (4)	35	No	CO	Death[f]	2, 5	M3
5	Ly	28	4/19/1995	70	No	A	Success	7, 8	WT
			3/1/1996 (11)	98	CO	P	Success	7	M3
6	Co	35	11/16/1995	2	D	P → CO[e]	Success	6	M1
			5/6/1996 (6)	1	D	CO	Success	6	M1
7	CF	41	2/3/1998	7	CO	P	Success	6	M3
			7/22/1998 (5)	7	P	C/P	Success	6	M3
8	La	28	11/24/1990	53	No	T	Success	6, 10	WT
			7/29/1991 (8)	18	No	CO	Success	7	WT
9	Co	25	12/8/1992	0	No	CO	Success	5	WT
			11/5/1993 (11)	0	No	CO	Success	7	WT
10	Co	35	3/22/1993	10	No	CO → P[e]	Success	18	WT
			10/28/1994 (7)	0	P	CO → P[e]	Death[f]	6	WT
11	Ly	23	3/30/1994	22	No	CO → A[e]	Success	4, 7	M3
			3/28/1995 (12)	26	P	D+T → A[e]	Success	5	M3
12	Ly	46	9/21/1994	61	No	CO	Success	15	WT
			10/21/1996 (25)	16	P	P+A	Success	3	WT
13	Ly	43	10/12/1994	50	No	CO	Success	1, 2	M2
			3/25/1996 (17)	5	PM/SD	P+A	Success	1, 3	M2

[a]PCP, *Pneumocystis carinii* pneumonia; DHPS, dihydropteroate synthase; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

[b]Co, Copenhagen (Denmark); CF, La Chaux-de-Fonds (Switzerland); La, Lausanne (Switzerland); Ly, Lyon (France).

[c]A, atovaquone; CO, cotrimoxazole; C/P, clindamycin/primaquine; D, dapsone; D+T, dapsone and trimethoprim; P, pentamidine; P+A, pentamidine and atovaquone; PM/SD (pyrimethamine/sulfadoxine inhibitors of dihydrofolate reductase (DHFR) and DHPS, respectively); T, trimetrexate (an inhibitor of DHFR).

[d]WT, wild type (Thr55 Pro57); M1, mutant 1 (Ala55 Pro57); M2, mutant 2 (Thr55 Ser57); M3, mutant 3 (Ala55 Ser57 double mutant).

[e]Switch of molecules because of toxicity for patients 3, 6, and 11 and because of toxicity and treatment failure for patients 1 and 10.

[f]Caused by PCP.

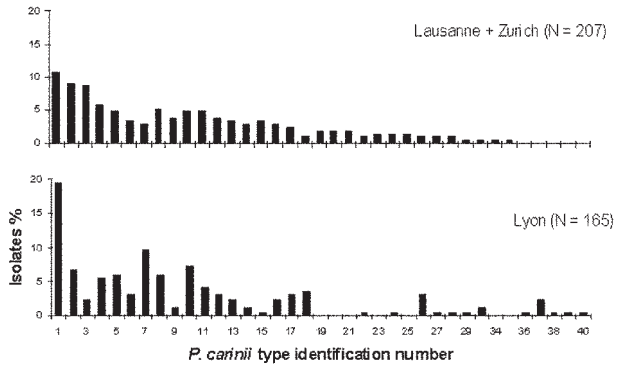

Figure. Frequency distribution of *Pneumocystis carinii* types observed in different locations. Each type, co-infecting or not, was considered as one isolate.

lap in hospitalization dates, or similar zip codes for home address suggested transmission of type 6 between these patients.

A change of *P. carinii* DHPS genotype between the two episodes was observed in three reactivation cases, either from wild type in the first episode to DHPS mutations in the second one (patients 1 and 5) or from DHPS with a single mutation (at position 57) in the first episode to a double mutation in the second one (patient 2). In two patients (3 and 4), the DHPS mutant strain was selected out of a mixture of wild-type and DHPS mutant strains. Because both episodes of each patient were caused by the same *P. carinii* types and because all patients received co-trimoxazole or dapsone as treatment, maintenance therapy, or both, these results strongly suggest that selection of *P. carinii* DHPS mutations occurred within the patients. The results of tests on patients 3 and 4 isolates highlight the fact that some patients may harbor genetically different strains of *P. carinii* and that the mutant strain may be readily selected when drug pressure is exerted. In the two remaining patients (6 and 7), the *P. carinii* DHPS mutant found in the bronchoalveolar lavage specimen from the second episode was already present in the first episode.

The wild-type DHPS allele was more frequently observed in the six reinfection cases than in the reactivation cases (8 wild-type alleles among 12 genotypes versus 4 among 16, Table). This finding is probably related to the fact that, with the exception of the second episode of patient 13, patients who were reinfected had no prophylaxis or did not receive sulfa drugs for prophylaxis.

In all the second episodes caused by reactivation, mutant DHPS strains were observed (7/7), compared to only two of six second episodes caused by reinfection (Table). This observation suggests an association between mutant DHPS and second episodes attributable to reactivation ($p<0.02$, Fisher exact test).

Conclusions

Our study suggests that *P. carinii* DHPS mutants may be selected in vivo (within a given patient) under the pressure of co-trimoxazole or dapsone and that DHPS mutations may be associated with reactivation of *P. carinii*. Whether DHPS mutations are induced by the pressure of the drug or preexisting and selected out by the pressure of the drug remains to be determined. Physicians should be alert to the increased risk for drug resistance during recurrence of PCP infection, although the impact of DHPS mutations on retreatment with sulfa or sulfone drugs remains to be determined. De novo selection of *P. carinii* DHPS strongly favors the hypothesis that *P. carinii* is developing sulfa and sulfone resistance.

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VIM- and IMP-Type Metallo-β-lactamase–Producing *Pseudomonas* spp. and *Acinetobacter* spp. in Korean Hospitals

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We determined the occurrence of acquired metallo-β-lactamase (MBL)–producing bacteria in Korean hospitals. Among the isolates nonsusceptible to imipenem that were collected from 28 hospitals from 2000 to 2001, 44 (11.4%) of 387 *Pseudomonas* spp. and 38 (14.2%) of 267 *Acinetobacter* spp. produced MBL and had alleles of bla_{VIM-2} or bla_{IMP-1}. MBL-producing isolates were detected in 60.7% of the hospitals.

Carbapenems are often used as a last resort for treating serious infections attributable to multidrug-resistant gram-negative bacilli because these drugs are stable even to extended-spectrum and AmpC β-lactamases. However, gram-negative bacilli with acquired metallo-β-lactamase (MBL), IMP-1, emerged and spread during the early 1990s in Japan (1). IMP-1 and its variants were then detected in other countries (2).

Another type of acquired MBL, VIM-1, was first reported in *Pseudomonas aeruginosa* in Italy (3), followed by reports of VIM-2 in France and Greece. VIM-2 was detected in *P. aeruginosa* in a Korean hospital isolated as early as 1995 (4). The occurrence of the VIM enzyme has continued to evolve: VIM-3 was reported in Taiwan (5), and VIM-4 in the United States (6).

The bla_{IMP} and bla_{VIM} genes are horizontally transferable because they are inserted in integrons, and some of these integrons are located on conjugative plasmids (7). Because of its ability to spread, carbapenem resistance related to IMP and VIM β-lactamase production has become a serious concern (8). Laboratory personnel and physicians must consider the therapeutic and infection-control implications of not detecting carbapenemase-producing bacteria (9). A large number of VIM-2–producing *Pseudomonas* spp. have been detected in a Korean hospital since 1995 (4), but the occurrence of MBL-producing isolates has not been studied at other Korean hospitals, despite the high prevalence of carbapenem-resistant *P. aeruginosa* and *Acinetobacter* spp. (10). The aim of our study was to determine the occurrence of acquired MBL-producing *P. aeruginosa* and *Acinetobacter* spp. among isolates collected by Korean Nationwide Surveillance of Antimicrobial Resistance Group hospitals. The MBL types produced and the sources of the MBL-positive isolates were also investigated. In addition, pulsed-field gel electrophoresis (PFGE) patterns were compared to determine intra- and inter-hospital spread of resistant strains.

The Study

Nonduplicate, imipenem-resistant isolates of 387 *Pseudomonas* spp. and 267 *Acinetobacter* spp. were collected from 2000 to 2001 from 28 hospitals in the Korean Nationwide Surveillance of Antimicrobial Resistance Group hospitals located in six cities or provinces. The identification of the species and the imipenem susceptibility were confirmed at the coordinating laboratory by using conventional tests (11) or ATB 32 GN system (bioMerieux, Marcy-l'Etoile, France) and by using the disk diffusion test (12), respectively.

MBL production was screened by using the Hodge test and the imipenem-EDTA double disk synergy test (13). The bla_{IMP-1} and bla_{VIM-2} alleles were detected by polymerase chain reaction (PCR), and three of the positive isolates were confirmed by sequencing, as described previously (4). *Xba*I-digested genomic DNA of *P. aeruginosa* isolates was separated by PFGE using the CHEF-DR-II system (Bio-Rad Laboratories, Hercules, CA) (4). The pattern was analyzed visually and by using UVIBand and Map software (UVItec Ltd., Cambridge, UK).

Some of the *Pseudomonas* and *Acinetobacter* isolates collected were not fully resistant to imipenem but showed intermediate resistance when retested. Among the isolates not susceptible to imipenem, 44 (11.4%) of 387 *Pseudomonas* spp. (42 *P. aeruginosa* and 2 *P. putida*) and 38 (14.2%) of 267 *Acinetobacter* spp. were considered MBL producers on the basis of positive results by the

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Table 1. Detection of metallo-β-lactamase–producing isolates among imipenem-nonsusceptible isolates of *Pseudomonas* spp. and *Acinetobacter* spp.

Organism	City/province	No. hospitals (%)		No. isolates (%)	
		Tested	Positive	Tested	Positive
Pseudomonas spp.	Seoul	11[a]	4 (36.4)	144	12 (8.3)
	Kyungki	2	2 (100)	40	6 (15.0)
	Kangwon	2	1 (50.0)	57	2 (3.5)
	Chulla	4	4 (100)	108	24 (22.2)
	Kyungsang	2	0 (0)	38	0 (0)
	Total	21	11 (52.4)	387	44 (11.4)
Acinetobacter spp.	Seoul	11[a]	4 (36.4)	107	12 (11.2)
	Kyungki	3	0 (0)	29	0 (0)
	Kangwon	3	2 (25.0)	41	8 (19.5)
	Chulla	3	1 (12.5)	25	13 (52.0)
	Kyungsang	2	1 (50.0)	53	1 (1.9)
	Chungchung	2	2 (100)	12	4 (33.3)
	Total	24	10 (41.7)	267	38 (14.2)

[a]Four were tertiary-care hospitals.

Hodge test and imipenem-EDTA double disk synergy test (Table 1). MBL-producing *Pseudomonas* spp. and *Acinetobacter* spp. were detected in 11 (52.4%) of 21 and 10 (41.7%) of 24 hospitals that were located in four of five and five of six cities or provinces, respectively. We detected the bla_{VIM} allele by PCR from all 42 isolates of MBL-producing *P. aeruginosa* and 2 isolates of *P. putida*. The bla_{VIM-2} and bla_{IMP-1} alleles were detected in 27 (71.1%) and 11 (28.9%) of 38 *Acinetobacter* isolates, respectively (Table 2). Nucleotide sequencing for three representative PCR-positive isolates confirmed the presence of the bla_{VIM-2} gene in one isolate each of *P. aeruginosa* and *Acinetobacter* spp., and the bla_{IMP-1} gene in one isolate of *Acinetobacter* spp.

The MBL-producing strains were isolated mainly from intensive-care unit patients (31.7%) and other inpatients (50.0%); five (6.1%) were from emergency service and other outpatients (Table 3). Overall, MBL-producing isolates were mainly obtained from specimens of sputum (50.0%) and urine (29.3%). However, the proportion of MBL-producing isolates was relatively higher among urine isolates: 17.3% for *Pseudomonas* spp. and 29.2% for *Acinetobacter* spp. We obtained one MBL-producing *Acinetobacter* isolate from each of the following specimen types: blood, spinal fluid, pleural fluid, and venous catheter tip (Table 4).

Table 2. Detection of bla_{VIM-2} and bla_{IMP-1} allele from metallo-β-lactamase–producing *Pseudomonas* spp. and *Acinetobacter* spp. by polymerase chain reaction

Organism	No. isolates (%)		
	Tested	bla_{VIM-2} positive	bla_{IMP-1} positive
Pseudomonas aeruginosa	42	42 (100)	0 (0)
P. putida	2	2 (100)	0 (0)
Acinetobacter spp.	38	27 (71.1)	11 (28.9)
Total	82	71 (86.6)	11 (13.4)

The PFGE of the *Xba*I-digested genomic DNA of 39 isolates of *P. aeruginosa* showed 22 patterns (data not shown). Six isolates from one hospital had an identical pattern. Thirteen isolates (33.3%) belonged to another identical pattern—six from one hospital, two from each of two hospitals, and one from each of three hospitals, which were located in a city and two provinces.

Conclusions

In this study, >10% of all imipenem-nonsusceptible isolates of *Pseudomonas* spp. and *Acinetobacter* spp. were attributable to MBL production (Table 1), and these MBL-producing isolates were detected in 62.5% of the participating hospitals. Our finding indicates that MBL-producing *P. aeruginosa* is more prevalent in Korea than in other countries (2) and that MBL-producing *Acinetobacter* spp. is increasing. The percentage of hospitals with MBL-producing isolates might have been higher if a larger number of imipenem-nonsusceptible isolates had been collected for this study.

VIM-2 was the only type of acquired MBL identified initially in Korea. VIM-2–producing *P. aeruginosa* was isolated at almost the same time in Europe (7) and Korea (4). However, IMP-1–producing isolates were rare until 2000 in Korea. Only one and three IMP-1-positive *P. aeruginosa* and *Acinetobacter* spp., respectively, have been isolated at the coordinating laboratory (4, unpub. data). In our study, 11 (28.9%) of 38 MBL-positive isolates of *Acinetobacter* spp. were IMP producers (Table 2). This increase suggests the possible introduction of IMP-producing strains of *Acinetobacter* spp. from Japan, where 28 isolates of bla_{IMP-1}-positive *Acinetobacter baumannii* were reported in a hospital as early as 1994 to 1996 (14).

Rasmussen and Bush (15) predicted that an increase of MBL-producing organisms was inevitable, given the more frequent use of carbapenems. Imipenem has been used for

Table 3. *bla*$_{VIM-2}$ and *bla*$_{IMP-1}$ allele-positive *Pseudomonas* spp. and *Acinetobacter* spp. isolated by service

Organism	No. isolates (%)				
	Outpatient	Inpatient	Intensive-care unit	Others	Total
Pseudomonas spp.	3 (6.8)[a]	26 (59.1)	11 (25.0)	4 (9.1)	44 (100)
Acinetobacter spp.	2 (5.2)[b]	15 (39.5)	15 (39.5)	6 (15.8)	38 (100)
Total	5 (6.1)	41 (50.0)	26 (31.7)	10 (12.2)	82 (100)

[a]Two were emergency service patients, and one was a urology patient.
[b]One was an emergency service patient, and one was a pediatric patient.

only 9 years in Korea, but the imipenem-resistance rate of *P. aeruginosa* has rapidly risen from 6% in 1996 to 19% in 2001. A study by the Korean Nationwide Surveillance of Antimicrobial Resistance Group showed that the mean imipenem-resistance rates of *P. aeruginosa* in 1997 did not differ substantially depending on hospital size, (i.e., 17% in medium hospitals [<1,000 beds] and 18% in large hospitals [≥1,000 beds]). The mean resistance rates to imipenem were not lower than those to ceftazidime in 2000, i.e., 21% versus 18% in large hospitals and 20% versus 19% in medium hospitals (data not shown).

