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EMERGING INFECTIOUS DISEASES

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Emerging Viral Diseases: An Australian Perspective

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With a few exceptions, emerging diseases in Australia are similar to those in other industrialized countries (1-8). Most exceptions are either vector-borne or zoonotic viral diseases, the major focus of this update. The continuing emergence of antibiotic resistance is a worldwide problem. In Australia, antibiotic resistance is being reported from a growing number of organisms (9-15), often necessitating new case management practices and guidelines (16,17). Also, like other countries, Australia has had a number of foodborne (18-22) and waterborne (23-25) epidemics in the past few years; the major difference is that the higher incidence of enterohemorrhagic *Escherichia coli* linked to outbreaks of hemolytic uremic syndrome is associated with serotype O111:H- rather than serotype O157:H-, which is more common in other countries. Major waterborne epidemics or contamination of reservoirs due to *Cryptosporidium parvum* have occurred over the past 3 years in the Eastern States of Australia (23-25), with the largest and most recent being a problem of contamination (in association with *Giardia lamblia*) in the Sydney water supply between July and September, 1998. However, despite this contamination, no increases in the number of cases of diarrheal disease were reported, perhaps because the inhabitants of Sydney were advised to boil the water before drinking it (25).

The distribution and incidence of most of the recently described viral diseases (e.g., human herpesviruses 6–8 and hepatitis C and E viruses) in Australia are similar to those reported in other industrialized nations (3). Recent data for hepatitis G in selected Australian populations also support this contention (26).

Vector-Borne Viral Diseases

Australia has more than 70 arboviruses, but relatively few cause human disease (27,28). The most common arbovirus causing human disease is Ross River virus, an alphavirus, which causes an epidemic polyarthritis. Although Ross River virus incidence has increased over the past decade, the virus is not emerging; its increased incidence is probably due to increased awareness and recognition by general practitioners, improved diagnostic reagents, and increasing encroachment of human habitation into or near wetlands and other areas conducive to mosquito breeding. The only indigenous virus that can be called "emerging" is Barmah Forest virus, also an alphavirus and also the cause of an epidemic polyarthritis-like disease. Associated with human disease only since 1988 and increasing in incidence as diagnostic reagents have become available and clinicians have become aware of it, the virus has spread into new geographic areas, such as Western Australia (29,30). The two mosquito-borne diseases of particular concern, however, are "imports"—Japanese encephalitis (JE) and dengue viruses.

Japanese Encephalitis Virus

The first outbreak of JE in the Australian region occurred in Torres Strait, northern Australia, in 1995 (31). Three cases (two of which were fatal) were reported from Badu Island in central Torres Strait, 2,000 km from the nearest

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focus of JE virus activity in Bali. Seroepidemiologic studies showed that the virus was relatively widespread in the central and northern islands with subclinical human cases on four islands and seropositive pigs on nine islands. Ten virus isolates were obtained during the outbreak: two from subclinical human infections (31) and eight from *Culex annulirostris* mosquitoes collected on Badu Island (32). Sequencing studies showed that these isolates (most closely related to a 1970 isolate from Kuala Lumpur and a 1981 isolate from Bali) (33) were almost identical, suggesting that the outbreak originated from a single source. These studies also showed that all isolates had the same 11 nucleotide deletion in the 3' untranslated region immediately downstream from the stop codon of the open reading frame (34), which provided a signature for comparing any future isolates. After the Badu Island outbreak, inactivated vaccine was offered to all the inhabitants of the northern and central Torres Strait islands (35). During 1996 and 1997, JE virus activity was detected through seroconversions in sentinel pigs on Saibai Island, which is in the north and only about 4 km from the Papua New Guinea coast (36) (J. Lee, D. Phillips, J. Hanna, unpub. results).

Seroepidemiologic studies found that virus activity has been widespread in Western Province, Papua New Guinea, since at least 1989, with seropositivity rates of 21% at that time among the Daru-speaking people. Results also showed that seropositivity rates were increasing in the Upper Fly River area and that the virus was spreading geographically (37). Indeed, recent results indicate that JE may have spread to Vanimo on the northern coast by April 1998 and to parts of Milne Bay Province in eastern Papua New Guinea (J. Lee, J. Wangi, P. Siba, G. Tau, unpub. results). The first four clinical cases of JE in Papua New Guinea were observed in 1997 and 1998, with two deaths. All cases were from the Kiunga area of Western Province (J. Oakley, S. Flew, C.A. Johansen, D. Phillips, R.A. Hall, J.S. Mackenzie, unpub. results). Anecdotal evidence suggests that the cases may have resulted in part from the large mosquito numbers associated with the severe drought in 1997. The first JE virus strain isolated in Papua New Guinea was obtained from *Cx. annulirostris* mosquitoes collected at Lake Murray in Western Province in 1997. Sequence studies have shown that this isolate was almost identical to the 1995 Torres Strait isolates, including the acquisition of the 11 nucleotide deletion (C.A. Johansen, R. Paru, S.A. Ritchie, A. Van den Hurk, M. Bockarie, J.S. Mackenzie, unpub. results).

A second outbreak of JE occurred in Torres Strait in March 1998, with one human case from Badu Island and sentinel pigs seroconverting on a number of islands. Shortly afterwards, the first human case on mainland Australia was reported in a fisherman who acquired the infection near the mouth of the Mitchell River in southwest Cape York (38). Extensive seroepidemiologic investigations found no further human infections in communities on Cape York, but domestic pigs had seroconverted both near Mitchell River and near Bamega at the northerly tip of Cape York. Two virus isolations were obtained from pig sera collected near Bamega, and one isolate was obtained from a sentinel pig on Mabuiag Island in Torres Strait (J. Hanna, S. Hills, D. Phillips, J. Lee, unpub. results). Mosquitoes were collected at a number of sites on Cape York as well as on Badu Island. No viruses were isolated from the Cape York mosquitoes, but approximately 44 isolates were obtained from Badu Island—43 from *Cx. annulirostris* mosquitoes and one from *Aedes vigilax* mosquitoes (S.A. Ritchie, A. Van Den Hurk, C.A. Johansen, D. Phillips, A. Pyke, J.S. Mackenzie, unpub. results). Nucleotide sequencing studies have shown that the mosquito and pig isolates from Mabuiag and Cape York were closely related to each other, as well as to the 1997 Papua New Guinea Lake Murray and the 1995 Badu Island isolates, including all isolates sharing the 11 base "signature" deletion, which indicated a single virus source for the virus activity in Northern Australia and Papua New Guinea. The focus of activity is probably in Papua New Guinea (C.A. Johansen, A. Drew, D.A. Phillips, A. Pyke, J.S. Mackenzie, unpub. results).

JE virus activity in northern Australia began in 1998. Sentinel animal sites are being established to investigate whether the virus has become enzootic in the wildlife. Australia has both the mosquito vectors (*Cx. annulirostris*) and vertebrate hosts (ardeid birds and pigs) for the virus to become established. In addition, large areas of wetland habitats in Cape York would be conducive to virus enzootic cycles and would increase the potential for the virus to move south to more populous areas of Australia (39).

Dengue Viruses

Despite a 120-year history, dengue does not appear to be endemic in Australia. Several epidemics over the past decade have been initiated from virus introduced by viremic travelers (27,28,40). Imported cases of dengue in travelers are regularly diagnosed throughout Australia, with 30 to 60 cases reported annually, and growing in number. In most parts of Australia where the local mosquitoes are unable to transmit dengue viruses, these cases pose no risk, but in areas of north Queensland where *Ae. aegypti* is common and travel between Australia and countries in the Asian-Pacific area is frequent, local transmission and epidemic activity are major risks. The potential for local transmission of dengue viruses is confined to an area of Queensland corresponding to the geographic range of *Ae. aegypti*, extending from the islands of Torres Strait in the north, to Mount Isa and Boulia in the west, possibly to Roma in the south, and to Gladstone on the east coast (41). Despite this relatively broad geographic range, epidemic activity over the past 2 decades has been restricted from Torres Strait south to Cairns, Townsville, and Charters Towers. The major epidemics over the past 5 years have included a large outbreak of dengue type 2 in 1992 to 1993, principally in Townsville and Charters Towers with more than 2,000 cases, and with the first case of dengue hemorrhagic fever this century (42,43); an outbreak of dengue type 2 in 1996 to 1997 on a number of Torres Strait islands and Cairns, with more than 200 serologically confirmed cases (44,45); and an outbreak of dengue type 3 in 1997 to 1998 largely restricted to Cairns with 239 confirmed cases (46; S. Ritchie, S. Hills, pers. comm.) and also a few cases of dengue type 2. This latter outbreak also included a case of dengue hemorrhagic fever and the first case of dengue encephalopathy in Australia (J. Hanna, unpub. obs.). Nucleotide sequencing of dengue 2 isolates from Australia and a comparison with isolates from elsewhere in the Asian-Pacific region indicated that the 1992-93 isolates were most closely related to an Indonesian virus, whereas the 1996-97 isolates were most closely related to viruses originally isolated in Burkina Faso. This latter finding is of interest because a large outbreak of dengue type 2 occurred on a number of Pacific Islands before and during the Australian outbreak, but the South Pacific viruses were quite distinct from the Australian viruses (45).