Acinetobacter spp. are also common nosocomial pathogens with multidrug resistance. The imipenem resistance rate of this organism isolated in Korea was found to be much lower than that of *P. aeruginosa,* but its resistance rate rose from 4% in the first quarter to 20% in the third quarter of 2002 at the coordinating laboratory (data not shown).

In our study, MBL-producing *Pseudomonas* spp. and *Acinetobacter* spp. were isolated mainly from sputum and urine specimens, and most (81.7%) isolates were from inpatients and intensive-care unit patients. Therefore, proper treatment of respiratory secretions and urine from intensive-care unit patients is considered an important aspect of preventing the spread of MBL-producing organisms. The presence of *P. aeruginosa* isolates with identical PFGE patterns among those collected not only from certain hospitals but also from different hospitals suggests that clonal spread is at least a part of the cause of intra- and inter-hospital dissemination of MBL-producing isolates. The presence of VIM-2-producing *Serratia marcescens, Enterobacter cloacae*, and *Achromobacter xylosoxidans* subsp. *denitrificans* (unpub. data) in other hospitals also suggests horizontal transfer of the resistance determinants.

Cornaglia et al. reported that five of seven patients infected with MBL-producing *P. aeruginosa* died, although the cause of death was difficult to establish with certainty (16). Clinical studies on the infection are rare because isolation of MBL-producing gram-negative bacilli increased only recently. We anticipate difficulties in treating patients infected with MBL-producing gram-negative bacilli, which can hydrolyze, in vitro, all available β-lactams, except aztreonam for which clinical efficacy is unknown.

Our study indicates the urgent need for action to prevent further spread of MBL-producing organisms. Previous experiences with penicillin-nonsusceptible pneumococci, methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, and extended-spectrum β-lactamase–producing *Klebsiella pneumoniae* indicate that once resistant bacteria can become widespread they cannot be controlled (10). Our first task is to detect MBL producers among clinical isolates (9). Although the National Committee for Clinical Laboratory Standards document (12) does not contain procedures for detection, simple procedures are available (13).

The prevalence of *bla*$_{VIM-2}$ allele-positive *P. aeruginosa* and *bla*$_{IMP-1}$ allele-positive *Acinetobacter* spp. is increasing possibly because of clonal and horizontal spread of the resistance determinant in Korean hospitals. Sputum and urine from inpatients and intensive-care unit patients were found to be the main sources of MBL-producing isolates. Laboratories not only in Korea but also in other countries with carbapenem-resistant organisms must be prepared to screen MBL-producing isolates to determine the clinical impact and prevent further spread of MBL-producing organisms.

Table 4. *bla*$_{VIM-2}$ and *bla*$_{IMP-1}$ allele-positive *Pseudomonas* spp. and *Acinetobacter* spp. isolated by source

Source	No. (%) of isolates with metallo-â-lactamase						% positive by source
	Pseudomonas spp.		*Acinetobacter* spp.		Total		
	Tested	Positive	Tested	Positive	Tested	Positive	
Sputum	200	22 (11.0)	143	19 (13.3)	343	41 (12.0)	50.0
Urine	98	17 (17.3)	24	7 (29.2)	122	24 (19.7)	29.3
Wound	49	2 (4.1)	71	7 (9.9)	120	9 (7.5)	10.9
Other[a]	18	3 (16.7)	29	5 (17.2)	47	8 (17.0)	9.8
Total	387	44 (11.4)	267	38 (14.2)	654	82 (12.5)	100

[a]Others included one *Acinetobacter* isolate of specimens from blood, spinal fluid, pleural fluid, and a venous catheter tip.

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Leishmaniasis in Germany

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In 2000, a reference center was created to systematically record leishmaniases in Germany. We analyzed 58 cases of leishmaniases imported during a 2-year period. These findings will serve as a baseline for the sandfly vector's anticipated northward move because of global warming and as an advisory for immunocompromised persons traveling to leishmaniasis-endemic areas.

Leishmaniases compose a spectrum of protozoal diseases currently endemic in 88 countries in Asia, Africa, the Americas, and southern Europe. The geographic distribution of leishmaniases has widened, and the disease is reported in areas in which leishmaniasis was previously nonendemic (1). Apart from cutaneous, mucocutaneous/mucosal, and visceral leishmaniases, HIV-1–associated leishmaniasis acquired in southern Europe and in other parts of the world have been observed in increasing numbers (1,2).

Leishmaniasis is not notifiable in Germany. In September 2000, a national advice and reference center was created at the Institute of Tropical Medicine in Berlin; the aim of the center was to monitor the frequency, origin, and type of leishmaniases seen in Germany; to advise physicians; and to improve information for travelers to disease-endemic areas. The healthcare professionals were informed about the reference center by the Robert-Koch-Institute, the center for surveillance of infectious diseases in Germany, as well as through the Journal of the German Medical Association, which is received by every registered physician (3). Leishmaniasis was diagnosed if parasites were detected in smears, culture, histologic sections, by polymerase chain reaction (PCR) of lesion biopsy specimens, bone marrow, or peripheral blood. For detection of *Leishmania*-specific antigen the small subunit (ssu rRNA), the internal transcribed spacer (ITS-1) region of the ribosomal RNA genes, or both, were amplified by PCR (4). *Leishmania* complexes and species were determined by digestion of the ribosomal ITS-1 PCR product with restriction enzymes (4).

Within 2 years, 70 cases of leishmaniases (43 cutaneous or mucocutaneous/mucosal; 27 visceral) were reported. For 58 case-patients (35 cutaneous or mucocutaneous/mucosal; 23 visceral), data were available on the age, sex, residence, travel destination, possible exposure location, reason for travel, duration of stay, duration and type of symptoms, concomitant diseases or therapies, type of diagnosis, and treatment received.

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Cutaneous and Mucosal Leishmaniasis

Of the 35 patients with cutaneous or mucocutaneous/mucosal leishmaniasis, 30 were German tourists (Table 1). The male-to-female ratio was 1.5:1. Ten had contracted cutaneous or mucocutaneous/mucosal leishmaniasis in Europe, 11 in Central and South America, 6 in Asia, and 3 in Africa. Two persons had been infected during work stays of 1 to 4 months in French Guyana, one each in Peru and Libya, and one patient had immigrated from Afghanistan.

The median duration of lesions until the diagnosis of leishmaniasis was made was 4 months (range 3 weeks to 2 years). Sixteen patients had more than one lesion (median 2, range 1–6 lesions). Seventeen lesions were located in the face, including mouth and nose, 28 on the upper extremities and 21 on the lower extremities. Lesions were ulcerated in 39 cases, papular-nodular in 24, and plaque-like in 3. Parasites were detected in 13 of 20 smears, in 9 of 10 cultures, and in 14 of 16 histologic sections; by using PCR, *Leishmania*-specific DNA was detected in 16 of 16 biopsy specimens.

Patient 1 had lesions in the mouth caused by *L. infantum*. She was under continuous immunosuppressive treatment for severe bronchial asthma. Patient 2 had received methotrexate and steroids for treatment of systemic collagenosis for several weeks. Both patients were tested for leishmanial infection in the blood; in both patients, the *Leishmania*-specific PCR of the buffy coat of the blood was positive. Patient 22 had mucocutaneous leishmaniasis of the nasal septum. She had been treated for a skin lesion caused by *L. braziliensis* 3 years earlier.

Visceral Leishmaniasis

A total of 18 of the 23 visceral leishmaniasis patients were German tourists; 3 were immigrants from Angola, Iran, and Togo; and 2 were visitors from Italy and Portugal (Table 2). The male-to-female ratio was 6.7:1.

The median time between symptom onset and the correct diagnosis was 4 months (range 1–16 months). All case-patients had fever, 17 (74%) had splenomegaly, 11 (48%) hepatomegaly, 20 (87%) anemia, 17 (74%) leukopenia, and 8 (35%) thrombocytopenia.

Bone marrow smears indicated *Leishmania* in 18 of 20, bone marrow culture in 6 of 7, bone marrow histologic sections in 7 of 8, PCR of the bone marrow in 8 of 9, and PCR of the buffy coat of the blood in 7 of 7 cases. Additionally, antibodies were detected in medium to high concentration by an immunofluorescence test, enzyme-

Table 1. Characteristics of patients with cutaneous and mucosal leishmaniasis, Germany

No.	Exposure	Status	Sex	Age (y)	*Leishmania* species	Treatment	Outcome
Europe							
1	France	Tourist	F	64	*L. donovani* complex	No treatment	No cure
2	Italy	Tourist	F	24	*L. donovani* complex	Liposomal amphotericin B	Improved
3	Malta	Tourist	M	22	*L. donovan*i complex	Perilesional pentavalent antimonials	Cured
4	Malta	Tourist	M	39	*L. donovan*i complex	Perilesional pentavalent antimonials	Cured
5	Malta	Tourist	F	57	n.d.	Perilesional pentavalent antimonials	Cured
6	Malta	Tourist	M	62	n.d.	Perilesional pentavalent antimonials	Cured
7	Spain (Majorca)	Tourist	F	5	n.d.	Pentamidine isethionate	Cured
8	Spain (Majorca)	Tourist	M	13	*L. donovan*i complex	Perilesional pentavalent antimonials	Cured
9	Spain	Tourist	F	31	*L. donovani* complex	No treatment	Unknown
10	Spain	Tourist	M	33	*L. donovan*i complex	Antibiotic	Cured
Americas							
11	Belize	Tourist	M	36	*L. braziliensis* complex	Liposomal amphotericin B	Unknown
12	Belize	Tourist	F	32	n.d.	IFN-gamma	Cured
13	Bolivia	Tourist	M	35	*L. braziliensis* complex	Liposomal amphotericin B	Unknown
14	Brazil	Tourist	M	25	n.d.	Systemic pentavalent antimonials	Cured
15	Brazil	Tourist	M	39	*L. braziliensis* complex	Liposomal amphotericin B	Unknown
16	Brazil	Tourist	M	33	*L. braziliensis* complex	Liposomal amphotericin B	Cured
17	Ecuador	Tourist	F	29	*L. braziliensis* complex	Liposomal amphotericin B	Cured
18	Ecuador	Tourist	M	36	n.d.	Liposomal amphotericin B	Cured
19	French Guyana	Work stay	M	28	*L. braziliensis* complex	Liposomal amphotericin B	Cured
20	French Guyana	Work stay	M	22	*L. braziliensis* complex	Liposomal amphotericin B	Cured
21	Guatemala	Tourist	M	31	*L. mexicana*	Ketoconazole	Cured
22	Peru	Work stay	F	25	*L. braziliensis* complex	Liposomal amphotericin B	Cured
23	Peru	Tourist	F	33	*L. braziliensis* complex	Liposomal amphotericin B	Cured
24	Peru	Tourist	M	35	*L. braziliensis* complex	Liposomal amphotericin B	Cured
Asia							
25	Afghanistan	Immigrant	M	21	*L. tropica*	Aminosidine ointment	No cure
26	Afghanistan	Tourist	F	12	n.d.	Aminosidine ointment	Cured
27	United Arab Emirates	Tourist	F	44	n.d.	Perilesional pentavalent antimonials	Cured
28	Syria	Tourist	M	5	n.d.	Perilesional pentavalent antimonials	Unknown
29	Syria	Tourist	F	3	n.d.	Perilesional pentavalent antimonials	Unknown
30	Turkey	Tourist	F	33	n.d.	Antibiotic	Cured
31	Turkey	Tourist	M	37	*L. donovani* complex	Liposomal amphotericin B	Improved
Africa							
32	Egypt	Tourist	M	25	*L. tropica*	Aminosidine ointment	No cure
33	Egypt	Tourist	F	27	*L. tropica*	Aminosidine ointment	Cured
34	Kenya	Tourist	M	50	n.d.	Perilesional pentavalent antimonials	Cured
35	Lybia	Work stay	M	34	n.d.	Aminosidine ointment	Cured

[a]n.d., not done; IFN, interferon.

linked immunosorbent assay (ELISA), or both, in 14 of 15 cases. Species was identified in 7 of 18 visceral cases contracted in southern Europe and indicated *Leishmania* belonging to the *L. donovani* complex, which implicated infection with *L. infantum*.

Six cases of visceral leishmaniasis occurred in children 2 months of age to 11 years of age. Four German tourists and two immigrants had long-known HIV infection (median duration 3 years, range 8 months–6 years). All HIV–coinfected patients had CD4-cell counts below 200/µL (median 108, range 23–185 CD4 cells/µL) when the diagnosis of visceral leishmaniasis was made. Of the remaining 11 patients, 1 had a thymoma with impaired T-helper-1 cell function, 2 had received intermittent immunosuppressive therapy (methotrexate and steroids) for rheumatologic disease, and 2 patients had their spleens removed. Three patients were in an impaired general condition because of combinations of diabetes, hypertonus, hypercholesterolemia, and emphysema. In the remaining three patients (53–68 years of age), apart from hypertonus in one, no impairing condition was detected.