After the 1992-93 outbreak, a Dengue Fever Management Plan was developed to reduce the potential for epidemic activity from imported cases. The plan has been extremely successful, and a number of imported cases have been recognized early and were contained before they could cause an epidemic (47,48). However, importation, either by continual movement of people between Papua New Guinea and Torres Strait or by movement of people for work, education, or recreation between Papua New Guinea and north Queensland, will always be a major route of entry of the virus.

Vector importations occur frequently, with a number of reports for both *Ae. aegypti* and *Ae. albopictus* (27), including two recent importations of *Ae. albopictus* into Townsville in 1997 (49) and Cairns in 1998 (S. Ritchie, pers. comm.).

Novel Zoonotic Viral Diseases

In the past 4 years, three newly described zoonotic viral diseases have been reported from Australia; two of these diseases are caused by the paramyxoviruses Menangle and Hendra (formerly equine morbillivirus), and the third is caused by Australian bat lyssavirus.

Menangle Virus

An apparently new virus in the family *Paramyxoviridae* was isolated from stillborn piglets with deformities at a large commercial piggery in New South Wales (51). The farrowing rate in the piggery decreased from an expected 82% to 60%; the number of live piglets declined in 27% of the litters born; the proportion of mummified and stillborn piglets, some with deformities, increased; and occasional abortions occurred. Virus was isolated from lung, brain, and heart tissues of infected piglets, and shown to be morphologically similar to viruses in the family *Paramyxoviridae*. No disease was seen in postnatal animals of any age, but a high proportion of sera (>90%) from animals of all ages contained high titers of neutralizing antibodies against the virus. Tests performed at the Australian Animal Health Laboratory confirmed that the virus, named Menangle virus, was unrelated to other known paramyxoviruses, including viruses known to infect pigs (51).

Serum from two workers—one at the affected piggery and one at an associated piggery that had received weaned pigs from the original piggery—had high titer, convalescent-phase

neutralizing antibodies to the new virus. Both workers had an influenzalike illness with rash during the pig outbreak, but extensive serologic testing showed no evidence of any alternative cause; therefore, the illness is believed to have been caused by the new virus (52).

A large breeding colony of gray-headed and little red fruit bats roosted within 200 m of the affected piggery. In a preliminary study, 42 of 125 serum samples collected from fruit bats in New South Wales and Queensland had neutralizing antibodies to the new virus. In addition, antibodies were found in sera collected in 1996, before the outbreak, and from a colony of fruit bats 33 km from the piggery (51). Therefore, the fruit bats are believed to be the primary source of virus causing the outbreak. Sera collected from wild and domestic animals near the affected piggery were seronegative.

The geographic range, normal host species, and genetic relationship of this new virus to other paramyxoviruses remain unknown. Nevertheless, Menangle appears to cause fatal disease and malformations in prenatal pigs and may be associated with influenzalike illness in humans.

Hendra Virus

Hendra virus was first recognized in 1994 after an explosive outbreak of severe, fatal respiratory disease affecting race horses and humans. Twenty race horses in the Brisbane suburb of Hendra were infected; 13 died. The trainer and stable hand were also infected, and the trainer died (53-55). A second incident occurred in Mackay, a coastal town approximately 1,000 km north of Brisbane. Two horses and a farmer died, the latter from severe meningoencephalitis (56-58). The death of the horses and the initial infection of the farmer occurred in 1994 and preceded the Brisbane outbreak; the virus is believed to have then entered a latent phase for 1 year before reactivating to cause fatal encephalitis. No connection was found between the Brisbane and Mackay incidents (56). Experimental studies have shown that in horses and cats, after subcutaneous, intranasal, and oral administration, the virus causes fatal pneumonia (59). In guinea pigs, subcutaneous administration is also fatal, but the infection is more generalized. Black fruit bats (*Pteropus alecto*) infected by subcutaneous, intranasal, or oral routes contract a subclinical infection and generate an antibody response (M. Williamson, unpub. results). Endothelial cell tropism and formation of syncytia in blood vessels are common pathologic findings in both overt and subclinical infections (B.T. Eaton, M. Williamson, unpub. obs.).

Extensive seroepidemiologic studies found no evidence of Hendra virus among horses, other farm animals, or more than 40 species of wildlife in Queensland (56,60; P.L. Young, K. Halpin, H. Field, unpub. results). However, working on the hypothesis that if outbreaks at two distant sites were connected, the most likely wildlife source would be either birds or fruit bats, P.L. Young and colleagues subsequently showed that fruit bats (flying foxes), members of *Megachiroptera*, were the natural hosts on serologic grounds and by virus isolation, with widespread evidence of infection in four species of fruit bat: the black (*Pteropus alecto*), grey-headed (*P. poliocephalus*), little red (*P. scapulatus*), and spectacled (*P. conspicillatus*) fruit bats (61,62; P.L. Young et al., unpub. results). Indeed the virus was antigenically and genetically indistinguishable from the earlier horse and human isolates. Thus, it is now clearly established that Hendra virus is a fruit bat virus and is widely distributed throughout the range of pteropid bats in Australia, with serologic evidence of infection in an average of 42% of wild-caught bats, the number of seropositive animals varying with species (53% of 229 *P. alecto,* 47% of 195 *P. poliocephalus*, 12% of 115 *P. scapulatus*, and 41% of 99 *P. conspicillatus*) and age, but not with geographic distribution (H. Field, unpub. results). Serologic evidence of infection of fruit bats has also been reported from Papua New Guinea. Two species of antibody-positive bats (*Dobsonia moluccense*, *P. neohibernicus)* were identified from Madang on the north coast of Papua New Guinea (K. Halpin, H. Field, J.S. Mackenzie, M. Bockarie, P.L. Young, P.W. Selleck, unpub. results), and bats of four more species (*D. andersoni, P. capistratus, P. hypomelanus*, and *P. admiralitatum*) were identified in Port Moresby and New Britain (H. Field, S. Hamilton, L. Hall, F. Bornacosso, K. Halpin, P.L. Young, unpub. results).

Morphologic features (63) and preliminary sequencing data of the M and F genes (64) suggested that Hendra virus was a member of the *Paramyxoviridae*, although it had unusual surface projections of two distinct lengths, 15 nm and 18 nm (63). The entire genome of the virus has now been sequenced (65;66; L.F. Wang, B.T.

Eaton and colleagues, unpub. results) and has revealed a gene order and P gene organization characteristic of members of the *Paramyxovirus* and *Morbillivirus* genera (65). Comparison of its deduced amino acid sequences with those of other family members confirm that Hendra virus is a member of the subfamily *Paramyxovirinae,* more closely related to members of the *Paramyxovirus* and *Morbillivirus* genera than the *Rubulavirus* genus. Overall, homology with other members of the subfamily is lower than that observed within an individual genus (L.F. Wang, B.T. Eaton, unpub. results). Hendra virus has several distinguishing features, including a genome that is 15% larger than that of other members, with each of the six transcription units containing a very long 3' untranslated region (L.F. Wang, B.T. Eaton, unpub. results). The P/V/C gene has a fourth open reading frame located between those of the C and V proteins and potentially encoding a small basic protein similar to those of some members of the *Rhabdoviridae* and *Filoviridae*; its long 3' untranslated region is a common feature of the *Filoviridae* (65). The sequence of the N gene has also recently been described (66), and like the P/V/C gene, has a 3' untranslated region approximately tenfold longer than other members of the *Paramyxovirinae*. Although the deduced amino acid sequence of the N protein was slightly more homologous to members of the *Morbillivirus* genus than to those of other *Paramyxovirinae* genera, the level of identity was much lower than that observed within the *Morbillivirus* genus.

Three other findings differentiate Hendra from most other members of the *Paramyxoviridae*: the wide host range (59), the cleavage site of the F protein, and the orientation of the cell surface from which virus is released (B.T. Eaton, W. Michalski, and M. Williamson, unpub. results). An accumulating body of evidence—size of genome, comparative sequence analyses, coding capacity for a small basic protein in the P gene, morphologic features, host range, and various biologic properties, together with the wildlife host of the virus—suggests that the virus had been misnamed—it was neither an equine virus nor a morbillivirus—although the name was relevant when the virus was first isolated. It has therefore been suggested that the virus be renamed Hendra and be classified in a new genus within the *Paramyxovirinae* (59,65,66). A number of aspects of the ecology of the virus remain to be determined. For instance, despite the obvious ubiquity of the virus in the fruit bat population and the extremely close relationship between bat caregivers and bats, there is no evidence of seroconversions among caregivers, despite their close contact with up to 1,000 bats per year (50). Specimens of persons who have died of either pneumonia or encephalitis of unknown etiology were virus-negative (C. Allan, J.S. Mackenzie, L.A. Selvey, unpub. results). Furthermore, all human infections appear to have been transmitted by horses. Thus, the virus appears to have low transmissibility to humans; it also appears to be linked with pregnancy: the index case of the Brisbane outbreak was a pregnant mare, a pregnant mare was involved in the Mackay incident, both incidents occurred during the birthing season of flying foxes, and virus was first recovered from uterine fluid of a pregnant animal (3). Thus, a number of questions remain about the ecologic, biologic, and pathologic characteristics of Hendra virus: 1) the infectivity and virulence of the virus and why it seems extremely difficult to transmit naturally between and within some susceptible host species, 2) classification of the virus, 3) role of pregnancy to transmission of the virus, 4) role of prior infection in horses in human infection, 5) method of transmission between fruit bats and from fruit bats to horses, 6) tropism of the virus, and 7) potential for producing a latent infection in humans.