Discussion

Information on single cases and a small case series of imported leishmaniases in Germany is available, but systematic reporting on frequency, type, and origin of leish-

Table 2. Characteristics of patients with visceral leishmaniasis, Germany

No.	Exposure	Status	Sex	Age	Risk factor	Leishmania species	Treatment	Outcome
1	Italy	Tourist	M	2 y	Child	n.d.	Liposomal amphotericin B	Cured
2	Italy	Tourist	F	5 y	Child	n.d.	Liposomal amphotericin B	Cured
3	Italy	Tourist	M	11 y	Child	n.d.	Liposomal amphotericin B	Cured
4	Spain	Tourist	F	8 mo	Child	n.d.	Liposomal amphotericin B	Cured
5	Spain	Tourist	M	9 mo	Child	*L. donovani* complex	Liposomal amphotericin B	Cured
6	Iran	Immigrant	M	7 y	Child	*L. donovan*i complex	Liposomal amphotericin B	Cured
7	Spain	Tourist	M	43 y	HIV	*L. donovan*i complex	Liposomal amphotericin B; maintenance therapy: HAART plus liposomal amphotericin B once monthly	No relapse for 6 months
8	Spain (Ibiza)	Tourist	M	48 y	HIV	*L. donovani* complex	Liposomal amphotericin B; maintenance therapy: HAART plus liposomal amphotericin B once monthly	No relapse for 8 months
9	Portugal	Visitor	M	29 y	HIV	n.d.	Liposomal amphotericin B; maintenance therapy: HAART plus liposomal amphotericin B once monthly	unknown
10	France	Tourist	M	31y	HIV	*L. donovan*i complex	Liposomal amphotericin B; maintenance therapy: HAART plus liposomal amphotericin B once monthly	Relapse after 4 months; retreatment with liposomal amphotericin B; No relapse for 3 months
11	Angola	Immigrant	M	40 y	HIV	*L. donovani* complex	Systemic pentavalent antimonials; maintenance therapy: HAART plus pentavalent antimonials once monthly	unknown
12	Togo	Immigrant	M	37 y	HIV	*L. donovani* complex	Liposomal amphotericin B	Cured
13	Italy (Sicily)	Visitor	M	31 y	Thymoma	n.d.	Liposomal amphotericin B	Cured
14	Italy (Ischia)	Tourist	M	67 y	Methotrexate/steroids	*L. donovani* complex	Liposomal amphotericin B	Cured
15	Italy (Sicily)	Tourist	M	68 y	Methotrexate	n.d.	Liposomal amphotericin B	Cured
16	Italy (Ischia)	Tourist	M	70 y	Splenectomy	n.d.	Liposomal amphotericin B	Cured
17	Spain	Tourist	M	51 y	Splenectomy	*L. donovani* complex	Liposomal amphotericin B	Cured
18	Greece (Korfu)	Tourist	M	66 y	Diabetes mellitus Hypertonus	n.d.	Liposomal amphotericin B	Cured
19	Spain	Tourist	M	52 y	Hypertonus Hypercholesterolemia	n.d.	Liposomal amphotericin B	Cured
20	Greece (Korfu)	Tourist	M	45 y	Diabetes mellitus Emphysema	*L. donovani* complex	Systemic pentavalent antimonials	Cured
21	Tunisia	Tourist	M	53 y	Hypertonus	*L. donovani* complex	Liposomal amphotericin B	Cured
22	Malta	Tourist	F	55 y	-	n.d.	Liposomal amphotericin B	Cured
23	China	Tourist	M	67 y	-	n.d.	Liposomal amphotericin B	Cured

[a]n.d., not done; HAART, highly active anti-retroviral therapy.

manial infections in Germany did not exist until 2000 (5–7). Our recent surveillance is dependent on passive consultation and reporting and therefore may have selection bias because if visceral leishmaniasis, a potentially fatal disease that requires hospitalization, is suspected, advice on diagnosis and treatment is sought more often than for the skin infection. We assume that our system captures approximately half of the visceral leishmaniasis cases and approximately one third of the classical cutaneous cases imported to Germany.

A total of 47% of all cases, but 78% of the visceral cases were contracted in the European Mediterranean area and Portugal, and most of the infections indicated a species of the *L. donovani* complex, most probably *L. infantum*, as

the probable causative agent. Thirteen infections (22%) were acquired on the Mediterranean islands of Ibiza, Ischia, Majorca, Malta, Korfu, or Sicily.

This distribution reflects the fact, that the Mediterranean countries, Spain, Italy, and the Mediterranean islands, in particular, are the favorite vacation areas for Germans. Annually, Germans take 18 million vacations to the European Mediterranean area (including 8 million to Spain and 6 million to Italy) with a median duration of 2 weeks. Sixty percent of travel to Italy and 90% of travel to Spain are to *Leishmania*-endemic areas.

While leishmaniasis has always been endemic in the Mediterranean countries, the maximum northern latitude for sandfly survival is speculated to move further to the North, beyond Germany (1) because of global warming. If this scenario is correct, the imported cases may serve as a potential substrate for the sandfly vector. Dogs that are imported as pets from the disease-endemic areas of southwestern Europe or that contract the infection when accompanying their owners for vacation are another potential substrate (8).

Infections with *L. infantum* in a child, as well as in a horse who had never left Germany, have recently been described and have led to speculations about an autochthonous focus (9,10). Also recently, the first sandfly species, *Phlebotomus mascittii* Grassi, 1908, was detected in southern Germany, although its potential as a vector of *Leishmania* remains to be demonstrated (11).

As expected, visceral leishmaniasis is often manifested in persons with impaired immunocompetence because of young age, HIV infection, immunosuppressive therapy and, in our analysis, in older persons with concomitant diseases.

Notably, 12 (67%) of 18 of the visceral cases contracted in the European Mediterranean area were in adults, thus confirming a change in age groups affected. Formerly, visceral leishmaniasis was known mainly as a disease of children (1,2). This change may partly be explained by the increased proportion of *Leishmania* and HIV–co-infected persons and partly by increased travel activities of otherwise immunocompromised persons, including elderly persons.

Furthermore, even in patients with cutaneous leishmaniasis, dissemination of parasites has to be excluded in case of impaired immunocompetence (e.g., immunosuppressive treatment). In these cases, *Leishmania*-specific PCR of the buffy coat of the peripheral blood is a sensitive method for detecting parasite spread beyond the skin.

Parents of small children and persons with reduced immunocompetence should be informed about their increased susceptibility to infection with *Leishmania* when traveling to disease-endemic areas. Measures to reduce the exposure to sandflies, such as clothes, repellents, and mosquito nets as well as collars impregnated with repellents for accompanying dogs, should be recommended.

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Probable Dengue Virus Infection among Italian Troops, East Timor, 1999–2000

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To investigate the attack rate and risk factors for probable dengue fever, a cross-sectional study was conducted of an Italian military unit after its deployment to East Timor. Probable dengue was contracted by 16 (6.6%) of 241 army troops and caused half of all medical evacuations (12/24); no cases were detected among navy and air force personnel.

Dengue fever (DF), caused by dengue virus (DENV) serotypes 1 to 4, is an emerging public health problem in many tropical countries (1). Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), the severe manifestations of DENV infection, were first recognized in the 1950s in Southeast Asia and are today a leading cause of childhood illness and death in many tropical countries. More recently, DHF and DSS have emerged in Central and South America and in the Pacific region (2,3). DF is also recognized as an emerging health problem for international travelers (4,5) and for troops deployed to tropical countries (6,7).

In 1999, following a United Nations Security Council recommendation, the International Force for East Timor (INTERFET) was formed to restore peace on the island. In November 1999, INTERFET troops totaled 11,000 from 17 countries. The Italian Armed Forces contributed 640 soldiers.

DF is endemic in East Timor. The peak transmission periods for DF are July–August and December–January, corresponding to the rainy months (8). In 1998, at least 11% of hospital inpatient deaths in East Timor were attributed to DHF (9). In October 1999, a localized outbreak of DF in a western district was attributed to serotype 3 (9) and serotype 2 was isolated in December 1999 (10). Serotypes 2 and 3 were also responsible for DF cases among Australian troops returning from East Timor in January–February 2000 (11).

During deployment, a high attack rate of febrile illness consistent with DF was reported among Italian troops. A seroepidemiologic survey was therefore conducted in February 2000 among soldiers returning home, in an attempt to determine the cause of this outbreak and to define infection rates and risk factors for infection.

The Study

All Italian troops eligible for deployment are routinely vaccinated against diphtheria/tetanus, tetravalent meningococcal meningitis, measles/mumps/rubella, hepatitis A and B, polio (with inactivated virus), typhoid fever (orally), and yellow fever (YF). In this situation, troops were also vaccinated against Japanese encephalitis (JE) (Nakajama strain, 3 doses on days 0, 7, and 14) just before landing in East Timor.

DF prevention consisted of the use of personal protection measures against mosquitoes (repellents applied to the skin; permethrin-treated bed nets and uniforms) along with environmental mosquito control. Adulticide spraying was conducted weekly by pesticide-dispersal units but only within the campsite and in its nearest surroundings, which were also inspected daily to reduce or eliminate breeding sites of vectors.

Italian troops were deployed in East Timor from late September 1999 to mid-February 2000, and all 640 participating military personnel were eligible for inclusion in the study. Army soldiers were permanently based on the ground and operated in Dili and surrounding areas, while air force and navy personnel had only logistical tasks and their presence in Dili was episodic, since they were mainly aboard ship or based in Darwin (Australia).

A seroepidemiologic survey was conducted February 15–28, 2000, among troops returning to Italy after their 3-month period of duty in East Timor. After informed consent was obtained, peripheral blood specimens were drawn and a written questionnaire administered. The questionnaire asked for personal health data, including all symptoms experienced during deployment and information about compliance with personal protection measures. Immunization status and clinical data concerning febrile illness cases consistent with DF were obtained from standardized records kept by medical personnel. Soldiers and navy/air force personnel were studied according to their serologic status and disease status during deployment.

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All specimens were screened for antibodies to dengue virus serotype 2 (DEN-2), yellow fever virus (YVF), and West Nile virus (WNV) by hemagglutination-inhibition test (HI). All serum specimens positive for DEN-2 were tested by neutralization test (NT) for DEN-2. Additionally, serum samples from participants who had experienced an acute clinical syndrome suggestive of DF were directly tested by NT for antibodies to DEN-2. Serum specimens negative for DEN-2 were then tested for neutralizing antibodies to dengue virus serotypes 1, 3, and 4 (DEN-1, DEN-3 and DEN-4).

The HI test was performed by the method of Clarke and Casals (12) and NT as 90% plaque reduction neutralization test (PRNT) on Vero cells. Briefly, serum specimens (twofold dilutions) and virus (10^2 PFU) were incubated overnight at 4°C, injected onto monolayers of Vero cells, and overlaid with 1% Tragacanth gum (Sigma-Aldrich S.r.I., Milan, Italy). Seven days postinfection, cells were washed with saline and stained with 1% crystal violet in 20% ethanol (DEN-2 and DEN-3) or by immunodetection assay (DEN-1 and DEN-4) as described (13). Vero cells were propagated in minimum essential medium with Earle's salts (EMEM), supplemented with nonessential amino acids, 10% fetal calf serum, 100 IE/mL of penicillin G, and 100 IE/mL of streptomycin.

The following viruses were used in the study: DEN-1 (Hawaii), DEN-2 (NGB), DEN-3 (H87), DEN-4 (H241), YF (Asibi), and WN (Bratislava). Viruses were injected into suckling mice by the intracerebral route. For NT, viral stocks were prepared as 10% brain suspension in Hank's saline+7.5% bovine serum albumin (Sigma-Aldrich). For HI, test antigens were prepared by sucrose-acetone extraction from mouse infected brains (12). Monoclonal antibodies specific for DEN-1 or broadly reactive with flaviviruses were purchased from ATCC (ATCC HB112, ATCC HB47) and used as mouse ascitic fluid after injection into adult BALB/c mice.

Undetermined febrile illness was defined as an acute clinical syndrome with temperature >38.5°C, unrelated to diarrhea, malaria, or other identified infections. Suspected dengue (14) was defined as an undetermined febrile illness of 2–7 days' duration, associated with two or more of the following manifestations: headache, retroorbital pain, myalgia, arthralgia, cutaneous rash. Antibody levels ≥1:1,280 dilutions by HI (1,15) for DEN-2 and ≥1:20 dilutions by NT to at least one of the four DENV serotypes were considered supportive serologic evidence of a recent dengue infection. Probable dengue (1,14) was defined as a case compatible with the clinical description of suspected DF and serologic findings supportive of a recent dengue infection.

The prevalence of undetermined febrile illness, suspected dengue, and probable dengue was compared by chi-square test among army and navy/air force personnel. Since navy and air force personnel had a limited exposure to the environment of East Timor, risk factors for probable dengue were studied only in the army contingent. A univariate analysis was first performed by Fisher exact test; each risk variable was crossed with the prevalence of probable dengue. Significance was tested at a level of α=0.05.

A multiple logistic regression model was used to determine the relationship between the outcome of probable dengue and a set of explanatory variables, and test the significance of each variable while simultaneously accounting for demographic and risk factors. The following variables were included in the model: age, rank, previous deployments in dengue-endemic areas, YF/JE vaccination, night guards, skin repellents/permethrin-treated uniforms/bed nets use, and operational versus logistic tasks. To identify a subset of variables significantly related to probable DF, the stepwise procedure was performed with the likelihood ratio test, by using at each step the p value of 0.05 as entry criterion and the p value of 0.10 as removal criterion. Univariate statistical analysis was performed with EpiInfo 6.04d software (Centers for Diseases Control and Prevention, Atlanta, GA, January, 2001) and multivariate analysis by SPSS 11.0 software (SPSS Inc., Chicago, IL).

Conclusions

Of 640 eligible participants (280 army, 93 air force, and 267 navy), 595 (93%) were included in the study: 241 army, 88 air force, and 266 navy personnel (Table 1). Serum specimens and questionnaires were obtained within 2 weeks after the troops' return, in late February 2000.

Some (14.5%) of the troops had previously been deployed to DF-endemic areas, primarily Somalia and Mozambique in 1992–1994. According to their immunization status versus YF and JE viruses, 100 (41.5%) of the 241 army soldiers had received vaccinations against YFV and JEV, 119 (49.4%) had been vaccinated against JEV only, 2 (0.8%) against YF only, and 20 (8.3%) had not been vaccinated.

Undetermined febrile illness was more frequently reported ($p<0.01$) among army soldiers than among navy and air force personnel: 85 (35.3%) of 241 versus 13 (3.7%) of 354 , respectively. All participants with suspected dengue (n=30), with serologic results supportive of a recent dengue infection (n=27), and with a probable case of dengue n=16), belonged to the army group (Table 2).

The 16 participants with probable dengue showed also a significant increase ($p<0.01$) in HI antibody titers to YFV (≥1:1,280 in 15/16 infected soldiers vs. 14/225 uninfected soldiers) and WNV (≥1:1,280 in 10/16 vs. 6/225). The average interval between the onset of clinical manifestations suggestive of DF and the date when blood samples

Table 1. Characteristics of Italian military personnel, East Timor, 1999–2000

Feature	Army	Navy	Air Force	Total
No. participants	241	266	88	595
Mean age (years ± sd)	27 ± 7	28 ± 7	35 ± 7	–
Time of deployment	22/Sep/99–16/Feb/00	21/Oct/99–19/Feb/00	19/Sep/99–17/Feb/00	–
Mean duration of deployment (days ± sd)	100 ± 25	109 ± 14	41 ± 23	–
Person months	803	968	102	1,873
Presence in East Timor for ≥90 days (no. soldiers)	241	0	0	241
Episodical presence in East Timor (no. soldiers)	0	266	88	354

were taken was 36±25 days. All 16 case-patients with probable DF had a fever >38.5°C; a saddle-back fever pattern was recorded for 5 (31.3%). Other reported symptoms included myalgia and rash in 13 (81.3%); headache in 11 (68.8%); retroorbital pain in 9 (56.3%), and adenopathy in 3 (18.8%). No patients had DHF/DSS.

The mean duration of probable DF cases was 7±3 days. Moreover, 12 of the 16 patients with probable DF were evacuated because of their clinical status. Univariate analysis of risk factors for probable DF suggested a possible protective effect of JEV vaccination and personal protection measures (Table 3). However, logistic regression analysis identified only a subset of variables significantly related to probable dengue, whose risk was higher among soldiers on duty in operational rather than logistic units, and lower among participants with regular use of bed nets (Table 4).

Since most of soldiers had been previously vaccinated with a flavivirus vaccine (YFV, JEV, or both), their immune response to an eventual dengue infection was expected to be a secondary (anamnestic) response, with high-titer antibodies cross-reacting with several DENV serotypes, as well as other flaviviruses (15). Thus, in spite of the lack of paired serum specimens, high antibody titers to DEN-2 by HI (≥1:1,280) (1,16) and to any of the four dengue virus serotypes by NT (≥1:20), after an average of 36 days from the onset of clinical manifestations compatible with dengue infection, may be considered supportive serology of a recent flavivirus infection, likely acquired during deployment.