Australian Bat Lyssavirus

Australia had been considered free of rabies and rabieslike viruses until 1996 when a new lyssavirus, closely related to classic rabies virus, was first identified in a fixed brain specimen from a young black flying fox (*P. alecto*), with unusual neurologic symptoms. Since this original isolation, a further 42 isolates have been obtained from all four species of fruit bat, with most isolates from black and little red (*P. scapulatus*) flying foxes, and four isolates from an insectivorous bat (*Microchiroptera*), the yellow-bellied sheathtail bat (*Saccolaimus flavicentris*) (P. Daniels, R. Lunt, unpub. results). The isolates were from as far apart as Melbourne and Darwin, but most were from Queensland. Antibodies to rabies virus (REFIT assay) have been detected in 2.6% of 345 bat nonrandom samples submitted to the Australian Animal Health Laboratory (P. Daniels, R. Lunt,

unpub. results). Antibody has been detected in both infected and apparently healthy bats, but whether this reflects the ability of bats to recover from infection or become latently infected with the virus is not known.

Analysis using nucleocapsid-specific monoclonal antibodies showed a strong relationship between this new lyssavirus and serotype 1 rabies virus (67). Indeed, rabies vaccine may elicit a protective immune response in humans, indicating the antigenic similarity of Australian bat lyssavirus and classic rabies virus (P.K. Murray, pers. comm. to the Lyssavirus Expert Committee [68]). Phylogenetic studies of the N protein sequences indicated that the Australian virus was genetically distinct from classic rabies (genotype 1) and was, therefore, a previously unrecognized member of the *Lyssavirus* genus and represented a new genotype, genotype 7 (67).

Two human infections have been attributed to Australian bat lyssavirus. One fatal rabieslike infection was in a female bat caregiver from Rockhampton, Queensland (69,70). An isolate of Australian bat lyssavirus obtained post-mortem was antigenically and genetically similar to the virus from the insectivorous yellow-bellied sheathtail bat (A.R. Gould, R. Lunt, P. Daniels, unpub. results). A second death has recently been reported in Queensland (J. Hann, J. Faoagali, G. Smith, I Serafin, J. Northill, unpub. obs.). The infection was in a 27-year-old woman from Mackay, who died 2 years after a bat bite (by a large flying fox). Polymerase chain reaction (PCR) testing of RNA extracted from saliva and nuchal biopsy proved vital to the antemortem diagnosis. Immunofluorescence staining of postmortem samples confirmed the diagnosis. Preliminary sequencing of the amplicon has indicated that the virus is very similar to other lyssaviruses isolated from flying foxes but clearly distinct from a virus isolate from a yellow-bellied sheathtail bat (I. Serafin, G. Smith, J. Hanna, B. Harrower, J. Northill, A. Westcott, unpub. obs.). More extensive sequencing of the human and bat isolates is under way. Measures to prevent further human infection have been implemented (68,71).

As with Hendra virus, a number of questions remain about the ecology and biology of Australian bat lyssavirus. The finding of well, antibody-positive bats, which suggests that bats can either recover from infection or that they can be silently infected, needs to be investigated, particularly with respect to infectivity and possible transmissibility. More information is needed on the geographic and host range of the virus ecology within bat communities and risk for transmission to terrestrial animals.

These novel zoonotic viruses appear to have frugivorous bats as their natural vertebrate hosts. While little is known of the viral fauna of fruit bats (or indeed of most wildlife species) in Australia, the occurrence of these three zoonotic viruses from bats over 3 years suggests that further prospective studies of diseases of wildlife are warranted. Indeed, two paramyxoviruslike viruses unrelated to any other known paramyxoviruses (K. Halpin, P.L. Young, unpub. results) have recently been isolated from flying foxes.

Conclusions

The vector-borne and zoonotic diseases in this editorial encompass three patterns of emergence: known diseases increasing in incidence or geographic range (e.g., dengue and JE virus, respectively); new infectious agents as etiologic agents of known diseases (e.g., Australian bat lyssavirus as a cause of a rabieslike illness); and new infectious agents causing previously unrecognized diseases (e.g., Hendra virus). All three patterns demonstrate the need (and international responsibility) for ongoing surveillance and monitoring. In Australia, surveillance is the legislative responsibility of the individual states and territories. A Communicable Diseases Network Australia-New Zealand was established in 1989 to improve the control of communicable diseases in Australia by coordinating national surveillance activities and responses to outbreaks and by training public health staff. In 1996, Australia developed a National Communicable Diseases Surveillance Strategy to provide a national framework to monitor infectious diseases and plan and prioritize interventions. Components of the strategy include improvements to the national surveillance infrastructure, better monitoring of diseases and surveillance data, and better response to outbreaks. The strategy is being implemented and may provide the mechanism for a national response to new and reemerging diseases.

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The Economic Impact of *Staphylococcus aureus* Infection in New York City Hospitals

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We modeled estimates of the incidence, deaths, and direct medical costs of *Staphylococcus aureus* infections in hospitalized patients in the New York City metropolitan area in 1995 by using hospital discharge data collected by the New York State Department of Health and standard sources for the costs of health care. We also examined the relative impact of methicillin-resistant versus -sensitive strains of *S. aureus* and of community-acquired versus nosocomial infections. *S. aureus*-associated hospitalizations resulted in approximately twice the length of stay, deaths, and medical costs of typical hospitalizations; methicillin-resistant and -sensitive infections had similar direct medical costs, but resistant infections caused more deaths (21% versus 8%). Community-acquired and nosocomial infections had similar death rates, but community-acquired infections appeared to have increased direct medical costs per patient ($35,300 versus $28,800). The results of our study indicate that reducing the incidence of methicillin-resistant and -sensitive nosocomial infections would reduce the societal costs of *S. aureus* infection.

Each year approximately two million hospitalizations result in nosocomial infections (1). In a study of critically ill patients in a large teaching hospital, illness attributable to nosocomial bacteremia increased intensive care unit stay by 8 days, hospital stay by 14 days, and the death rate by 35% (2). An earlier study found that postoperative wound infections increased hospital stay an average of 7.4 days (3).

Staphylococcus aureus was the most common cause of nosocomial infections reported in the National Nosocomial Surveillance System between 1990 to 1996 (4). The leading cause of nosocomial pneumonia and surgical site infections and the second leading cause of nosocomial bloodstream infections (4), *S. aureus* also causes community-acquired infections (e.g., osteomyelitis and septic arthritis, skin infections, endocarditis, and meningitis). More than 95% of patients with *S. aureus* infections worldwide do not respond to first-line antibiotics such as penicillin or ampicillin (5). Additionally, methicillin-resistant strains of *S. aureus* (MRSA) are common. First reported in the 1960s (6), MRSA has become increasingly prevalent since the 1980s (7,8) and is now endemic in many hospitals and even epidemic in some, with resistance in approximately 30% of all *S.aureus* infections (8).

Vancomycin is the only drug that can consistently treat MRSA. However, beginning in 1989, hospitals have reported a rapid increase in vancomycin resistance in enterococci (VRE) (9). Increased vancomycin use helps select for VRE, and even a small increase in incidence of VRE infection could lead to cross-resistance in *S. aureus*, since genes conferring vancomycin resistance might be transferred from VRE (10). In 1996, Japan reported the first case of *S. aureus* infection with intermediate resistance to vancomycin (11). In 1997, two unrelated cases of *S. aureus* infection with intermediate resistance to vancomycin were reported in the United States (Michigan and New Jersey) (12). In both cases, patients had been treated with multiple courses of vancomycin for repeated MRSA infections over the 6 months before the *S. aureus* infection with intermediate resistance to

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vancomycin; additionally, VRE colonization had been diagnosed 7 months before the *S. aureus* infection with intermediate resistance to vancomycin in the New Jersey patient. The emergence of *S. aureus* infection with intermediate resistance to vancomycin in the United States suggests that *S. aureus* strains are constantly evolving and full resistance may develop (12).

The various ways of controlling MRSA (13) are still being debated. The elimination of endemic MRSA in hospitals is difficult and costly (14-17). In general, infection control in the United States is less stringent than in Canada and in some European countries, where identification of known carriers, prospective surveillance of patients and hospital workers, and use of nasal mupirocin have helped control drug-resistant *S. aureus* infection rates (18).

Knowledge of the scope of the problem is helpful for hospital administrators, insurers, and medical personnel who make policy decisions on control measures to prevent the spread of MRSA and the emergence of vancomycin-resistant *S. aureus*. However, the economic cost of *S. aureus* infections is not well known. Many studies focus on the cost of nonorganism-specific nosocomial infections (2,19,20). Moreover, the reported cost of a nosocomial infection varies because of the wide range of study populations, sites of infection, and methods used (16,21). The few investigations into the cost of *S. aureus* infections have focused on the differential cost of MRSA and MSSA infections (22,23) and are case studies of outbreaks in single hospitals. Thus, they do not provide perspective on the scope of the problem for a population over time.