Overall, 6.6% of army soldiers contracted probable dengue. No cases of probable DF were detected in the low-exposure group of navy and air force personnel. The high attack rate of probable dengue among the army contingent may be due to several reasons. First, DF and DHF/DSS are epidemic throughout Southeast Asia (3), including Indonesia (17); in particular, the incidence of DF markedly increased in East Timor in 1998–1999 (18). Secondly, the multinational deployment to East Timor took place during the rainy season (December–January), when the risk of infection is high.

Approximately 60% of troops with supportive serologic evidence of a recent dengue infection showed the clinical manifestations of classic DF, 20% had milder symptoms, and 20% were asymptomatic. This finding agrees with the U.S. troops' experience in Somalia in 1993, where >85% of all DENV infections were symptomatic (6). In contrast, the overall ratio of inapparent to clinical DENV infections is quite high in persons living in disease-endemic areas, as in Indonesia, where it has been reported to be as high as 9.3 (17).

Performing duties outside the camp was associated with a significantly higher risk of infection, probably because vector control activities were regularly carried out within the compound. Regular use of bed nets was the only personal protection measure that significantly decreased the risk of contracting probable dengue. This finding is not new (6) and may have been because some of the troops were frequently on duty at night and thus slept during the day when the biting activity of dengue vectors is highest. Otherwise, the regular use of repellents (applied to the skin) and permethrin-treated uniforms seemed to decrease the risk for dengue infection, but the differences between those who did not follow these practices and those who did were not significant statistically.

DF is therefore an emerging problem for troops deployed to dengue-endemic areas, mainly because of the lack of effective preventive measures, the high attack rate,

Table 2. Clinical and serologic findings of recent dengue infection among Italian troops

	Serologic findings of recent dengue infection[a]		
Clinical assessment	No. supportive (%)	No. not supportive (%)	Total no. (%)
Undetermined febrile illness[a]	6 (22.2)	49 (22.9)	55 (22.8)
Suspected dengue[b]	16 (59.3)	14 (6.5)	30 (12.4)
Asymptomatic	5 (18.5)	151 (70.6)	156 (64.7)
Total	27 (100)	214 (100)	241 (100)

[a]All military personnel with supportive serologic findings belonged to the army contingent (N=241). Probable dengue cases are represented by the 16 soldiers with clinical manifestations compatible with DF (suspected dengue) and serologic findings supportive of a recent dengue infection.

[b]Cases are defined in the section "Materials and Methods."

Table 3. Risk factors associated with probable dengue (univariate analysis)

Demographic and risk factors	No. cases of probable DF/no. soldiers exposed (%)	OR 95% CI	p value[b]
Age, <26 y			
Yes	10/124 (8.1)	1.62	
No	6/117 (5.1)	(0.51 to 5.61)	0.26
Lower rank (enlisted men vs. NCOs/officers)			
Yes	9/145 (6.2)	0.84	
No	7/96 (7.3)	(0.27 to 2.76)	0.47
Previous deployments in dengue-endemic areas			
Yes	3/44 (6.8)	1.04	
No	13/197 (6.6)	(0.18 to 4.01)	0.59
YFV vaccination			
Yes	5/102 (4.9)	0.60	
No	11/139 (7.9)	(0.16 to 1.95)	0.26
JEV vaccination			
Yes	9/219 (4.1)	0.09	
No	7/22 (31.8)	(0.03 to 0.34)	<0.01
Night guard at least once a week			
Yes	6/142 (4.2)	0.39	
No	10/99 (10.1)	(0.11 to 1.25)	0.06
Skin repellents, regular use (at least once a day)			
Yes	9/209 (4.3)	0.16	
No	7/32 (21.9)	(0.05 to 0.56)	<0.01
Use of permethrin-treated uniform			
Yes	9/186 (4.8)	0.35	
No	7/55 (12.7)	(0.11 to 1.17)	0.05
Bed nets, regular use (every night)			
Yes	9/223 (4.0)	0.07	
No	7/18 (38.9)	(0.02 to 0.26)	<0.01
On duty in operational vs. logistic units			
Yes	15/179 (8.4)	5.55	
No	1/62 (1.6)	(0.82 to 238.67)	0.05

[a]DF, dengue fever; OR, odds ratio; CI, confidence interval; NCOs, noncommissioned officers; YFV, yellow fever virus; JEV, Japanese encephalitis virus.
[b]Fisher exact test.

the high symptomatic/inapparent infection ratio, and the long period of being unfit for duty after the acute phase of the disease. DF may thus seriously disrupt the readiness of a military unit. Moreover, previously infected soldiers redeployed to disease-endemic areas may be at increased risk for DHF/DSS complications. Persons previously infected by a DENV serotype may be at higher risk of developing DHF/DSS, if they are subsequently infected by a different serotype. Such risks should be taken into account while planning international peace-keeping operations, and the risk of DHF among previously dengue-infected military personnel should be evaluated.

Cross-reaction by antiflavivirus antibodies induced by JEV vaccine may otherwise afford some cross-protection against DF. JEV vaccine (Nakajama strain) seems to decrease the attack rate of DHF and reduce the severity of cases for a short time (19). More recently, researchers have noted that prior vaccination of hamsters with a live, attenuated JEV vaccine strain (not licensed for human use) and a St. Louis encephalitis virus wild strain seems to reduce the severity of a subsequent WNV infection (20). Our data suggest that prior vaccination with the commercially available JEV inactivated vaccine for human use (Nakajama strain) may have some protective effect against subsequent probable DF. The decrease was, however, not significant, according to the multiple logistic regression model we used.

Table 4. Risk factors associated with probable dengue by multivariate analysis[a]

Risk factors	OR estimate	p value
On duty in operational vs. logistic units	11.29	<0.05
Bed nets, regular vs. nonregular use	0.04	<0.01

[a]OR, odds ratio.

Our data suggest that effectiveness of routine protective measures against vector mosquitoes is far from satisfactory. A tetravalent dengue vaccine is needed to effectively reduce the risk for DF and DHF/DSS among troops deployed to tropical areas as well as to protect long-term international travelers to dengue-endemic countries.

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HIV Epidemic among Young Thai Men, 1991–2000

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Characterization of the HIV epidemic in Thailand has benefited from the systematic testing of young men upon entry into the military. These data, which have shown that public health measures can reverse an HIV epidemic, have been reanalyzed with current geographic information systems methods. The resulting maps are, thus far, the best means of visualizing the geography of the dynamic HIV epidemic in Thailand.

In few nations is the HIV epidemic characterized as well as it is in Thailand. As elsewhere, sentinel surveillance of high-risk populations is performed and more general populations are partially assessed by HIV testing of blood donors and pregnant women. In addition, Thailand has benefited from another measure to assess the HIV epidemic in the general population. Since very early in the epidemic, young men have been tested for HIV at the time they entered into military service. Military medicine and public health leaders recognized that, in addition to using this information to benefit individual men, maintaining this information in a confidential database linked to area of residence would allow monitoring of national demographic trends in the epidemic. Data generated in this way have provided key evidence that public health policy has reversed the trend of increasing HIV infections (1), preventing an estimated 200,000 HIV infections by 2000 (2).

The HIV prevalence data on young Thai men is provided to public health policy makers on a regular basis. It has also been published in the scientific literature (3,4) with data presented that uses provinces as the scale of analysis. More recently, this unique dataset has been analyzed by using geographic information systems (GIS). GIS methods allow enhanced visualization of the trends, both geographic and chronologic, within this well-documented HIV epidemic. Using a GIS approach, we present the Royal Thai Army dataset, expanded to include the decade of 1991–2000 and data precision refined to the district level.

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Methods

The methods of recruiting young men into the Thai military have been described previously (3,4). Approximately 50,000–60,000 men, mostly 21 years of age, are selected each year by lottery in their home province (the province in which they are listed on the house registration). The system produces a representative national sampling of Thai men. Because of their young age, HIV prevalence in these annual recruit classes may serve as a proxy for HIV incidence. Induction into the military occurs in May (M) and November (N) of each year. At the time when blood is collected, recruits provide information about the location of their main residence (including province and district) during the previous 2 years (3). Although the actual locations where infections occurred are unknown, residential data enables analysis of the association between HIV prevalence and this key geographic marker.

To refine the HIV prevalence analyses, geographic localization uses districts as the first administrative subunit of provinces. When data were analyzed by using annual classes grouped at the district level, calculations of the percentage testing positive for HIV were statistically unreliable because the number of men tested in some rural districts was so small. Therefore, we merged data in two ways to decrease variability attributable to the small sample size of the prevalence figures: classes were combined across time, and districts were combined across space. Sixteen classes of men recruited from 1991 to 2000 were combined temporally into four larger classes, each representing discrete 2-year periods: 1) N91, M92, N92, M93; 2) N94, M95, N95, M96; 3) N96, M97, N97, M98; and 4) N98, M99, N99, M00. Data on classes recruited from the M91, N93, M94, and N00 lotteries were not available for analysis (M91, before full implementation; N93 and M94, protocol under revision; N00, completed after dataset closed). However, even after combining classes into these 2-year periods, a number of districts still had numbers too low for statistical reliability.

We also merged some districts with neighboring districts so that each had a minimum denominator of 20 in the HIV prevalence calculation. Numbers of $\geq$20 persons provide minimal, but acceptable, reliability in the percentage-positive calculations. Districts with <20 were combined with other districts according to the following protocol, following a sequence of priorities: we combined districts if they were in the same province, had historic connections (formerly part of single larger district), had similarly small numbers tested, had similar demographics, and had similar topography or other geographic features.

For the GIS analysis, data tables provided in Excel files (Microsoft Corp., Redmond, WA) by the Armed Forces

Research Institute of Medical Sciences were joined to district-level GIS maps obtained from the Thai Environmental Institute and National Statistical Office. We used Arcview 3.2a software (ESRI, Redlands, CA) to create dot density and choropleth maps. (A choropleth map uses shades or colors to demonstrate the geographic distribution of a range of values.)

Results and Discussion

Figure 1 provides visual evidence of the geographic distribution of the epidemic. Each of the 10,043 dots represents a recruit who tested positive for HIV infection (positive for HIV-1 by enzyme immunoassay and Western blot) among the 442,923 recruits tested. Arcview software uses an algorithm that allows the random placement of dots within a geographic area (in this case a district). By creating these dots at the district level but presenting them at the province level, we show the vivid geographic pattern of infection while illustrating the human impact of the epidemic. The dot distribution does not distinguish between population density and HIV prevalence. Nevertheless, these absolute numbers provide important information regarding the potential HIV-related impact on the healthcare system and various other social structures.

The evolution of the HIV epidemic over the course of the decade is seen in the four maps shown in Figure 2. These maps use gradients of color shading to distinguish the percentage of men who tested positive for HIV during each 2-year period. The initial high prevalence of HIV in men from the upper north region is illustrated, as is the decline in prevalence over time (which likely occurred as a result of public health interventions). The relative persistence of HIV prevalence in some districts in the far south of the country is also shown, suggesting that locale-specific features of the epidemic need to be well understood in that area, potentially leading to changes in public health interventions.

Previous analyses conducted at the province level identified regional variation quite clearly, but at a less detailed level (3,4). The maps from our study indicate considerable variation even to the district level of analysis, which suggests important local factors may be at work in determining rates of transmission. The HIV epidemic in Thailand seems to have largely missed some districts and devastated others, even within the same province. Chiang Mai and Chiang Rai Provinces are examples of this phenomenon. These maps also show that, although the growth of the epidemic has slowed in most parts of the country, the epidemic has not decreased in some districts in south Thailand. Choropleth maps best demonstrate the changing prevalence of HIV among recruits from each district. The dot map best demonstrates the intensity of the epidemic within and across provinces. Both types of imagery are required to describe and understand the epidemic and to suggest locales in which public health initiatives are needed. In areas where the intensity of the epidemic is highest, health and social services may require additional resources to serve persons with the disease. In areas where the prevalence rates are highest or are increasing, socially and culturally appropriate interventions may be needed to strengthen and focus HIV programs.

Figure 1. Dot density map of young men who tested positive for HIV at time of entry into the Royal Thai Army, Thailand, November 1991–May 2000. Each dot represents one man. Location of dots based on recruit's residence during the previous 2 years. Data on recruits entering in November 1993 and May 1994 are not available.

These GIS maps are the most accurate maps available to date and are built upon the unique HIV prevalence datasets collected over a decade by the Royal Thai Army. GIS mapping at this descriptive level enhances visualization of these data (5,6). More sophisticated methods of multivariate GIS visualization could enhance this analysis even more but would require the Royal Thai Army to assess recruits at induction on other variables, especially risk factors, or to use recruits' home addresses rather than districts or provinces of residence. Nonetheless, changes in time and place of chronic viral infection can be added to

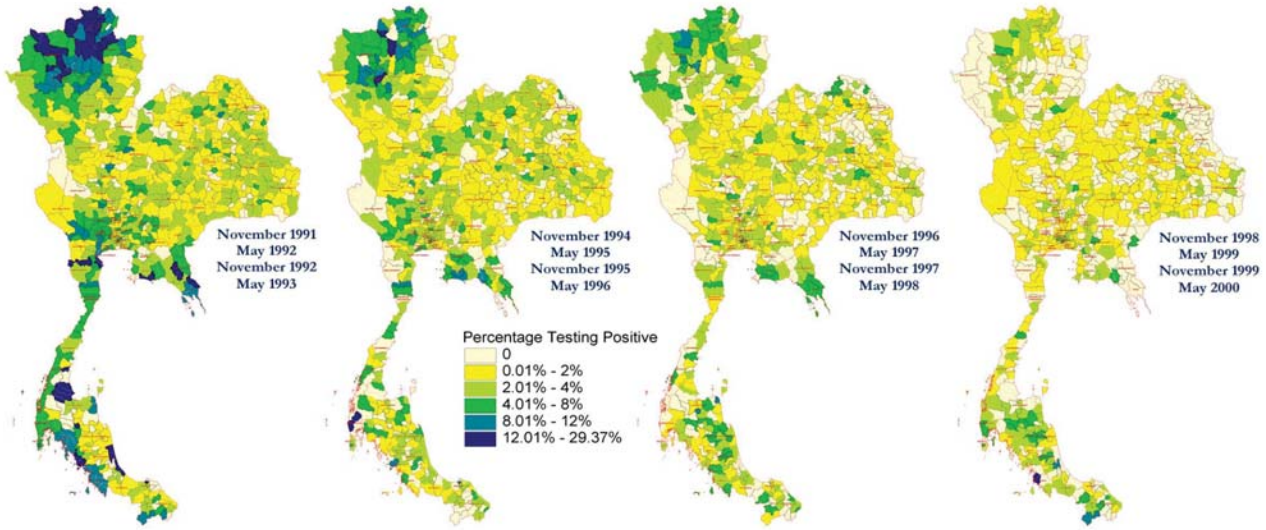

Figure 2. Choropleth maps of HIV prevalence in four classes of young men at time of entry into the Royal Thai Army, Thailand, 1991–2000. Location determined by residence during the previous 2 years. Prevalence is stratified by color and localized to district or group of districts so that calculations are based on >20 men.

the recognized areas of GIS application in epidemiology, transmission of vector-borne and water-borne diseases, and environmental health (7). The maps also illustrate the point that political borders may have no relationship to epidemiologic boundaries. Differences in HIV prevalence often occur, not only between regions and provinces but also within provinces and across provincial borders. When an epidemic can be visualized at this level of detail and accuracy, researchers can formulate and address questions regarding the bases of these differences. At the same time, policy makers can better direct intervention strategies and more finely assess the outcomes of these interventions. Coordination of data collection and joint GIS analysis by neighboring countries would enhance regional understanding of the emergence and spread of HIV epidemics and monitoring of control programs.