We estimated the incidence, death rate, and cost of *S. aureus* infections associated with hospitalization in the New York City metropolitan area in 1995. We selected this geographic region because of its high prevalence of multidrug-resistant infections (24,25). We also compared the relative contributions of nosocomial versus community-acquired infections and methicillin-sensitive (MSSA) versus methicillin-resistant *S. aureus*.

The Study

Data

The 1995 Statewide Planning and Research Cooperative System (SPARCS) Administratively Releasable File was the primary source of data (26). SPARCS is a database of all hospital discharges in New York state, as reported by hospitals to the State of New York Department of Health, and the Administratively Releasable File contains discharge information on hospital location, patient characteristics (age, sex, race, ethnicity), and visit characteristics (primary diagnosis, secondary diagnoses, primary procedure, secondary procedures, length of stay, total charges, patient status, and disposition). We analyzed data for hospitals in the following New York City metropolitan area counties: Bronx, Dutchess, Kings, Manhattan, Nassau, Orange, Putnam, Queens, Richmond, Rockland, Suffolk, Ulster, and Westchester. Data on infection incidence or resource use not in SPARCS were obtained through a comprehensive literature search or estimated by a clinical panel consisting of four physicians specializing in infectious disease. Other sources for cost information were the 1995 Medicare Fee Schedule (27) for physician fees and the 1995 Red Book (28) for outpatient pharmaceutical average wholesale prices.

Definitions

We identified patients with the most common types of hospital-associated *S. aureus* infections: pneumonia, bacteremia, endocarditis, surgical site infections, osteomyelitis, and septic arthritis (Table 1) from SPARCS, which uses the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) diagnosis codes (29). With the exception of ICD-9-CM code 482.4 (staphylococcal pneumonia) and 038.1 (staphylococcal septicemia), these codes are not organism-specific.

To identify *S. aureus* infections, we used the nonorganism–specific codes in conjunction with an additional ICD-9-CM code to identify the bacterial agent (i.e., 041.11 bacterial infection due to *S. aureus* in conditions classified elsewhere and of unspecified site). Patients with multiple infections were counted only once in the overall incidence rate. Their primary or first occurrence of a diagnosis of interest was used.

Because source of infection (nosocomial versus community-acquired) is not reported in SPARCS, we assumed that specific types of disease were either nosocomial or community-acquired on the basis of the clinical panel opinion (Table 2).

Table 1. ICD-9-CM codes used to identify infections in Statewide Planning and Research Cooperative System

Type of Infection	ICD-9-CM[a]	Description
Pneumonia	482.4	Pneumonia due to staphylococcus
Bacteremia	038.1	Staphylococcal septicemia
	790.7	Bacteremia
	996.62	Infection and inflammatory reaction due to internal vascular device, implant, and graft
Endocarditis	421.0	Acute and subacute bacterial endocarditis
	996.61	Infection and inflammatory reaction due to cardiac device, implant, and graft
Surgical site infection	998.3	Disruption of operation wound
	998.5	Postoperative infection
Osteomyelitis	730.01-730.09	Acute osteomyelitis
	730.10-730.19	Chronic osteomyelitis
Septic arthritis	711.00-711.09	Pyogenic arthritis
	996.66	Infection and inflammatory reaction due to internal joint problems

[a]International classification of diseases, 9th Revision, Clinical Modification, 1995.

Table 2. Definitions of nosocomial or community-acquired *Staphylococcus aureus* infections

Type of Infection	Nosocomial	Community-acquired
Pneumonia	Secondary diagnosis[a]	Primary diagnosis[a]
Bacteremia	Catheter- or surgery-associated infections[b]	Noncatheter- and nonsurgical-associated infections
Endocarditis	Prosthetic valve infections	Natural valve infections
Surgical site infection (SSI)	All SSIs	None
Osteomyelitis	None	All
Septic arthritis	Prosthetic joint infections	Natural joint infections

[a]ICD-9-CM 482.4 as the primary diagnosis vs. 482.4 as one of several other diagnoses.
[b]ICD-9-CM 996.62, or 038.1 associated with a surgical ICD procedure code, or 790.7 associated with a surgical ICD procedure code.

Modeling the Incidence Rate

ICD-9-CM code 041.11 (bacterial infection due to *S. aureus*) is not widely used by reporting hospitals. Therefore, the incidence of *S. aureus* infections based on the counts of 041.11 in SPARCS would underestimate the number of cases. We estimated the incidence of *S. aureus* infections (except pneumonia) as follows (Table 3): the total incidence of each type of infection (e.g., endocarditis) in SPARCS was multiplied by the

Table 3. Incidence of *Staphylococcus aureus* infections from research or clinical panel

Type of infection	Description	*S. aureus* %	Reference
Bacteremia	Staphylococcal septicemia	50	30
	Bacteremia	15	31,32
	Infection and inflammatory reaction due to internal vascular device, implant, and graft	16	4
Endocarditis	Acute and subacute bacterial endocarditis	30	Clinical panel
	Infection and inflammatory reaction due to cardiac device, implant, and graft	14	33
Surgical site infection	Disruption of operation wound and postoperative infection	20	4
Osteomyelitis	Acute and chronic osteomyelitis	50	34,35
Septic arthritis	Pyogenic arthritis	11 (age <5 yr) 33 (age 5-18 yr) 55 (age >18 yr)	33
	Infection and inflammatory reaction due to internal joint prosthesis	25	33

estimated percentage attributable to *S. aureus* (determined by research or clinical panel opinion) to give the total number of infections due to *S. aureus*. The incidence of pneumonia was equated with the occurrence of the ICD-9-CM code 482.4 (staphylococcal pneumonia). For ICD-9-CM code 038.1 (staphylococcal septicemia), we assumed that only 50% of infections were attributable to *S. aureus* (with the remainder attributable to *S. epidermidis*) (30).

Modeling Death Rates

The death rates attributable to bacteremia, endocarditis, or community-acquired pneumonia were assumed to be equal to the death rates found when these infections were coded as a primary diagnosis in SPARCS and 041.11 was used as a secondary diagnosis. For nosocomial pneumonia, however, we assumed that the attributable death rate was a percentage of the actual death rate—for ventilator-associated pneumonia patients, death rate is a function of both the severity of underlying disease and the pneumonia. A series of matched-cohort studies have demonstrated that the death rate attributable to ventilator-associated pneumonia is 0% to 57% of the actual death rate (36-39). On the basis of this research and expert panel judgment, nosocomial pneumonia in ventilator-associated pneumonia patients (identified by ICD-9-CM V46.0 or V46.1) was assumed to have an attributable death rate of 50% of the death rate found in SPARCS (30,40). We assumed that the attributable death rate of nonventilator-associated pneumonia was the death rate found in SPARCS. On the basis of the low death rate found in SPARCS (approximately 2%), we assumed that no deaths were attributable to osteomyelitis, septic arthritis, or surgical site infections.

Modeling Direct Medical Costs

Direct medical costs were defined as hospital costs attributable to *S.aureus* infection, professional fees incurred during hospitalization, and costs of other infection-related medical services provided after discharge. For each infection, total direct medical costs were calculated by multiplying the average direct medical cost per patient by the incidence of disease. Average hospital costs attributable to *S. aureus* per patient were assumed to be equal to the average hospital charge from SPARCS when the infection (e.g., pneumonia, bacteremia) was coded as a primary diagnosis and 041.11 was used as a secondary diagnosis. Professional fees incurred during hospitalization include physician visits and consultations for evaluation and management, as well as radiologic, surgical, and anesthesiologic costs. The average frequency of physician services per patient was based on clinical panel estimates. Costs of these services were based on 1995 Medicare Payment Rates for the Long Island, New York, area as an intermediate point between New York City costs and those of outlying counties.

Costs of medical services after discharge include those of postdischarge complications (e.g., abscesses, aneurysms) requiring rehospitalization, home-based intravenous antibiotic therapy, and outpatient oral antibiotic therapy. The average frequency of other medical services provided per patient was based on clinical panel estimates. Costs of hospital readmission were based on SPARCS charges; costs of home-based intravenous therapy were based on literature estimates (40,41); and costs of outpatient medications were based on average wholesale prices (25).

Modeling MRSA and MSSA *S. aureus* Infections

SPARCS does not identify MRSA or MSSA infections, and a code for infection with a drug-resistant organism (V09) is rarely used. Therefore, we modeled the comparative incidence, death rate, and cost of MRSA and MSSA. We computed the incidence of MRSA and MSSA infections by using the estimate that 29% of infections were due to MRSA (8). The clinical panel estimated that 10% of community-acquired infections were due to MRSA (includes infections acquired at long-term care facilities).

The number of deaths for MRSA and MSSA infections was estimated as follows: the clinical panel estimated a risk ratio for death rates of MRSA and MSSA infections, and deaths due to MRSA and MSSA infections were calculated from the estimated risk ratio and the overall number of deaths due to *S. aureus* infection. We estimated the direct medical cost per patient for MRSA and MSSA infections as follows: differences in resource use for those with MRSA and MSSA infections were identified by the clinical panel; these differences were converted into differences in cost using a method similar to that described above for modeling direct medical costs; and average costs for MRSA and MSSA infections were

calculated by using the average cost for an *S. aureus* infection and the average difference in cost between MRSA and MSSA infections.