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Taenia solium Cysticercosis, Irian Jaya, Indonesia

To the Editor: Cysticercosis, a tissue infection caused by accidental ingestion of eggs released from humans harboring the pork tapeworm, *Taenia solium* (TsCysti), is one of the most serious reemerging parasitic diseases worldwide (1). Taeniasis is an intestinal infection caused by the adult stage of the large tapeworm. Carriers of *T. solium* acquire infection through eating undercooked pork contaminated with cysticerci (larvae). Although most Indonesian people are Muslim and do not eat pork, infection with *T. solium* has occurred in some areas or islands where most local people are Christian or Hindi.

The area most affected by this infection is Irian Jaya, Indonesia, the western half of New Guinea Island (2–4). In field surveys conducted in 2000 and 2001, we found that 5 (8.6%) of 58 local people and 7 (11%) of 64 local dogs living approximately 1 km from the local capital city, Wamena, in Jayawijaya District, harbored adult tapeworms and cysticerci of *T. solium*, respectively (5,6). We have further seroepidemiologic data from 1996 and molecular confirmation of subcutaneous nodules (SCN) as cysticerci of the *T. solium* Asian genotype. We believe this organism is an emergent problem in Irian Jaya.

We previously reported that TsCysti was highly endemic in Jayawijaya District, Irian Jaya (2–6). A total of 96 local people ≥18 years of age from Assologaima, Jayawijaya District, were chosen at random and examined by serologic testing and by administering questionnaires in February 1996 after the local and Indonesian governments gave their ethical approval. The 96 persons were divided into three groups on the basis of a history of epileptic seizures (ES, n=17), physical examination of SCN by palpation (n=32), or good health (including no ES or SCN; n=47). A total of 14 subcutaneous nodules removed from 14 men in both ES and SCN groups were confirmed to be cysticerci of *T. solium* by morphologic observation and to be *T. solium* Asian genotype by mitochondrial DNA analysis with cytochrome *c* oxidase subunit 1 gene (3,7). For serologic analysis, we conducted an enzyme-linked immunosorbent assay (ELISA) that used glycoproteins purified from cyst fluid of *T. solium* cysticerci by preparative isoelectric focusing (fractions of pH 9.1) (8) in 2001.

On the basis of serologic results, 12 (70.6%) of 17, 20 (62.5%) of 32, and 12 (25.5%) of 47 of ES, SCN, and healthy groups, respectively, were infected with the larval stage of *T. solium*. Serologically positive rates increased to 83.3% (10/12) of people with subcutaneous nodules in the ES group. A follow-up study of seropositive persons in the healthy group in 1997 showed that five of eight persons had ES (two persons), headache (one person), or SCNs in upper arm (two persons). Seropositive persons in all three groups (ES, SCN, and health) were considered to be infected with TsCysti. Persons of the SCN and healthy groups who showed optical density values higher than the cut-off value were considered to have asymptomatic TsCysti cases.

The local persons we examined ranged from 18 to 29 years of age (n=30), 30–44 years of age (n=36), and ≥45 years of age (n=30). Seropositive persons (n=12) from the ES group (n=17) were 18 to 29 years of age (40.0%, 2/5), 30–44 years (71.4%, 5/7), and ≥45 years (100%, 5/5). The prevalence of TsCysti did not vary statistically by sex (males 53.6% [37/69] versus females 33.3% [9/27], Pearson's chi-square test, p=0.074).

That 14 persons confirmed to have subcutaneous cysticerci of *T. solium* were seropositive strongly suggests that the serologic test (ELISA) is highly reliable for detecting TsCysti in patients, whether their infection is symptomatic or asymptomatic. In contrast, one of the following scenarios was expected for cases in three of five persons in the ES group who did not have SCN and were seronegative: 1) the case was not due to TsCysti, 2) the case was caused by TsCysti but without antibody response, rather common in cases of a solitary cyst, or 3) the case was caused by TsCysti with calcified cysts and without antibody response. Twelve (approximately 40%) of seronegative persons from the SCN group (n=32) were expected to have cases of TsCysti without antibody response or to have calcified cysts without antibody response. Cases without antibody response would be most expected because of the heavily contaminated environment in Irian Jaya (3–6). However, further evaluation with computed tomography or magnetic resonance imaging scans is necessary. Based on serologic results and mitochondrial DNA confirmation of *T. solium* Asian genotype (3,7), we concluded that 47.9% (46/96) of local people examined at random, 53.6% of men (37/69) and 33.3% of women (9/27) ≥18 years of age had TsCysti.

An additional 30 local people in non–TsCysti-endemic Merauke District underwent serologic testing. One woman had an exceptionally high antibody titer. She was a transmigrant from another island (South Sulawesi Province). Although Paniai, Jayawijaya, and Manokwari Districts are contaminated with *T. solium* taeniasis and cysticercosis (2–4), no additional critical evidence exists to show that Merauke District has already been contaminated with this parasite.

Taeniasis and cysticercosis may have been accidentally introduced into Irian Jaya in 1969 when the country was governed by Indonesia, since the governing body came from Bali, the only area in Indonesia where

TsCysti was exclusively endemic (2). The contaminated areas in Irian Jaya have increased from the central area (Paniai), to the east (Jayawijaya) (3), and then to the west (Manokwari), where 54 TsCysti cases have been reported (Papua Province Health Office Services, 1997, unpub. data). We wanted to know if taeniasis/cysticercosis had been introduced into the eastern half of New Guinea Island, called Papua New Guinea (PNG) (9). We had already serologically confirmed that 16 (3.0%) of 541 local residents and Irianese refugees in Alice River villages along the border in PNG had asymptomatic TsCysti (Ito et al., unpub. data). Follow-up surveys will be crucial in several other districts including Merauke District in Irian Jaya, PNG, and other islands such as Timor Island, where most of the population is Christian and many suspected cases have recently been reported by the District Health Office Services (10). Schoolchildren should also be checked so that cases can be detected and treated early. Sustainable education of the local community in Irian Jaya, Indonesia, and Papua New Guinea is also necessary.

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Recombinant Vaccine–Derived Poliovirus in Madagascar

To the Editor: Between October 2001 and April 2002, five cases of acute flaccid paralysis associated with vaccine-derived poliovirus (VDPV) type 2 isolates were reported in the southern province of the Republic of Madagascar. The first patient, an 11-year-old child from the urban district of Toliara, first experienced paralysis on October 29, 2001. Three other children, 6, 9, and 14 months of age from Ebakika village, in a rural district of Taolagnaro (250 miles east of Toliara), showed signs of poliomyelitis between March 21 and March 26, 2002. The last case-patient, a 20-month-old child from Ambanihazo village (6 miles north of Ebakika), came into contact with one of the three case-patients in Ebakika in March 2002, and symptoms developed on April 12, 2002 (1). None of the patients had been fully vaccinated against poliomyelitis.

Nine type 2 poliovirus (PV) strains were isolated. A restriction fragment length polymorphism (RFLP) assay, with three different genomic regions amplified by reverse transcription–polymerase chain reaction (RT-PCR) and four different restriction enzymes (*Hin*fI, *Dpn*II, *Rsa*I, and *Dde*I) were used to characterize the PV isolates at the molecular level (2). The RFLP profiles of all of the isolates in the two capsid protein regions were identical to that of the type 2 strain of the oral polio vaccine (OPV) in the VP1-2A region (nucleotides 2,872 to 3,647) but slightly different in the VP3-VP1 region (nucleotides 1,915 to 2,883). The observed differences allowed us

to distinguish two groups (isolates from Toliara and isolates from Taolagnaro) and two subgroups (isolates from March and isolates from April). The RFLP profiles of isolates in the noncapsid region, at the 3′-terminal end of the genome (polymerase 3D and 3′ noncoding regions: nucleotides 6,535 to 7,439) also confirmed the presence of two separate groups. These last profiles were completely different from those of the three reference vaccine strains, suggesting recombination with other enteroviruses.

Partial genomic sequencing confirmed these observations. The entire VP1 region (903 nucleotides) of the type 2 PV strains from Toliara and Taolagnaro differed from the type 2 OPV strain by 1% and 2.5% nucleotides, respectively. This difference may indicate that the two strains had been multiplying or circulating for approximately 1 and 2.5 years, respectively. Taolagnaro strains are closely related to each other (<1% nucleotide difference) but appear to be very different from Toliara strains (2.9% nucleotide difference), indicating the existence of two genetic lineages. The sequencing of the noncapsid region (440 nucleotides corresponding to nucleotide positions 6,705 to 7,144 of the Sabin 2 genome) confirms the existence of two lineages derived from different recombination events with two nonidentified enteroviruses of the phylogenetic cluster C. This cluster, based on sequence similarity, includes some coxsackieviruses and all PV strains (3).

We tried to identify the donor strains for sequences in the 3' terminal end of these recombinant strains by aligning the nonidentified sequences with homologous enterovirus sequences available in a nucleotide sequence database (FASTA, version 3.3 applied to GenBank) (4). The highest percentages of nucleotide sequence identity were those with PVs and with most other cluster C enteroviruses available in the database (87% to 91% nucleotide identities). No wild PV strains have been isolated in Madagascar since 1997 despite surveillance and investigation of viral causes of acute flaccid paralysis cases (5). Thus, that the detected VDPVs were the product of recombination between OPV strains and two nonpolio enteroviruses is more likely than that they were the product of OPV strains and two different undetected wild PV strains. However, we cannot exclude the possibility that wild PVs were imported or circulating silently for a while.

In response to the outbreak, the local health authorities conducted house-to-house vaccination with OPV. Further field investigations were carried out to determine the extent to which VDPV had spread and to search actively for other cases. Data analysis is in progress.

As with the other epidemics in Egypt and Hispaniola, VDPV circulated in a province of Madagascar with low OPV coverage (6,7). Because a high OPV coverage rate helps prevent the circulation of both VDPVs and wild PVs, obtaining and maintaining high rates of immunization coverage are essential (8). Moreover, two recombinant VDPV lineages in Madagascar indicate that recombination is frequent between OPV and cluster C enteroviruses. Similar recombinant VDPVs have been implicated in the epidemics in Hispaniola and in the Philippines (6,9). Determining whether the neurovirulence and transmissibility of these VDPVs could be the result of

the recombination with nonpolio enteroviruses is important. These VDPVs have major implications for the cessation of immunization with OPV after certification that wild PV has been eradicated.

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West Nile Virus Infection in Crocodiles

To the Editor: Recently West Nile virus (WNV) infection has been reported in three alligators (*Alligator* sp.) from central Florida (1) and one captive crocodile monitor (*Varanus salvadori*) with neurologic signs from the District of Columbia and Maryland area (2). These first reports of the virus in American reptiles highlight the possible role of this group of vertebrates in the WNV life cycle. To our knowledge, WNV in a reptile was reported only once before in a serosurvey conducted in Israel from 1965 to 1966, in which 22 reptiles and 96 amphibians were tested for hemagglutination-inhibiting antibodies against several viruses, including WNV; one turtle (*Clemmys caspica*) was seropositive (3). Experimental infection of the lake frog (*Rana ridibunda*) with a Russian strain of WNV resulted in high levels of viremia (4). At present, the role of reptiles and amphibians in the life cycle and epidemiology of WNV is not known.

We report, for the first time, WNV infection in crocodiles (*Crocodylus niloticus*). To assess the potential role of crocodiles in the life cycle of WNV in Israel, serum specimens were collected from 20 healthy crocodiles on a commercial farm in the Negev Desert, in southern Israel (31°14′N, 34°19′E). The crocodiles came from two separate breeding farms (32°03′N, 35°26′E and 30°18′N, 35°07′E) in the Syrian-African Rift Valley, which is on the main route of bird migration from Africa to Europe. Five males and 15 females, 1–2.5 years of age, were examined. Blood was withdrawn from the crocodiles' ventral caudal vein, separated by centrifugation, and kept at –20°C until analyzed. Neutralizing antibody titers were determined against WN-goose-98 (5) and attempts to isolate the virus were performed by using Vero cell culture (6) and by using direct reverse transcription–polymerase chain reaction (RT-PCR) on the serum specimens. To eliminate the possibilities of nonspecific reaction, all serum samples were concurrently tested for the only other flavivirus known to be present in Israel; Israeli turkey meningoencephalitis virus (ITV) (7). Because ITV does not produce cytopathic effects (CPE) in Vero cells, virus neutralization was conducted on BHK cells for both WNV and ITV by using WN-goose-98 and ITV (vaccine strain). In this case, the virus stocks ($10^{-4.2}$ 50% tissue culture infective dose) were diluted 1:400, and virus neutralization titers were checked 3 days later.

Viral RNA was extracted from serum samples with the QIAamp RNA blood kit (QIAGEN, Valencia, CA) , according to the manufacturer's protocol and resuspended in 30 µl of RNase-free water. The primer pair WN240-Kun848 (respective genome positions 5′: 848 and 1,645) was used to synthesize an 800-bp product in the E gene region (8,9). The resulting DNA fragment was visualized on

1.5% agarose gel stained with ethidium bromide.

The seroprevalence rate in the first set of virus neutralization assays in Vero cells was 14/20 (70%, with titers ranging from 1:20 to 1:320 [3x1:20, 3x1:40, 3x1:80, 2x1:160, 3x1:320]). No differences were discernible in either the seroprevalence rate or in the average titers of crocodiles from two different breeding farms. In BHK cells, a similar seroprevalence rate was observed, with titers ranging from 1:40 to 1:1,280 (3x1:40, 2x1:80, 1x1:160, 4x1:320, 3x1:640, 1x1:1280). All serum samples, except one, were <1:10 against ITV virus, which had a titer of 1:640 against WNV and 1:10 against ITV. Viremia was not detected in any of the 20 samples in Vero cell culture or by RT-PCR.

These results demonstrate a high rate of infection with WNV in crocodiles in Israel. The crocodiles may have been exposed to the virus during the summer at their present location, since no difference in prevalence was seen between the two groups (which differed only in the farm of origin) and since the younger crocodiles had been hatched in the spring of 2002. Furthermore, a cross-reaction with the other prevalent flavivirus in Israel, ITV, was ruled out. Preliminary results from an equine seroprevalence study (involving 800 horses over a 3-year period) of virus neutralization antibodies to WNV collected during fall 2002, indicate that most horses sampled in Israel's Arava Valley (a desert in the Syrian-African Rift near the Jordanian and Egyptian borders) and the Gulf of Aqaba/Eilat (30°59′N, 35°18′E to 29°34′N, 34°57′E) (85%, 79/90) were positive (A. Steinman and S. Tal, unpub. data,). WNV was also isolated from mosquitoes in the same region (10). The seroprevalence of WNV antibodies among horses and local birds from the Negev Desert is not known nor is the time when the horses acquired WNV infection. However, the isolation of WNV from mosquitoes (10) and the presence of antibodies to WNV in young crocodiles demonstrate arboviral activity in this region in the summer of 2002, although clinical cases were few. That virus was not isolated from crocodiles in late November (past outbreaks of WNV in Israel mainly occurred between August and October) (6,11).