Incidence, Death Rate, and Attributable Costs

S. aureus Infection

Of 1,351,362 nonobstetrical hospital discharges in SPARCS for New York City in 1995, an estimated 13,550 (1.0%) were discharges of patients with *S. aureus* infections (Table 4). The total direct medical costs incurred by these patients was an estimated $435.5 million—average length of stay nearly 20 days, direct cost of infection, $32,100 (Table 4). The number of deaths was estimated at 1,400 (a 10% death rate). In contrast, the hospital charges for the average hospital stay in SPARCS (for all nonobstetrical discharges) were $13,263—average length of stay 9 days, death rate 4.1%. Thus, patients with *S. aureus* infection had approximately twice the cost, length of stay, and death rate of a typical hospitalized patient.

Pneumonia and bacteremia represented most *S. aureus* infections and accounted for 60% of the total direct medical costs and 97% of the number of deaths. Endocarditis caused the longest stay (26 days) and highest direct cost per patient ($47,200); surgical site infection caused the shortest stay (14 days) and lowest direct cost per patient ($21,810). Hospital charges were an average of $29,000 (90% of the total costs); professional fees were an average of $2,300 (7%); and postdischarge costs represented $800 (3%) (Table 5).

Table 4. Incidence, length of stay, costs, and death rates of *Staphylococcus aureus* infections by type of infection

			Direct Medical Cost			
		Length of stay	Total	Per patient	Deaths	
Type of infection	Incidence	(days)	($M)	($)	Total	%
Pneumonia	3,600	22.2	128.3	35,400	890	25
Bacteremia	4,400	18.0	137.0	31,300	470	11
Endocarditis	550	25.9	25.8	47,200	40	7
Surgical site infection	2,300	13.6	50.5	21,800	ND[a]	ND
Osteomyelitis	2,000	23.9	68.4	35,000	ND	ND
Septic arthritis	700	22.0	25.5	35,100	ND	ND
Total or average	13,550	19.8	435.5	32,100	1,400	10

[a]ND=no data.

Table 5. Direct medical charges—average hospital facility charges, professional fees, and postdischarge costs per case

	Hospital charges		Professional fees		Postdischarge costs		Total
Type of infection	$	(%)	$	(%)	$	(%)	$
Pneumonia	33,400	(94)	2,000	(6)	ND[a]		35,400
Bacteremia	27,900	(89)	2,100	(7)	1,300	(4)	31,300
Endocarditis	41,700	(88)	4,300	(9)	1,200	(3)	47,200
Surgical site infection	20,200	(93)	1,600	(7)	ND		21,800
Osteomyelitis	30,000	(86)	3,200	(9)	1,800	(5)	35,000
Septic arthritis	30,600	(87)	3,100	(9)	1,400	(4)	35,100
Average	29,000	(90)	2,300	(7)	800	(3)	32,100

[a]ND=no data.

Nosocomial Infection

Nosocomial infections accounted for 46% of the total incidence of *S. aureus* infections (6,300 infections), while community-acquired infections accounted for 54% (7,250 infections) (Table 6). Community-acquired pneumonia as a primary diagnosis accounted for 12% (1,500) of the total cases. If community-acquired pneumonia is assumed to be mostly acquired in long-term care facilities, most infections (58%) were acquired institutionally. The cost attributable to community-acquired infections ($35,300) was approximately $6,500 higher on a per patient basis than the cost attributable to nosocomial infections ($28,800). The death rates attributable to community-acquired and nosocomial infections were similar (10.5% and 10.1%).

MRSA Infection

MRSA infections accounted for 21% (2,780) of the total *S. aureus* infection incidence (29% of 6,300 nosocomial infections plus 10% of 7,250 community-acquired infections), while MSSA infections accounted for 79% (10,770) of total infections (Table 6). The attributable cost of a patient with MRSA was approximately $2,500 higher than the attributable cost of a patient with MSSA ($34,000 versus $31,500). The higher cost of MRSA infections is due to the higher cost of vancomycin, longer hospital stay, and the cost of patient isolation procedures. For nosocomial infections alone, the cost attributable to MRSA was approximately $3,700 higher on a per patient basis than the cost attributable to MSSA infections ($31,400 versus $27,700). The death rate attributable to MRSA infections was estimated at more than 2.5 times higher than that attributable to MSSA infections (21% versus 8%).

Table 6. Incidence, length of stay, costs, and deaths of *Staphylococcus aureus* infections by source of infection and degree of resistance

Source of infection	Incidence	Direct medical cost: Total ($M)	Direct medical cost: Per patient ($)	Deaths: Total	Deaths: (%)
Nosocomial	6,300	181.0	28,800	640	10
Community	7,250	254.5	5,300	760	11
Pneumonia	1,500	51.7	34,900	380	25
Non-pneumonia	5,750	202.8	35,400	380	7
MRSA[a]	2,780	94.5	34,000	590	21
MSSA[b]	10,770	339.4	31,500	810	8

[a]Methicillin-resistant strains of *S. aureus*.
[b]Methicillin-sensitive strains of *S. aureus*.

Sensitivity Analyses

Although assumed to be underused in SPARCS, the ICD-9-CM code 041.11 represents a lower boundary of the total incidence of *S. aureus* infection. In SPARCS, code 041.11 was used 7,366 times associated with a diagnosis of interest (e.g., endocarditis) and represented a total cost of $236.4 million and a death rate (740 deaths) of 2% (Table 7). The upper boundary of the total cost of *S. aureus* infections was calculated by assuming that all hospital charges and deaths of patients with *S. aureus* infections were attributable to the infection, representing a total cost of $599 million and a death rate of 14.5% (1,960 deaths). We conducted sensitivity analyses (varying the percentage of nosocomial MRSA; percentage of patients isolated; difference in length of stay between patients with MRSA and MSSA; attributable length of stay for patients with ventilator-associated pneumonia; number of *S. aureus* catheter infections; and percentage of *S. aureus*–caused bacteremia, septicemia, and postoperative infections) and found that the difference in cost per case between MRSA and MSSA infections was $1,700 to $5,100.

Table 7. Sensitivity analyses

	Direct medical cost ($)	Deaths
Study results	435.5	1,400
Lower boundary: only 041.11 cases	236.4	740
Upper boundary: all costs attributable	599.0	1,960

Comments

Our sensitivity analysis shows that we did not vastly over- or underestimate the direct medical costs of *S. aureus* infections in New York City. However, the study had several limitations; it was retrospective, and the data sources were not validated by other means (e.g., interviews or chart review). Therefore, coding errors in this database may affect the results. The clinical panel estimates we used to model differences between MRSA and MSSA may lead to some inaccuracy in those difference estimates. Thus, our comparison of costs and deaths between MRSA and MSSA should be viewed as a best approximation in the absence of case-control data or a multivariate analysis of a well-defined patient population.

Our estimates of the cost per infection are generally higher than estimates in studies reviewed by Jarvis (19). A major reason may be our focus on New York City, where costs are much higher than in other areas of the United States. In addition, earlier studies have used only hospital costs. Our perspective was societal; therefore, we included physician fees and outpatient costs, as well as hospital charges. Finally, most of these studies focused on non–organism-specific nosocomial infections; *S. aureus* infections may have a higher average cost per

episode than infections of other organisms (42).

On the other hand, we used conservative estimates for certain costs. Medicare prices for professional services are generally lower than commercial rates. Also, we did not account for postdischarge complications that did not lead to hospitalization. Additionally, our societal estimates did not include the cost of dying or lost productivity associated with these illnesses.

Despite its limitations, this study shows that hospitalizations associated with *S. aureus* are serious and have high medical costs and death rates. The average length of stay attributable to *S. aureus* infection for these patients was very high, 20 days—nearly three times the average for any other type of hospitalization (43). The increased length of stay in turn leads to increases in direct medical costs, with an average cost per case of $32,100 in 1995.

Treating an MRSA infection costs 6% to 10% more than treating an MSSA infection ($2,500 to $3,700 per case). This cost difference does not reflect MRSA's greater virulence; rather, it reflects the increased cost of vancomycin use and isolation procedures (if used). These estimates are slightly lower than the difference of $5,104 found by Wakefield et al. (21), perhaps because they focused on the cost of serious *S. aureus* infections, while our analysis examined all hospitalizable *S. aureus* infections.

Patients with MRSA infections have a high average attributable death rate of 21% versus 8% for an MSSA infection. Some of the death rate difference may be related to the underlying condition of patients who become infected with MRSA (e.g., older patients, drug users, sicker patients, patients previously exposed to other antibiotics) (44) and to the lack of effectiveness of vancomycin itself in curing MRSA. (Vancomycin has a narrow therapeutic index that allows little room for increasing blood concentration without incurring substantial losses in tolerance [45]).

Both MSSA and MRSA infections are associated with high costs and large numbers of deaths in the New York City metropolitan area. The costs and deaths associated with *S. aureus* infections may dramatically increase if the newly isolated *S. aureus* infection with intermediate resistance to vancomycin spreads or if VRSA emerges. For example, after penicillin-resistant *S. aureus* appeared in the 1950s, the death rate of bacteremia increased from 28% to 50% at the University of Minnesota (Figure) (46). After methicillin was introduced, the death rate decreased (47). Efforts should be directed toward reducing the incidence of MRSA and MSSA nosocomial infections to reduce their economic impact on society.