WNV has been endemic in Israel since the early 1950s (12). More recently, in the summer of 2000, an extensive outbreak occurred, affecting hundreds of people (11), dozens of horses (6), and several flocks of geese (5). However, no deaths of crocodiles were reported. This contrasts with the report from Florida (1), where WNV was isolated from dead alligators, and where hundreds of cases of sudden death had been reported in previous years; these deaths are now suspected to result, at least in part, from WNV disease.

The role of various reptile species in the epidemiology of other arboviruses such as western equine encephalitis, eastern equine encephalitis, and Venezuelan equine encephalitis is well documented (13–15). At present, the role of reptiles and amphibians in the life cycle and epidemiology of WNV is not known, and further research is necessary.

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Dejà Vu

"I must have made this molecule before;
It is familiar to the core!"
Said a yeast cell emerging from mitosis,
With no experience yet in synthesis.
Then, guided by a transmigrant human gene,
It assembled that "alien" protein.

Boghos L. Artinian

fever virus. Med Parazitol (Mosk) 1985;3:49–50.
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Rickettsia aeschlimannii in Spain: Molecular Evidence in *Hyalomma marginatum* and Five Other Tick Species that Feed on Humans

To the Editor: *Rickettsia aeschlimannii* is a pathogenic spotted fever group rickettsia first isolated from *Hyalomma marginatum* ticks collected in Morocco in 1997 (1). Later found in *H. marginatum* ticks from Zimbabwe, Niger, Mali, and Portugal (2), *R. aeschlimannii* has also been found in a *Rhipicephalus appendiculatus* tick attached to the right thigh of a patient in South Africa (3). These data suggest a broad geographic distribution for *R. aeschlimannii* and the possibility that tick species other than *H. marginatum* may also be suitable vectors for this rickettsia.

The pathogenicity of *Rickettsia aeschlimannii* in humans has been demonstrated by Raoult et al. (4) in a French patient who became ill after returning from a trip to Morocco. The patient exhibited symptoms similar to those of Mediterranean spotted fever (MSF) produced by *R. conorii*, with a tache noire–like eschar on his ankle, fever (39.5°C), and a generalized maculopapular skin rash. The second documented and most recent case of human infection by *R. aeschlimannii* occurred in a South African man who was bitten by *R. appendiculatus*; an eschar also developed around the tick attachment site on this patient (3). He was aware of the risk for tick-transmitted disease, so he removed the tick and administered doxycycline; he did not develop additional symptoms.

Over the past 6 years, throughout the region of Castilla y León, northwestern Spain, we have collected and identified 3,059 ticks belonging to 15 species (unpub. data) that were attached to persons living in this territory. We have systematically analyzed the ticks by polymerase chain reaction (PCR) to detect those infected with *Borrelia burgdorferi*, *Anaplasma phagocytophila*, and *Rickettsia* spp. This procedure enabled us to identify, for the first time in Spain, *R. aeschlimannii* in 35 tick specimens belonging to *H. marginatum* and to another five species.

During the 6-year study, ticks found on patients who sought medical advice in the hospitals and healthcare centers of Castilla y León were removed and referred to our laboratory for identification and analysis. Each tick was first disinfected by immersion in 70% alcohol, rinsed in sterile water, and dried on sterile filter paper. We then extracted DNA in 5% Chelex-100, according to the method of Guttman et al. (5). In searching for *Rickettsia* spp., we proceeded as described by La Scola and Raoult (6): All DNA samples were first tested for a fragment of the rickettsial *glt*A gene (7), and then, in the *glt*A-positive samples, a fragment of the rickettsial *omp*A gene (8) was amplified, sequenced, and compared with gene databases for identification. The *glt*A amplicon was sequenced only when the *omp*A was not successfully amplified. To prevent DNA contamination and the carryover of amplified products, we used sterile tools at all times and carried out each step of the analysis (extracting DNA, preparing the reaction mixture, and amplifying and analyzing the PCR product) in separated work areas. Two negative controls (Milli-Q water and DNA from laboratory-reared noninfected ticks) were included in each amplification trial. These controls were never amplified.

We obtained 21 *omp*A amplicons (629–632 bp) from 21 ticks. One

amplicon, from a *Haemaphysalis punctata* tick, had 100% sequence identity with the *omp*A of *R. aeschlimannii* (GenBank accession no. U43800). The nucleotide sequences of the remaining 20 *omp*A amplicons shared >99% similarity with the *omp*A of *R. aeschlimannii*. These 20 amplicons were obtained from nine *Hyalomma marginatum*, five *Rhipicephalus bursa*, three *R. turanicus*, one *R. sanguineus*, and two *Ixodes ricinus* ticks.

In an additional 14 ticks (10 *H. marginatum*, 2 *R. bursa*, 1 *R. sanguineus,* and 1 *I. ricinus*) we sequenced 14 *glt*A amplicons (382 bp), which were 100% identical to the *glt*A of *R. aeschlimannii* (GenBank accession no. U59722). No other tick-borne pathogens were detected in the 35 *R. aeschlimannii*–containing ticks.

R. aeschlimannii has never been detected in Spain; therefore, our study constitutes its first citation in this country. Because *R. aeschlimannii* had been already detected in ticks in Portugal, we believe that its presence in Spain was expected and our finding is not surprising. However, the high number of tick species in which we found this rickettsia was unexpected: six species belonging to four genera. For these six species, the ratio between the specimens infected and the specimens analyzed (as well as the infection rates) were as follows: *I. ricinus* (3/1,320; 0.23%), *H. marginatum* (19/324; 5.86%), *H. punctata* (1/106; 0.94%), *R. bursa* (7/425, 1.64%), *R. sanguineus* (2/102; 1.96%), and *R. turanicus* (3/330; 0.91%). Although *H. marginatum* was the fourth most anthropophilic species in our study, this species simultaneously showed the highest number of infected specimens and the highest infection rate, making *H. marginatum* the main vector of *R. aeschlimannii* in our region. The next most important vectors are *Rhipicephalus* spp., and in particular *R. bursa*.

The 35 *R. aeschlimannii*–positive ticks were removed in the first 6 to 12 hours after attachment, before they could have ingested any blood, thus indicating that they were previously infected with the bacterium. Persons bitten by these specimens had never had symptoms of spotted fever, and they remained asymptomatic after the bite, suggesting that, as expected because of the rapid removal of the ticks, they did not acquire the infection.

Although MSF is endemic in Castilla y León (9), we only found one tick infected with *R. conorii* (0.03%) among the 3,059 analyzed (unpub. data), whereas *R. aeschlimannii* was much more prevalent in these same ticks (1.14%). Hence, in accordance with what was proposed by Raoult et al. (4) for MSF cases in Morocco, we suspect that many cases of MSF in Castilla y León may really have been due to *R. aeschlimannii*.

Our findings show that *R. aeschlimannii* is present in Castilla y León, the largest region in Spain, in six tick species that frequently feed on humans. Our observations expand the geographic distribution of this bacterium and the range of its potential tick vectors.

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Hantaviruses in São Paulo State, Brazil

To the Editor: Hantavirus pulmonary syndrome (HPS) is an emerging health problem in Brazil. This syndrome was first reported in 1993 in three persons living in a rural area of Juquitiba County; two of them died of acute respiratory failure (1). Although Juquitiba County is part of the metropolitan area of greater São Paulo City, patients lived in a recently deforested region. From 1993 through 2002, approximately 200 HPS cases were reported in Brazil, with a 40% case-fatality ratio (Ministry of Health of Brazil, Report on Hantavirus cases 1993–2002, unpub. data).

The wild rodent *Bolomys laziurus* is believed to be the most important hantavirus reservoir in the State of São Paulo, based on high levels of specific antibodies observed in serum from captured specimens (L.E. Pereira, Adolpho Lutz Institute, pers. comm., 2001). The economy of the inland region of Ribeirão Preto in the State of São Paulo, with its 3.5 million inhabitants, is based on the sugar cane agroindustry. The region has been almost completely deforested, with important consequences to the environment and wild rodent ecology. Twenty HPS cases were reported in Ribeirão Preto in the last 5 years, with a 60% case-fatality ratio. Review of medical records showed that a prodromic fever occurred in all 14 case-patients studied; dyspnea, cough, hypotension, and tachycardia occurred in about two thirds of patients; and hemorrhagic phenomena (hematuria, melena, and hypermenorrhea) in about one third. Thrombocytopenia was observed in all the patients, elevated hematocrit in about three fourths, and leukocytosis with neutrophilia and a left shift in the differential count in about two thirds. Serum creatinine levels were also increased (average level 2 mg/dL). Chest radiographs showed diffuse alveolar flocculant infiltrates in most cases (2,3). Laboratory diagnosis of HPS was made by serologic testing (enzyme-linked immunosorbent assay [ELISA]) in 18 cases and by reverse transcription–polymerase chain reaction (RT-PCR) in 11 cases; for 7 cases, both techniques were used. We performed a nucleotide sequence analysis of the N gene of hantavirus (residues 236–477) obtained from the blood of 11 of the 20 patients. This analysis showed that the infections were caused by Araraquara virus, a previously known hantavirus that had been detected by RT-PCR in the serum of an HPS patient living in a nearby county (4). Thus, Araraquara virus is the causative agent of a severe form of HPS, with a high death rate. This high death rate could also be related to the lack of adequate initial therapy provided by clinicians who probably did not immediately suspect HPS and may have not recommended hospitalization in intensive-care units. In addition, some hospitalized patients were in shock when first seen and were rehydrated with massive quantities of fluids, which may have aggravated pulmonary edema and contributed to death.

The occurrence of 10% of the Brazilian HPS reported cases in Ribeirão Preto indicates that this region is suitable for studying the epidemiology of hantavirus infections. A serologic survey conducted in the region in 1999, which included 567 primary-care patients from Ribeirão Preto, Guariba, and Jardinópolis Counties, found that 7 (1.23%) of them had immunoglobulin (Ig) G antibodies to Sin Nombre virus by ELISA and that 5 of those lived in Jardinópolis (population 30,000), a county where a fatal case of HPS occurred in 1999 (5). Thus, Jardinópolis County was chosen for a population-based survey. In May 2001, we obtained personal information and collected fingerprick blood samples from 818 participants, 15–70 years of age, living in urban and rural areas of the county. IgG antibodies to the N recombinant protein of Andes virus were detected by ELISA in the blood samples of 14.3% of the participants (5). Even though all HPS cases in Ribeirão Preto were associated with rural activity and rodent exposure, these serologic data suggest that hantavirus infections are common in Jardinópolis County, independent of sex, profession, or history of contact with rodents. None of the 14.3% participants with IgG antibodies to hantavirus had a history of HPS-like disease, and the ELISA test showed cross-reactions with most of the South American hantaviruses, including Araraquara. Persons living in the urban area had higher levels of antibodies to hantavirus than those from rural areas. In Ribeirão Preto, the physical boundaries of cities have expanded to incorporate other areas, encroaching upon rural areas with many popular subsidized housing complexes. Work-related and recreational rural activities in that region are also frequent, which makes it difficult to interpret these data. These results suggest that in this region of southeast Brazil, hantaviruses may be causing undiagnosed asymptomatic or clinically minor infections in addition to typical HPS. This finding envokes important questions. Is more than one hantavirus circulating in this region, causing mostly benign infections? Is Araraquara virus widespread, causing mostly inapparent infections and only rarely causing HPS? Would HPS be associated with some predisposing condition in the infected person? If more than one hantavirus is circulating in the region, could urban rodents be reservoirs?

Further studies are necessary to better understand the epidemiology and clinical signs and symptoms of hantavirus infection in the region of Ribeirão Preto. Such studies should

emphasize determining the reservoirs, the modes of virus transmission to people, and the possible distinct clinical forms of hantavirus infections.

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Israeli Spotted Fever *Rickettsia* in Sicilian *Rhipicephalus sanguineus* Ticks

To the Editor: Mediterranean spotted fever (MSF) is endemic in Italy, where it is a reportable disease. From 1992 to 1998, the Italian Ministry of Health was notified of approximately 8,500 cases of human rickettsioses presumed to be MSF. MSF occurs more commonly in some central (Lazio) and southern (Sardinia, Sicily, and Calabria) regions (1,2); in 1998, an average of 8.8 cases occurred for every 100,000 persons in Sicily, compared with the national average of 1.6 cases per 100,000 persons. *Rickettsia conorii* has been thought to be the only pathogenic *Rickettsia* of the spotted fever group in Sicily (3,4) or the western Mediterranean area. Recently, three different spotted fever group rickettsiae, including *R. helvetica*, were detected in *Ixodes ricinus* ticks from central and northern Italy. This finding suggests that bacteria other than *R. conorii* are involved in rickettsial diseases in Italy (5).

To investigate whether unusual tick-transmitted rickettsiae are also present in Sicily, we used molecular-sequence–based identification techniques to study two strains isolated from the hemolymph of *Rhipicephalus sanguineus* ticks collected in 1990 in western Sicily. These isolates had been previously identified by serologic tests as belonging to the spotted fever group rickettsiae. We obtained bacterial DNA and performed polymerase chain reaction (PCR) for *ompA* gene and restriction analysis under conditions previously described by Roux et al. (6). Our observation of a peculiar *Pst*I profile allowed a presumptive identification of one of the two tick isolates as belonging to the Israeli spotted fever rickettsiae, while the other showed a restriction profile corresponding to that of *R. conorii* strain Seven. To confirm the identification of the Israeli spotted fever *Rickettsia* isolate, we sequenced the PCR-amplified fragment of *omp*A gene (MWG-Biotech AG, Ebersberg, Germany) and aligned sequence data with homologous sequences of reference strains of the spotted fever group rickettsiae retrieved from the GenBank database. Sequence analysis showed 100% similarity with the homologous sequence of Israeli spotted fever *Rickettsia* reference strain ISTT CDC1 (GenBank accession no. U43797). The Israeli spotted fever *Rickettsia* belongs to the *R. conorii* complex (7,8) and was first isolated in 1974 from ticks and humans. Initially, Israeli spotted fever rickettsiae distribution appeared to be restricted to Israel (9), but more recently the organism has also been isolated from patients with MSF in Portugal (10). Our finding of Israeli spotted fever *Rickettsia* infection in a *R. sanguineus* tick, the main vector for MSF in Sicily, also suggests that the geographic distribution of Israeli spotted fever might be wider than previously thought, including not only Israel and the Iberian Peninsula but also Italy.

Molecular analysis of spotted fever group *Rickettsia* isolates from Sicilian MSF patients is under way to verify this hypothesis. Because initial signs and symptoms of Israeli spotted fever are particularly uncharacteristic, awareness of the presence of Israeli spotted fever *Rickettsia* in our geographic area may hasten provision of the appropriate treatment. The Sicilian *omp*A gene sequence described in this study has been

deposited in the GenBank database (accession no. AY197565).

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Co-feeding Transmission and Its Contribution to the Perpetuation of the Lyme Disease Spirochete *Borrelia afzelii*

In Reply: Richter et al. (1) have asked an important question: To what extent does the transmission of nonsystemic infections of the Lyme borreliosis spirochete (*Borrelia afzelii)* between co-feeding nymphal and larval *Ixodes ricinus* ticks apply to natural tick infestations on wild rodents? The authors conclude that the transmission of infections 3 days after inoculation by tick bite is >100 times less efficient than the transmission of infections that have lasted at least 14 days. That answer depends on a critical calculation based on experimental results combined with field observations. Unfortunately, this calculation is incorrect by a factor of approximately 20.