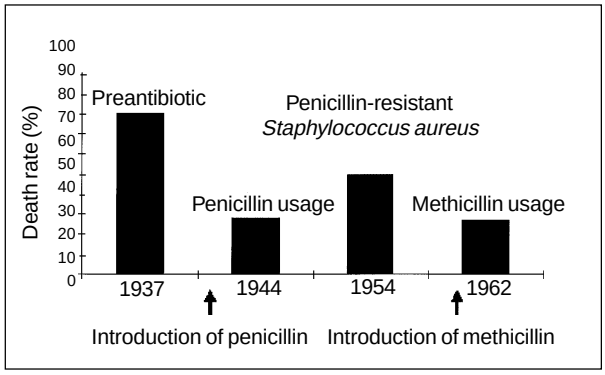

Figure. Death rate of staphylococcal bacteremia over time. (Data from 46, 47.)

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Socioeconomic and Behavioral Factors Leading to Acquired Bacterial Resistance to Antibiotics in Developing Countries

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In developing countries, acquired bacterial resistance to antimicrobial agents is common in isolates from healthy persons and from persons with community-acquired infections. Complex socioeconomic and behavioral factors associated with antibiotic resistance, particularly regarding diarrheal and respiratory pathogens, in developing tropical countries, include misuse of antibiotics by health professionals, unskilled practitioners, and laypersons; poor drug quality; unhygienic conditions accounting for spread of resistant bacteria; and inadequate surveillance.

Acquired bacterial resistance is common in isolates from healthy persons and from patients with community-acquired infections in developing countries, where the need for antibiotics is driven by the high incidence of infectious disease (1). Among isolates of diarrheal, respiratory, and commensal enteric pathogens (2-5), resistance is increasing, particularly to first-line, inexpensive, broad-spectrum antibiotics (Table 1). Furthermore, introduction of newer drugs (e.g., fluoroquinones) has been followed relatively quickly by the emergence and dissemination of resistant strains (5). The selection and spread of resistant organisms in developing countries, which can often be traced to complex socioeconomic and behavioral antecedents, contribute to the escalating problem of antibiotic resistance worldwide.

Table 1. Pathogens with a steadily increasing prevalence of acquired antibiotic resistance in developing tropical countries

Pathogen	Drug(s)	Country (years)	Ref.
Shigella flexneri, *S. dysenteriae*	ampicillin, tetracycline, sulfonamides (alone or with trimethoprim), nalidixic acid	Bangladesh (1983-1990) Brazil (1988-1993) Rwanda (1983-1993) Thailand (1981-1995)	(6) (7) (8) (5)
Vibrio cholerae	cotrimethoxazole, nalidixic acid, ampicillin	Guinea-Bissau (1987-1995) India (1993-1995)	(9) (10)
Salmonella typhi	ampicillin, chloramphenicol, cotrimethoxazole	Bangladesh (1989-1993)	(3)
Salmonella (nontyphoidal)	cotrimethoxazole	Thailand (1981-1995)	(5)
Enterotoxigenic *Escherichia coli*	cotrimethoxazole	Thailand (1981-1995)	(5)
Campylobacter	fluoroquinolones	Thailand (1987-1995)	(5)
Mycobacterium tuberculosis	isoniazid, streptomycin, rifampicin (primary resistance)	Kenya (1981-1990) Morocco (1992-1994)	(11) (12)

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Misuse of Antibiotics by Physicians in Clinical Practice

Antibiotic use provides selective pressure favoring resistant bacterial strains; inappropriate use increases the risk for selection and dissemination of antibiotic-resistant bacteria, which are placed at a competitive advantage. Therefore, one would expect that drugs more commonly affected by bacterial resistance in developing countries are generally inexpensive and popular broad-spectrum agents (2-5,13). However, the relationship between antibiotic use and the emergence and spread of resistance is complex. Antibiotic use in clinical practice alone cannot explain the high frequency of resistant organisms in developing countries (14,15). Nevertheless, excessive clinical use (a form of misuse) is at least partially responsible for the escalating rates of resistance, especially in hospital settings, worldwide. The unnecessary prescription of antibiotics seen in industrialized nations has also been documented in many developing countries, particularly in cases of acute infantile diarrhea and viral respiratory infections (16-22). Clinical misuse of antibiotics may be more common among private practitioners than among public health personnel—private practitioners charge higher fees, the demand for antibiotics seen in private patients is higher, and more drugs are available in private clinics than in public hospitals (23-25).

Several strategies have been proposed for combating the inappropriate use of antibiotics by clinicians (26). Antibiotic monitoring systems and hospital formularies or antibiotic treatment protocols often reduce antibiotic prescription rates (24,27). Adoption of a national essential drug list can limit the antibiotics available to prescribers (28,29). However, implementation of these strategies does not guarantee optimal antibiotic use by clinicians in developing countries because the irregular drug supply, availability of drugs from unofficial sources, and financial constraints also affect antibiotic choices (30-32).

Continuing medical education changes the attitude of clinicians. Studies of antibiotic misuse in Cuba and Pakistan (33,34) recommend continuing medical education for health workers as the single most important tool for combating antibiotic misuse. A study in Zambia has demonstrated the efficacy of education in reducing antibiotic prescription rates (35). However, education has not been successfully implemented in many developing countries, where too often, governments and health workers cannot afford the time and money required for continuing medical education (36).

Health workers in many developing countries have almost no access to objective health information (24). Pharmaceutical company representatives typically outnumber practitioners and often adversely influence their prescription habits (37), as reflected by sales of nonessential drugs and drug combinations (38). Drug labels and package inserts often fail to provide accurate information (39), and in industrialized countries, patients often pressure physicians to prescribe antibiotics (19).

Misuse of Antibiotics by Unskilled Practitioners

In many developing countries, well-trained health personnel are scarce and cannot serve the entire population, especially in rural areas. Community health workers and others with minimal training treat minor ailments (40). The qualifications and training of community health workers, as well as the quality of care they provide, vary from country to country. Unskilled personnel are less aware of the deleterious effects of inappropriate antibiotic use. For example, pharmacy technicians in Thailand prescribed rifampicin for urethritis and tetracycline for young children (41). Unqualified drug sellers offer alternative drugs when the prescribed drugs are out of stock or refill prescriptions without consulting the prescriber (42,43). In India, traditional healers often dispense antibiotics (44). A high proportion of patients in some developing countries are treated by untrained practitioners simultaneously with oral and injectable antibiotics administered with contaminated needles and syringes (45-47) for misdiagnosed noninfectious diseases (48).

Misuse of Antibiotics by the Public

In most developing countries, antibiotics can be purchased without prescription, even when the practice is not legal. In many African, Asian, and Latin American countries, antibiotics are readily available on demand from hospitals, pharmacies, patent medicine stalls (drugstores), roadside stalls, and hawkers (17,43,46,49-53). In rural Bangladesh, for example, 95% of drugs consumed for 1 month by more than 2,000 study participants came from local pharmacies; only

8% were prescribed by physicians (54). People are encouraged to buy from unofficial distributors because drugs often are not available in government hospitals (55). Drug vendors usually have little or no knowledge of the required dosage regimen, indications, or contraindications (43,45,55). In markets and public transport in West African countries such as Cameroon (49) and Nigeria (Okeke and Lamikanra, pers. obs.), the vendor (usually a medically untrained salesman) tries to convince potential buyers to purchase the drug, even if they are not ill.

To save time and keep drug-hunting to a minimum, a patient may start at a source more likely to stock the desired drug, forgoing the expertise of a doctor. Unofficial sources are generally more accessible than official sources. For example, in Nepal, retail drug outlets are four times as numerous as government health posts and hospitals (46). Alternate sources offer the option of purchasing small quantities of medicines, while hospitals require purchase of the complete 5- or 7-day antibiotic regimen (17,43,52). The purchase of small samples is exceedingly common, particularly for most customers, who buy without prescription (52). These subinhibitory antibiotic regimens predispose for selection of resistant bacterial strains.

Antibiotic use in developing countries is underestimated. The quantity of drugs distributed within a country is calculated under the assumption that each person purchases a complete regimen (56). However, medication can be purchased in small aliquots from roadside stalls, and distribution of locally produced or counterfeit antibiotics is not recorded. The motives for self-medication and antibiotic overuse by laypersons are similar to those for clinical abuse by health professionals: to cut costs and act expeditiously to treat confirmed or suspected bacterial infection (57). For example, 50% to 80% of Bangladeshi patients infected with *Shigella* admitted that they had taken at least one antibiotic in the 15 days before a hospital visit (58), as had 18% to 70% of pediatric patients with acute respiratory infection in two Chinese studies (20,59). The proportion of patients who self-medicate is probably higher, because patients are often reluctant to admit having taken antibiotics before visiting a hospital (60).

Common cultural beliefs about antibiotics include the notions that there is a pill for every symptom; antibiotics can heal many illnesses, including dyspepsia and headaches; and injections are more powerful than pills. The misuse of antibiotics frequently becomes integrated into the local culture (62) (e.g., antibiotics are used to prevent diarrhea after eating suspected contaminated foods or [by prostitutes] to prevent sexually transmitted diseases [52,63]).