When hairless laboratory mice were restrained within wire mesh tubes and larvae were allowed to attach at random over their bodies, 13.6% of these larvae became infected with *B. afzelii* if they fed 3 days after the attachment of a single infected nymph (i.e., transmission probability of 0.136, as used below). By contrast, 85.4% of larvae that fed 14 days after the nymph became infected (1). At three sites in Germany and France, over the period April–October in each of the years from 1993 through 1995, 17.6% of mice (*Apodemus flavicollis* and *A. sylvaticus*) and voles (*Clethrionomys glareolus*) fed larval and nymphal ticks together, while 1.5% fed nymphs alone. Of these nymphs, 26.4% were infected with *B. burgdorferi* s.l. before attachment. The probability of a larva's acquiring an infection equals the product of 1) the probability of transmission from host to larva and 2) the probability of the host's being infected, while the larva feeds, via an infected nymphal tick bite. For a short-lived (3-day) infection, the probability is $0.136 \times 0.176 \times 0.264 = 0.0063$; for longer-lived (14-day) infections, the probability is $0.854 \times (0.176 + 0.015) \times 0.264 = 0.0431$. The ratio is therefore 1:6.8. Richter et al. erroneously concluded that the ratio was 1:116 because they did not take into account the probability of wild rodents' acquiring a long-lived, "systemic" infection; the authors assumed the probability was 1. A greater proportion of garden dormice (*Eliomys quercinus*) carried ticks and so would yield much higher transmission probabilities but in almost the same ratio, 1:6.4.

In fact, how much of the increase from 13.6% transmission at day 3 to 85.4% at day 14 was due to the development of systemic infections (i.e., disseminated to parts of the hosts' bodies >2 cm from the infected tick bite) is not clear because the feeding sites of the larvae attached ad libitum on the hairless mice were not reported. In the original discovery of co-feeding transmission of *B. burgdorferi* s.l. (2), the infection prevalence in larvae feeding close to infected nymphs increased from 33% on day 2 to 96% on day 11 and 100% on day 14 (3; see Figure 2 therein) in the demonstrated absence of a systemic infection. Mice skin and ticks feeding at distant sites remained uninfected. Only after day 14 had a systemic

infection developed (2). Because spirochetes are not transmitted to the host until at least 17.6 h after an infected nymph starts feeding (4–6) and then disseminate only slowly from the feeding site (7), co-feeding in space rather than in time is the crucial feature in Lyme borreliosis (2,8) (so-called "extended co-feeding"[3]). Larvae that attach to hosts simultaneously with infected nymphs rarely acquire spirochetes (1,9), wherever they attach. This pattern is distinct from the more immediate and short-lived co-feeding transmission of tick-borne viruses (10–12). In both cases, however, the key feature is a nonsystemic infection.

Despite the uncertainties in Richter et al.'s study, their corrected ratio is very similar to that (1:5.7) calculated (3) with a "synthetic model . . . based on major assumed parameters" (sic) (1). In that model we assumed that 50% of larvae were likely to be feeding within 1 to 2 cm of any infected nymph, the distance over which co-feeding ticks can pick up nonsystemic infections (1,2), because in the wild very few rodents carry nymphs in the absence of larvae (1,13), and >95% of all immature stage ticks feed in aggregations, mostly on the ears and also around the eyes or on the snouts of mice and voles. Considerable risks exist in using laboratory experimental results to quantify the epidemiologic importance of nonsystemic infections in the wild because of differences between host species, unnatural spatial distributions of introduced ticks on hosts, and the subtleties of natural tick-host relationships. Coincident aggregated distributions of larvae and nymphs among their rodent hosts, whereby the same individual hosts carry the largest numbers of both stages, increase the number of larvae co-feeding with any infected nymph, and so augment the potential amplification of infection prevalence in ticks (13). Nevertheless, in the case of rodents, nonsystemic infections are soon rendered redundant by the much longer lived systemic infections. In contrast, in the case of host species in which systemic infections do not develop, the transmission of nonsystemic infections between co-feeding ticks is the only way in which infection prevalence can be amplified in feeding ticks. Field data suggest that this route of transmission occurs on wild Sika deer (*Cervus nippon*) (14). Natural experimental systems have confirmed that on sheep this transmission pathway exists and is sufficient alone to maintain enzootic cycles of Lyme borreliosis (8).

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Co-feeding Transmission and Its Contribution to the Perpetuation of the Lyme Disease Spirochete *Borrelia afzelii*

In Reply to Randolph and Gern: Although transmission between co-feeding vector ticks may perpetuate particular tick-borne viruses, this mode of transmission plays no role in the epizootiology of Lyme disease spirochetes (1,2). In their letter, Randolph and Gern defend their suggestion that tick-borne pathogens perpetuate effectively by direct passage from one feeding tick to another by criticizing our analysis (3). These researchers mainly address our comparison of the transmission efficiency between simultaneously feeding ticks with that between ticks feeding sequentially on a persistently infected rodent. Our experiments demonstrate that approximately six times as many larvae (85.4%) acquire Borrelia afzelii spirochetes from a systemically infected mouse than from a mouse on which an infected nymph is feeding simultaneously (13.6%) (1). In nature, however, larval ticks rarely co-feed with nymphs on mice or voles; only approximately one fifth (18.8%) of these hosts harbor both subadult stages simultaneously. And of the nymphs, only approximately one quarter (26.4%) are infected by Lyme disease spirochetes. As a result, the natural transmission efficiency between simultaneously feeding ticks would be only one twentieth (5%) of that observed in the laboratory. Multiplying the experimentally observed efficiency of co-feeding transmission (13.6%) by the likelihood of larval and nymphal ticks co-infesting small rodents, as well as by the prevalence of infected nymphal ticks, reduces the efficiency of co-feeding transmission in nature to <1%. Although Randolph and Gern commit several minor mathematical errors, their calculations support our argument that few larval vector ticks would acquire spirochetal infection directly from an infected nymph (3).

Randolph and Gern err, however, by applying the same mathematical modifications to the transmission efficiency by which larvae acquire spirochetes from a persistently infected host (3). Whereas the efficiency of co-feeding transmission observed in the laboratory must be modified to pay tribute to the rare event of larvae co-feeding with an infected nymph in nature, the efficiency by which larvae acquire infection from a persistently infected host is independent of such limiting parameters. Because a competent rodent host remains infectious to larval ticks throughout its life, the proportion of hosts infested by particular subadult stages of the vector is irrelevant. Thus, the transmission efficiency on a persistently infected host is unchanged in the laboratory and the field. Almost 85.4% of larvae feeding on mice or voles in nature would, therefore, acquire spirochetal infection—far more than by co-feeding. We are correct in stating that natural

transmission by sequentially feeding ticks is more efficient than transmission between co-feeding ticks.

Randolph and Gern suggest that we could have recorded the distance between the feeding ticks to clarify whether the increase from a 13.6% transmission efficiency between co-feeding ticks to a transmission efficiency of 85.4% from a persistently infected host is associated with the development of a systemic infection. Our experimental observation (Table 1 in our article [1]), as well as a study on the movement of spirochetes through their host's skin (4), conclusively demonstrates that the increase in transmission efficiency is due to the progressive dissemination of spirochetes from the site of inoculation. The likelihood of a larva's acquiring spirochetes from any site of its host's skin increases with the passage of time since the infected nymph attached. To compare the two modes of transmission in terms of efficiency (Table 2 in our article [1]), we permitted the larvae to attach randomly to their rodent hosts, just as they would do in nature.

In the epizootiology of Lyme disease spirochetes, "simultaneous" transmission between co-feeding ticks (<1%) is some two orders of magnitude less efficient than sequential transmission between ticks feeding on persistently infected reservoir rodents (85.4%). The two studies that relied on natural infestation densities and refrained from using artificial feeding capsules conclusively demonstrated that transmission of Lyme disease spirochetes between ticks feeding simultaneously and in close proximity contributes little to the perpetuation of this pathogen, either in North America or in Europe (1,2). We are correct in concluding that Lyme disease spirochetes are maintained in nature mainly by sequential attachment of ticks to persistently infected reservoir hosts.

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Conference Summary

West Nile Virus Southeast Conference[1]

On January 14–15, 2003, more than 60 scientists, public health officials, and clinicians from throughout the southeastern United States gathered in Atlanta to present data from the 2001 and 2002 West Nile virus (WNV) epidemics. The aim of the daylong conference, hosted by the Southeastern Center for Emerging Biological Threats at Emory University, was to assemble a diverse perspective on WNV by sharing knowledge, identifying key questions for research about the disease and prevention, and fostering collaborations between epidemiologists, veterinarians, laboratorians, and clinicians.

In the overview presentation, the experiences of state health officials, clinical spectrum and pathogenesis, laboratory diagnostics, veterinary issues, surveillance, and vector control were discussed. The 2002 epidemic produced the largest annual number of cases to date, and four novel modes of transmission were discovered: 1) transplanted organs or tissue, including blood; 2) breast milk from an infected mother; 3) percutaneous exposure to infected tissue or serum among laboratory and hospital workers; and 4) transplacental exposure to fetuses in utero, resulting in birth defects. In addition, WNV-associated acute flaccid paralysis was discussed; the paralysis is caused by localized infection of the anterior horn cells of the spinal cord, resulting in signs and symptoms similar to poliomyelitis.

State epidemiologists or their representatives presented information about the state epidemics in the southeastern United States. The experiences in four states (Florida, Georgia, Louisiana, and Mississippi) were common in their themes of expansion of WNV epidemics, concentrated nature of outbreaks, importance of protection from mosquito bites, limitations of diagnostic methods, and dynamics of WNV spread in the United States from 2001 to 2002. Significant differences also emerged regarding the observed benefits of mosquito control activities and the value of animal surveillance as an early detection system. Also, while the states unanimously agreed that collaboration among local, state, and federal public health agencies, academic research institutions, and other nongovernmental organizations is critical to responding effectively to WNV, the degree to which such collaborations actually occurred and the existence of previously established relationships varied.

Several clinicians discussed the pathogenesis and clinical aspects of the disease. The pathogenesis of WNV was reviewed; clinicians suggested that infections of the central nervous system can demonstrate any or all of three distinct characteristics: neuroinvasiveness (the ability to enter the nervous system), neurotropism (the ability to infect neural cells), and neurovirulence (the ability to cause neurologic disease). WNV possesses all three. The virus can enter the nervous system, as shown by the encephalitis that occurs in approximately 1 in 150 infected persons; it has been shown to infect neurons; approximately 10% of patients in which the virus has invaded the nervous system eventually die. One presenter suggested that the virus had changed in the last 60 years, evolving into a more virulent strain. The neurologic manifestations of the disease were described, including weakness and flaccid paralysis (which can occur even without fever or meningoencephalitis).

WNV infection in patients with HIV was also described. Two HIV-infected patients, with CD4 cell counts below 200 cells/μL were identified with WNV by the presence of immunoglobulin M antibodies to WNV. The first was a 50-year-old homeless man co-infected with tuberculosis. Despite treatment including intravenous acyclovir, the patient continued to deteriorate, and died 18 days after admission. An autopsy revealed meningitis. The second HIV-positive patient diagnosed with WNV was a 48-year-old man who arrived at the emergency room with fever, headache, and confusion; he reported feeling "slow," and indeed was slow to respond to questions. This patient improved rapidly and was discharged after 3 days.

Information about intracellular host-virus interactions was summarized. Studies in mice have identified a genetic allele that apparently confers resistance to flaviviruses. Although humans do not have a direct genetic homologue, studies to identify genetic differences to explain different clinical outcomes should be pursued. Results of a case-control study suggested that the greatest increases in risk were related to environmental factors favoring mosquito popula-

[1]Presentations: James J. Sejvar, Centers for Disease Control and Prevention (CDC); Mary Currier, Mississippi Department of Health; Raoult Ratard, Louisiana Department of Health & Human Services; Lisa Conti, Florida Department of Health; Susan Lance-Parker, Georgia Department of Human Resources; Richard T. Johnson, Johns Hopkins University School of Medicine; A. Arturo Leis, University of Mississippi Medical Center; Margo Brinton, Georgia State University; Sally Slavinski, Mississippi State Health Department; Carlos del Rio, Emory University; Sharif R. Zaki, CDC; Lillian Stark, Florida Department of Health; Elizabeth Howerth, University of Georgia; Brigid Elchos, Mississippi Department of Health; Jonathan Day, University of Florida; Tom Bevan, Georgia Institute of Technology; Mike Bunning, CDC; and David Stallknecht, University of Georgia.

tions. These findings in turn raised questions about factors involved in human susceptibility, risks of pesticide exposure, efficacy of mosquito control, the value of sentinel animals in surveillance, and the roles played by various species in virus transmission and amplification.

The ability to diagnose WNV in the laboratory emphasized the role of pathology, including histopathology, electron microscopy, immunohistochemistry, polymerase chain reaction, and virus isolation. Few of these tests are entirely sensitive or specific. The challenges involved in the pathologic diagnosis in animals because of the large diversity of infected species were discussed. The single most urgent concern, repeatedly emphasized by state public health officials and clinicians, is the need for a faster, simpler diagnostic test for WNV that would ease the amount of work by public health laboratories, assist physicians in correctly diagnosing infected patients, and improve surveillance by identifying subclinical cases.

The problems of vector control and the best application of these methods were also emphasized. The cost of these programs and proof of effectiveness will require careful research, including the identification of specific mosquito vectors and assessment of the long-term safety of pesticides and personal repellant applications. The most important principle in attempting emergency vector control is to consider it early, before the epidemic has evolved.

Data from in vitro studies evaluated the interaction of viral vectors and amplification hosts. These studies might elucidate the importance of birds, horses, and household pets in maintenance of epidemics. Because wild birds play a key role in the spread of WNV, the exact nature of that role must be clarified to predict the development and expansion of future epidemics.

The variation in changing epidemiology of the states' experiences to date with WNV, even within the southeastern United States, clearly demonstrates that research needs to be replicated in numerous localities; what succeeds in one state may not prove successful in another. Whether epidemics will continue to expand in size and geographic distribution or whether a more sporadic pattern of occurrence will emerge is still unclear. Controlling WNV in the southeastern United States will take a concerted, cohesive effort. The continued collaboration of the diverse scientists in this meeting will aid in this effort. Presentations from this conference are available on the Web site for Southeastern Center for Emerging Biological Threats (available from: URL: www.secenterbiothreats.org).

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Conference Summary

West Nile Virus and Wildlife Health

The West Nile Virus and Wildlife Health Workshop, hosted by the Smithsonian Institution, National Audubon Society, U.S. Geological Survey, and U.S. Department of Agriculture, was held February 5–7, 2003, at the Smithsonian Environmental Research Center in Edgewater, Maryland. The event was attended by more than 100 scientists, who heard 29 speakers and participated in strategy discussions during the 2-day meeting. The main focus of the conference was the present and future impact of West Nile virus on wildlife populations. Talks and discussions emphasized how basic research, public health, and land management can contribute to our understanding of the disease's impact and spread. A primary objective of this meeting was to develop future research priorities from both basic and applied perspectives.