Another cause of antibiotic abuse and selection for resistant bacteria is poor patient compliance. First, physician-patient interactions are often inadequate. They can be short (e.g., a mean of 54 sec was recorded in a Bangladeshi study [16]) and of poor quality (e.g., in Mexico, poor patient-physician communication was partially responsible for the noncompliance of patients with antibiotic regimens [21]). Second, because patients often travel long distances and incur large expenses for medical care, they are unlikely to return for follow-up visits. The reverse situation—the prescriber visiting his patient—is difficult logistically, especially in rural Africa (64). In addition, the patient may be unable to read medicine labels. Finally, because many drugs are expensive, indigent patients purchase incomplete regimens whenever possible and discontinue treatment when symptoms disappear but before the pathogen is eliminated (52).

Poor Quality of Antibiotics

Lack of Quality Compliance and Monitoring

Besides the risk for therapeutic failure, degradation products or adulterants in poor quality antibiotics can produce subinhibitory concentrations in vivo, which increase the selection of resistant strains. Drugs that do not comply with minimum standards are illegal in all countries. However, the quality of many antibiotics and other drugs in developing countries is often below standards in the formulary. In Nigeria for example, substandard ampicillin, ampicillin/cloxacillin, tetracycline, and oxytetracycline capsules have been detected (53,65-67). In many cases, therapeutic failure is the only indication of substandard drugs. Analytic laboratories to detect substandard drugs are uncommon, and when they exist, health workers, distributors, and consumers are often unaware of them.

Degraded Antibiotics

The shelf lives of drugs developed and marketed in temperate countries are determined

by storage temperatures. During distribution in tropical countries, conditions of transport and storage are poorly controlled, and the drugs may be degraded. Ballereau et al. (68) recorded temperatures of 26°C to 40°C and 30% and 90% humidity in Guinea-Bissau during a 2-year period (temperatures of greater than 25°C can degrade antibiotics). Many antibiotics, being heat- and moisture-labile, are particularly vulnerable. Of seven drugs that lost 10% or more of their active constituents when stored in pharmacies in Guinea-Bissau for 2 years, six were antimicrobial drugs (68). Drug consignments are exposed to such adverse conditions during shipment (69) or at tropical ports while they await lengthy port clearance. Drugs are often handled by untrained workers who may store them incorrectly. Hawkers and small traders in Nigeria frequently display large glass jars containing different types of antibiotic capsules mixed together, fully exposed to harsh sunlight and high ambient temperature and humidity. In a Nigerian study of eight batches of tetracycline capsules, only the batch obtained directly from the manufacturer was not excessively degraded and contained active drug levels within formulary limits (Table 2) (53,70). Studies conducted in Thailand and Nigeria demonstrated similar degradation of chloroquine and amoxicillin (67,70).

Table 2. Source and quality of tetracycline capsules in a Nigerian suburban town (compiled with data from [53])

Sample	Source	Tetracycline content (% of label claim)	Content of ATC[a] (%)	Bioavailability (%)[b]
C1	Manufacturer[c]	105.9	None detected	100
C2	Hospital	107.5	5.3	63.4[d]
C3	Roadside stall	104.5	1.1	80.5[d]
C4	Pharmacy	66.1	2.4	65.2[d]
C5	Patent medicine stall	84.5	1.9	87.6[d]
C6	Roadside stall	67.8	1.5	Not tested
C7	Patent medicine stall	89.6	1.8	Not tested

[a]Anhydrotetracycline, one of four tetracycline degradation products.
[b]Measured from cumulative excretion of tetracycline in the urine of five volunteers.
[c]Reference standard obtained from the manufacturer.
[d]Significantly different from C1 ($p = 0.01$, Wilcoxon signed rank test).

Expired Antibiotics

Some pharmacologically active drugs produced in industrialized countries have expired when distributed in developing countries—they were shipped at the end of the drugs' shelf lives or their clearance and distribution after transcontinental shipment were delayed. Expired drugs may receive new labels, be dumped without a label change, or be donated rather than sold (71-73). Tax deductions and the cost of liquidation are incentives for donating expired or near-expired drugs. Effective enforcement of the World Health Organization (WHO) guidelines on drug donations may curtail such practices (74).

Counterfeit Drugs

Some drugs sold in developing countries do not contain the concentration of active substances stated on their labels, even at the time of manufacture. These counterfeit drugs flourish, despite efforts of local regulatory agencies to stop their production and distribution (75-77). Approximately 65% of the 751 instances of counterfeit pharmaceuticals reported to WHO or to Interpol from 28 countries in the past 15 years were produced in developing countries (77). Counterfeit drugs include products with little or no active ingredients (e.g., in Nigeria, Indonesia, Brazil, Thailand, Bangladesh, Malaysia, and Francophone African countries [39,76,78,79]) or products for which excipients have been replaced by less expensive alternatives (e.g., substitution of ethylene glycol for propylene glycol in pediatric paracetamol formulations, which caused many deaths in Nigeria, Argentina, Bangladesh, India, and Haiti [76,78]). Counterfeit drugs, like other counterfeit materials, compete favorably in the markets of developing countries. The analytic facilities available to law enforcement agencies often cannot detect these drugs before they reach the patient. Multinational pharmaceutical companies, which probably possess the best analytic facilities for in-house quality assurance in developing countries, try to detect counterfeit drugs to protect their income and reputation; however, such efforts are directed primarily at counterfeits of these companies' own products. Because of the profusion of generic drugs in developing countries, a substantial proportion of counterfeit drugs go undetected.

Adulterated Drugs

Herbal preparations in developing countries are often adulterated with orthodox medicaments. For example, in one study, 24% of Chinese herbal preparations marketed in Taiwan contained one or more of such adulterants (80). Although the adulteration of such products with antibiotics has not been reported, such practices may be common (81). A Nigerian traditional healer, for example, admitted to 'augmenting' herbal preparations with tetracycline from commercially available capsules (82).

Bioinequivalent Antibiotics and Biopharmaceutic Interactions

In the last 2 decades, the importance of bioavailability has been underscored by the recognition that chemically equivalent generic drug formulations do not always deliver the expected amount of drug to the bloodstream. Slowly absorbed and acid-labile antibiotics are particularly prone to bioinequivalence and consequent therapeutic failure. In addition, poorly absorbed antibiotics remain in the gut to facilitate the selection of resistant organisms. The few published studies from the developing world have found bioinequivalence in antibiotic formulations, and the problem may be widespread (Table 2) (53,83). Inexpensive generic antibiotics commonly used in developing countries usually are not subject to bioavailability studies.

The bioavailability of an antibiotic formulation is modulated by conditions surrounding its administration; conditions unique to developing countries are rarely investigated. Drug combinations used in the tropics but rarely elsewhere may not be optimally absorbed. For example, coadministration of chloroquine and ampicillin lowers the bioavailability of ampicillin (84). A Nigerian meal lowered the biologic availability of orally administered nitrofurantoin (85). Chewing of Khat, a popular Yemeni stimulant, adversely affected the bioavailability of ampicillin and amoxicillin (86). By contrast, the Ayurvedic preparation Trikatu enhanced the absorption of several drugs (87). Whether traditional medicines with antimicrobial properties enhance antibiotic resistance is unknown.

Dissemination of Resistant Organisms

Crowding and Unhygienic Conditions

Residents of developing countries often carry antibiotic-resistant fecal commensal organisms (13,88). Visitors to developing countries passively acquire antibiotic-resistant gut *Escherichia coli,* even if they are not taking prophylactic antibiotics, which suggests that they encounter a reservoir of antibiotic-resistant strains during travel (89). Apparently healthy people in developing countries carry potentially pathogenic, antibiotic-resistant organisms asymptomatically (90). Several factors, such as urban migration with crowding and improper sewage disposal, encourage the exchange of antibiotic-resistant organisms between people and the exchange of resistance genes among bacteria, thereby increasing the prevalence of resistant strains. In Nigeria, resistant *E. coli* isolates from persons in an urban metropolis (Lagos) were significantly more likely to be resistant to ampicillin and streptomycin ($p \leq 0.05$), and possibly more resistant to sulphathiazole and tetracycline ($p \leq 0.10$), than isolates from residents of nearby smaller towns and villages (Table 3) (91). Moreover, strains isolated from Lagos were more likely to show resistance to 4 to 6 of 7 antibiotics tested, whereas strains from rural areas were in most cases resistant to only 0 to 3 antibiotics (91).

In 1991, 80% of residents of developing countries had no sanitary facilities for sewage disposal (92). Pipe-borne water, often scarce in developing countries, is not always potable. The

Table 3. Antibiotic resistance of *Escherichia coli* strains isolated from residents of an urban area (Lagos) or rural/suburban areas (southwest Nigeria) (from [91])

Antimicrobial agent	Percentage of resistant isolates: Urban (n = 30)	Rural/suburban (n = 44)
Ampicillin[a]	53	27
Chloramphenicol	13	14
Streptomycin[a]	63	32
Sulphathiazole[b]	73	48
Tetracycline[b]	87	64
Trimethoprim	53	41

[a]Significant differences between the two groups at $p \leq 0.05$ (Chi-square test)
[b]Significant differences between the two groups at $p \leq 0.10$ (Chi-square test)

development of sanitation and other facilities is not always proportionate to the rapid rises in urban populations (93,94). As urban migration continues, overcrowding increases and hygiene declines, increasing the probability of spread of antibiotic-resistant and commensal pathogens. Potable water, well-ventilated housing and proper waste disposal should reduce infections, the need for antibiotics, and subsequent development of antibiotic resistance.