The conference centered around four main themes: 1) host, vector, and pathogen interactions (disease ecology); 2) vertebrate behavior and ecology; 3) vector behavior and ecology; and 4) modeling and spatial statistics. We describe some of the findings from the meeting. For an in-depth summary of this meeting, please visit the conference website for meeting abstracts and a downloadable conference white paper (available from: URL: www.serc.si.edu/migratorybirds/migratorybirds_index.htm).

West Nile virus (WNV) has spread rapidly across North America since its probable introduction to the New York City area in 1999 (D.J. Gubler, Centers for Disease Control and Prevention, Fort Collins, CO). By December 2002, the Canadian provinces of Saskatchewan, Quebec, Ontario, Nova Scotia, and Manitoba reported dead birds that tested positive for WNV. By winter 2002, only four states in the continental United States remained free of confirmed WNV infection; the virus was expected to reach the West Coast later in the year. WNV has also found its way into tropical regions. One case in a person was reported in 2001 from the Cayman Islands. Additionally, resident birds from Jamaica (January 2002) and the Dominican Republic

(November 2002) have shown WNV antibodies. Recent reports note that the virus has also reached Mexico's Yucatan peninsula. Since 1999, WNV has killed thousands of birds and other wildlife, and the impact on regional wildlife populations is unclear.

A primary theme of the meeting was that we still have much to learn about how WNV is dispersed, transmitted, and amplified by competent vectors and still relatively unknown reservoir hosts. At the time of the conference, WNV had been detected in 37 mosquito species, 157 bird species, horses, 16 other mammals, and alligators (D.J. Gubler, Centers for Disease Control and Prevention, Fort Collins, CO). The *Culex* genus, particularly *Culex pipiens* in the northern United States and *C. quinquefasciatus* in the southern United States, appears to be the most important mosquito group for the avian vector amplification cycle. However, opportunistic mosquito species are probably important bridge vectors to humans, horses, and other deadend hosts. While the avian amplification cycle appears to the most dominant, other cycles may also be occurring at the same time (i.e., in mammal and ticks). Reptiles, amphibians, and associated mosquito vectors may also play important roles (M.J. Turell, The United States Army Medical Research Institute for Infectious Diseases, Fort Detrick, MD, and E. Jacobson, University of Florida, Gainesville, FL). Although mosquitoes appear to be the main vector, other ectoparasites such as ticks, louse flies, and fleas should also be examined as potential vectors.

Several scientists reported that transmission of WNV is more complicated than previously thought. The presence of WNV in avian reproductive organs suggests that vertical transmission may be a possibility (T.S. McNamara, Wildlife Conservation Society, Bronx, NY). WNV in the kidneys leads to cloacal excretion, which may lead to cloacal-oral mouth infection. Bird-to-bird transmission has been demonstrated in the laboratory and may be an important infection route among social birds like the American crow (R.G. McLean, U.S. Department of Agriculture, Fort Collins, CO). Evidence suggests that ingesting infected vertebrates and mosquitoes can infect birds.

The impact of WNV on animal populations is another unknown area. Data from individually marked populations of crows in New York State and Oklahoma (K.J. McGowan, Cornell University, Ithaca, NY; A. Clark, State University of New York-Binghamton, Binghamton, NY; and C.L. Caffrey, National Audubon Society, Ivyland, PA) show that these populations are experiencing important declines after the initial WNV outbreak. Analysis of breeding bird surveys and annual winter bird censuses (Christmas bird count) from a wide array of passerine bird species showed local declines in WNV "hotspots" but no declines at the range-wide scale that can be attributed to WNV (J. Sauer, United States Geological Survey, Laurel, MD; P.P. Marra, Smithsonian Environmental Research Center, Edgewater, MD; and W. Hochachka, Cornell University, Ithaca, NY).

Another important issue discussed at this conference was the secondary impact that pest management might have on organisms not pinpointed for WNV, especially in aquatic environments. This issue is especially important in nature reserves (W.K. Reisen, University of California, Davis, CA).

Modelers attending the meeting stressed the importance of standardizing sampling methods, such as the dead bird surveillance programs operated across the nation by many state health departments. These programs must consistently and conscientiously monitor sampling efforts and report the total sample sizes of dead birds collected, including the number of birds that test negative (D.J. Rogers, Oxford, UK). In addition, a better understanding of the real-world persistence of WNV antibodies in live bird surveillance programs would be useful for virus dispersal models.

Scientists at the meeting felt strongly that we need to closely monitor how WNV impacts organisms in tropical regions, including humans and the many endemic avian species already threatened or endangered. Species in Hawaii, many of which are still endangered after malaria's century-old invasion, should be of special concern. WNV is not the first and will not be the last virus to enter our borders. By developing techniques to survey, monitor, and control WNV in wildlife, we prepare ourselves for the next pathogen species. Our experiences with WNV emphasize the need to strengthen and integrate animal monitoring programs with basic research on population and disease ecology. A conference white paper, several review articles, and a list of research priorities are planned as products of this meeting.

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Upcoming Infectious Disease Conferences

July 7–10, 2003
ASM Conference on Bio-, Micro- and Nanosystems
American Society for Microbiology
New York, New York
Contact: ASM Conferences
Phone: 202-942-9261
Fax: 202-942-9340
Email: conferences@asmusa.org

July 13–16, 2003
The 2nd IAS Conference on HIV Pathogenesis and Treatment
Paris, France
Contact: JCD Conseil/IAS 2003
Phone: 33 1 40 64 2000
Fax: 33 1 40 64 2050
Website: http://www.ias2003.org

July 16–18, 2003
ISAAR 2003—4th International Symposium on Antimicrobial Agents and Resistance
Antimicrobial Treatment in the 21st Century: Current Challenges and Future Strategies
Seoul, Korea
Contact: Ms. Susan Chung
Phone: 82-2-3410-0327
Fax: 82-2-3410-0023
Email: susan@ansorp.org
Website: http://www.ansorp.org/isaar2003/intro.htm

July 16–19, 2003
11th Annual Conference: Boards of Health: Stewards of Public Health
National Association of Local Boards of Health
Baltimore, MD
Website: http://www.nalboh.org/confer/confmain.htm

Henri Rousseau—known as Le Douanier Rousseau (1844–1910) The Snake Charmer (1907). Oil on canvas, 169 cm x 189.5 cm
Musée d'Orsay, Paris, France. Credit: Réunion des Musées Nationaux/Art Resource, NY

As the Gods began one world, and man another,
So the snakecharmer begins a snaky sphere
With moon-eye, mouth pipe.
He pipes. Pipes green. Pipes water

Sylvia Plath, "Snakecharmer"

Critics called him "naïve," the term for painters with no formal training in art. Henri Rousseau, self-made late-bloomer from Laval, France, fit the definition. But his work proved that in art as in all ventures, training, though valuable, is not the key ingredient—not as key perhaps as talent, inspiration, or originality. Untrained in art but not uneducated by the standards of his day, Rousseau was a teacher and a military man. He was interested in politics and the realm of ideas. He knew music and poetry and even tried his hand as playwright. Dubbed "Le Douanier" (customs officer) after his main occupation outside art, he struggled in anonymity until near the end of his life, when he was discovered by Pablo Picasso and others and was recognized for his powerful individual style (1).

Like other naïve or primitive artists, Rousseau found art late in life. He took up painting as a hobby and soon retired from his job in the customs office to devote time to this new vocation. He copied the masters, struggled to learn their craft (particularly the academic style of Ingres), and aspired to paint like them. He exhibited often at the Salon des Indépendents in Paris, where artists could show their paintings without selection restrictions, and what he might have lost to technical clumsiness he seemed to make up in ingenuous charm. Although his work is difficult to categorize, he seems to have been influenced by his contemporary Paul Gauguin and his followers, the Nabis, who promoted directness of feeling and color harmony (2). Rousseau eventually found his own formal language and style, but what elevated his mature work to greatness were perhaps the very oblivion of convention, the freshness of approach, and the depth of discovery that comes from a truly unique perspective.

Rousseau's exotic compositions owe nothing to traditional art methods yet defy modern labels. The fantastic vegetation in his jungle paintings (for which he is best known) has no equivalent in nature. These exotic landscapes, oversized and filled with exuberant color, were entirely imaginary. Although often inhabited by half-concealed wild beasts and laced with conflict, they exuded an eerie stillness. The images, smooth, vivid, and clearly defined, were flat and fluid against dense but dimensionless greenery, and although unreal and extraordinary, were rendered in meticulous botanical detail.

The Snake Charmer, on this month's cover of Emerging Infectious Diseases, is one of Rousseau's finest and most celebrated works. Like his other jungle paintings, it is filled with lush greenery. Punctuated by an uncoiling reptile at arm's length, the thick vegetal screen that makes up most of the landscape is live with tension. The dark, undulating figure of the snake charmer dances ambiguously amidst a tangle of wildlife. Nature, framed by "a wave of flickering-grass tongues," (3) looms in the foreground immediate and tangible, yet dreamlike and distant as the moon. In a trance, the animals are guided (it seems as much by the glossy stream as by the snake charmer's reed) into a tight ecological web, where unbeknownst to them, they share more than the music.

Rousseau's imagination, like that of many of his contemporaries in Paris, succumbed to the allure of exotic lands, where plants grew larger than life, wild animals held unknown powers and magnetism, and humans lounged in "Eden's navel" (3) amidst all that was lost in the fall from grace. To explain the products of his inflamed imagination, Rousseau falsely claimed that he had visited Mexico. But unlike Gauguin, who went to Tahiti in search of inspiration, Rousseau traveled only vicariously and found his models in local gardens and the Paris zoo.

Exotic lands have become prosaic to us. What remains naïve and primitive is our knowledge of the forest's architecture and the perils of its convergence with human habitat. But like the uncoiling snake in Rousseau's painting, out of the impenetrable jungle comes knowledge about pestilences, piece by piece: a favorable environment, a stable population, a reservoir host, the agent. The emergence of West Nile virus in North America is a case in point. We, modern snake charmers, must pipe the pieces (bird, horse, reptile) into a knowable, harmonious fabric.

Polyxeni Potter

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EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.9, No.8, August, 2003

Upcoming Issue

For a complete list of articles included in the August issue, and for articles published online ahead of print publication, see http://www.cdc.gov/ncidod/eid/upcoming.htm

Look in the August issue for the following topics:

Porcine Reproductive and Respiratory Syndrome Virus: Origin Hypothesis

Detecting Bioterror Attack by Screening Blood Donors: Best-Case Analysis

Survival of *Batrachochytrium dendrobatidis* in Water: Quarantine and Disease Control Implications

Legionnaires' Disease Outbreak in Murcia, Spain

Enzootic Transmission of Yellow Fever Virus in Peru

NmcA Carbapenem-hydrolyzing Enzyme in *Enterobacter cloacae* in North America

Echovirus 13 Associated with Aseptic Meningitis, Spain

Molecular Characteristics of a Non–*Babesia divergens* Organism Causing Zoonotic Babesiosis in Europe

Multidrug-Resistant Tuberculosis in HIV-Negative Patients, Buenos Aires, Argentina Invasive Group A Streptococcal Disease: Risk Factors for Adults

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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.
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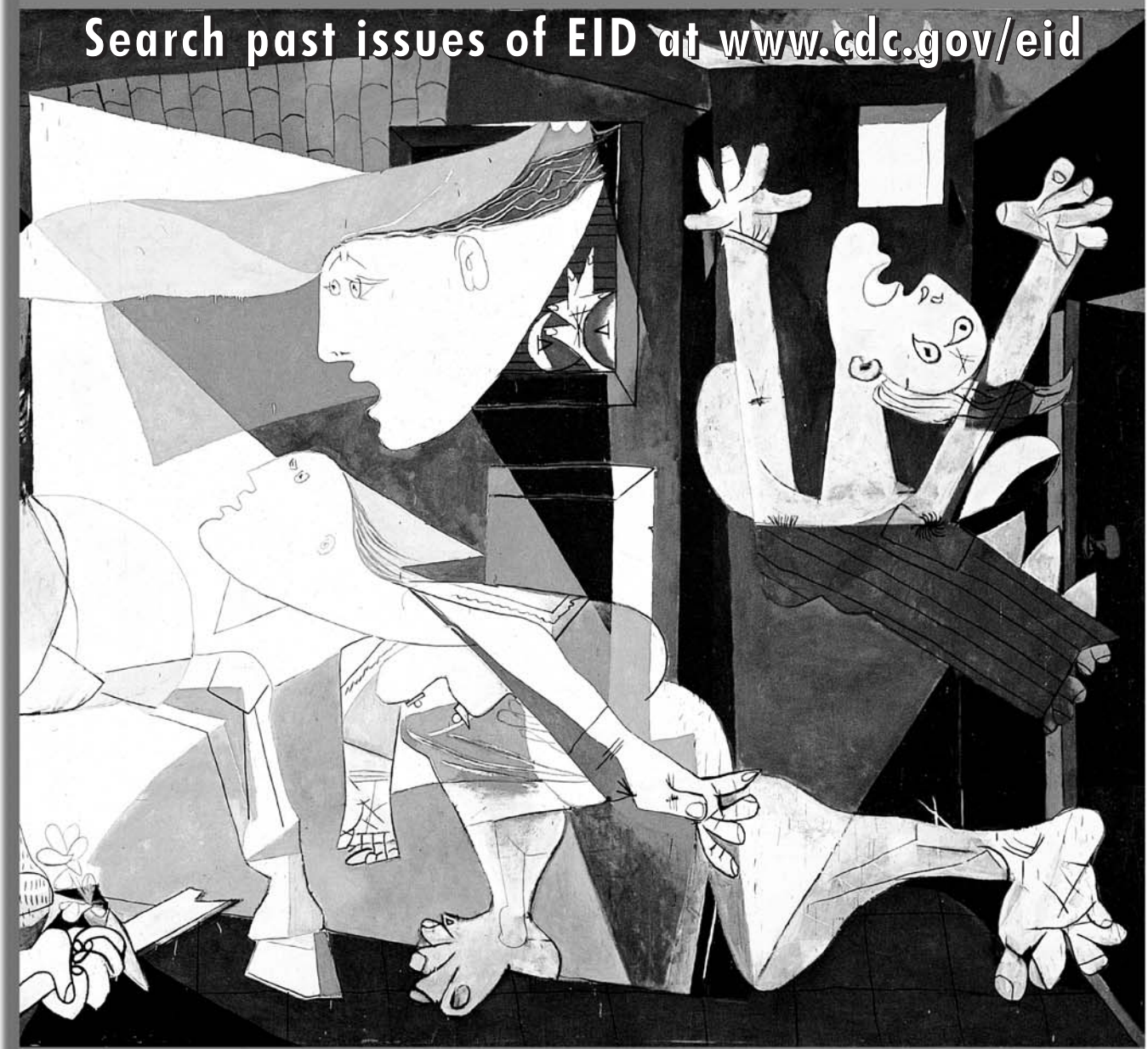

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Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy and Historical Reviews, Dispatches, Commentaries, Another Dimension, Letters, Book Reviews, and News and Notes. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

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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).

Manuscript Types

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author. 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) and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch. 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, nor should they contain figures or tables. References (not more than 10) may be included.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. (500–1,000 words) of emerging infectious disease conferences may provide references to a full report of conference activities and should focus on the meeting's content rather than on individual conference participants.