Because tropical conditions encourages the survival of bacteria, more pathogens and commensals are found in tropical environments than in temperate climates (95). The warm and humid tropical climate and the low levels of health care, hygiene, and sanitation contribute to a relatively high prevalence of infectious disease in developing countries.

Inadequate Hospital Infection Control Practices

Infection control practices in many hospitals in developing countries are rudimentary and often compromised by economic shortfalls and opposing traditional values (96). The resulting nidus of nosocomial pathogens and resistant organisms may be disseminated to the outside community. Improper disposal of hospital waste accentuates such spread. Untreated hospital waste in Uganda was often dumped into public sewers or thrown into rubbish heaps ravaged by scavengers (97).

Inadequate Surveillance

Susceptibility Testing and Surveillance

Information from routine susceptibility testing of bacterial isolates and surveillance of antibiotic resistance, which provides information on resistance trends, including emerging antibiotic resistance, is essential for clinical practice and for rational policies against antibiotic resistance. Bacterial infections are often treated after they become life-threatening, which encourages empirical selection of broad-spectrum antibiotics (98,99). The antibiotic susceptibility pattern of bacterial isolates in much of the developing world is unknown, and little guides empirical prescribing. Susceptibility testing cannot be done readily because equipment, personnel, and consumables are scarce and expensive (59,100). In most all infections, no clinical specimens are cultured. Where available, community-based antibiotic surveillance data may be useful to prescribers in the absence of patient-specific antibiotic-susceptibility results. For example, Ringertz et al. (101) demonstrated that resistance among respiratory pathogens was infrequent in parts of Ethiopia. This information would help local Ethiopian prescribers to treat such infections with inexpensive, broad-spectrum antibiotics.

National surveillance programs for antibiotic resistance, the norm in industrialized nations, are less common and less elaborate in developing countries (4). Current inferences about antibiotic resistance trends in developing countries are based on a small number of reports, generated by a handful of microbiology laboratories in urban areas—data not representative of a country, because wide variations in antibiotic resistance patterns may exist within countries (Table 3). Moreover, surveillance should be conducted regularly and continuously because resistance rates can vary in one region of a country over time (Table 1) (102).

Defective Antibiotic Susceptibility Assays

Well-standardized antibiotic susceptibility assays provide more reliable results (103). However, standard bacterial strains with which to assay new batches of antibiotics or antibiotic disks are not available in laboratories in many developing countries. Delayed transportation and breakdown of cold storage also affects the quality of antibiotics used as diagnostic reagents. Degraded antibiotic powders and antibiotic disks used for susceptibility testing lead to exaggerated estimates of bacterial resistance levels. The frequent recovery of bacteria resistant to the beta-lactams or tetracyclines in tropical countries could reflect, in part, the temperature and moisture lability of test reagents. Laboratory scientists in developing countries face difficulties in obtaining research supplies, which often require them to improvise by, for example, using injectable antibiotic formulations to measure MICs when standard antibiotic powders are not available. The report that clinical microbiologists in developing countries make their own disks from "local blotting papers" (104) illustrates how improvisation can lead to inconsistent laboratory results and unreliable data.

Economic and Political Factors

Lack of resources hampers implementation of most strategies against antibiotic resistance. Statistics from the World Bank show that developing countries spent $41 per person on health in 1990, compared with the $1,500 per person spent by industrialized countries. Disease prevalence as measured by disability-adjusted life years and by communicable disease in particular is much greater in developing than in industrialized countries (93,105-107). As a result of such gross underfunding, the drug supply is chronically inadequate or at best erratic in health facilities in many countries, including Nigeria (43,105,106).

Armed conflicts have recently led to a breakdown in health services and sanitation and rapid dissemination of resistant pathogens, particularly in sub-Saharan Africa and Asia (108,109,110). During an outbreak of cholera and bacillary dysentery in Rwandan refugees, resistance to multiple first-line antibiotics in clinical isolates of *Vibrio cholerae* and *Shigella dysenteriae* contributed to high death rates (109).

Even in developing countries not at war, political corruption and mismanagement of funds, personnel, and development programs have created large populations living in abject poverty and at high risk for infection (111). Medical expenses, days lost from work, and transportation costs account for substantial economic loss. The cost of medical treatment, even subsidized treatment, is beyond the means of many patients. Poorly paid health workers sometimes extort fees from patients (111). Thus, persons with communicable diseases, unable to afford medical treatment, may infect others. Poverty also interferes with patient compliance, which in turn promotes the emergence of antibiotic resistance during short-term therapy of acute infections and long-term therapy of chronic infections, such as tuberculosis (111).

Combating the Problem of Antibiotic Resistance

The recommendations of WHO for ensuring proper drug use (79) can be adapted to combat the escalation of community-acquired antibiotic resistance in developing countries. The misuse of antibiotics by health-care professionals, unskilled practitioners, and patients can be alleviated by auditing antibiotics, limiting antibiotic choice, developing prescription guidelines, and emphasizing continuing medical and public education. The quality of antibiotics can be improved by emphasizing quality compliance and monitoring antimicrobial drugs manufactured or dispensed. Such reforms will help control substandard drugs that are degraded, counterfeit, or bioinequivalent. Dissemination of resistant organisms in the community can be impeded by improved public sanitation and hygienic practices and upgraded hospital infection control. Finally, strategies to ensure that these recommendations are adopted and implemented under difficult economic and political conditions can be formulated. Antimicrobial resistance will continue to escalate in developing countries unless corrective measures are instituted.

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Campylobacter jejuni—An Emerging Foodborne Pathogen

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Campylobacter jejuni is the most commonly reported bacterial cause of foodborne infection in the United States. Adding to the human and economic costs are chronic sequelae associated with *C. jejuni* infection—Guillian-Barré syndrome and reactive arthritis. In addition, an increasing proportion of human infections caused by *C. jejuni* are resistant to antimicrobial therapy. Mishandling of raw poultry and consumption of undercooked poultry are the major risk factors for human campylobacteriosis. Efforts to prevent human illness are needed throughout each link in the food chain.

History

Awareness of the public health implications of *Campylobacter* infections has evolved over more than a century (1). In 1886, Escherich observed organisms resembling campylobacters in stool samples of children with diarrhea. In 1913, McFaydean and Stockman identified campylobacters (called related *Vibrio*) in fetal tissues of aborted sheep (1). In 1957, King described the isolation of related *Vibrio* from blood samples of children with diarrhea, and in 1972, clinical microbiologists in Belgium first isolated campylobacters from stool samples of patients with diarrhea (1). The development of selective growth media in the 1970s permitted more laboratories to test stool specimens for *Campylobacter*. Soon *Campylobacter* spp. were established as common human pathogens. *Campylobacter jejuni* infections are now the leading cause of bacterial gastroenteritis reported in the United States (2). In 1996, 46% of laboratory-confirmed cases of bacterial gastroenteritis reported in the Centers for Disease Control and Prevention/U.S. Department of Agriculture/Food and Drug Administration Collaborating Sites Foodborne Disease Active Surveillance Network were caused by *Campylobacter* species. Campylobacteriosis was followed in prevalence by salmonellosis (28%), shigellosis (17%), and *Escherichia coli* O157 infection (5%) (Figure 1).

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Disease Prevalence

In the United States, an estimated 2.1 to 2.4 million cases of human campylobacteriosis (illnesses ranging from loose stools to dysentery) occur each year (2). Commonly reported symptoms of patients with laboratory-confirmed infections (a small subset of all cases) include diarrhea, fever, and abdominal cramping. In one study, approximately half of the patients with laboratory-confirmed campylobacteriosis reported a history of bloody diarrhea (3). Less frequently,

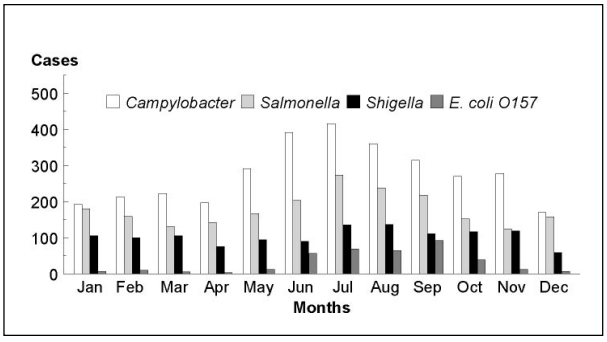

Figure 1. Cases of *Campylobacter* and other foodborne infections by month of specimen collection; Centers for Disease Control and Prevention/U.S. Department of Agriculture/Food and Drug Administration Collaborating Sites Foodborne Disease Active Surveillance Network, 1996.

C. jejuni infections produce bacteremia, septic arthritis, and other extraintestinal symptoms (4). The incidence of campylobacteriosis in HIV-infected patients is higher than in the general population. For example, in Los Angeles County between 1983 and 1987, the reported incidence of campylobacteriosis in patients with AIDS was 519 cases per 100,000 population, 39 times higher than the rate in the general population. (5). Common complications of campylobacteriosis in HIV-infected patients are recurrent infection and infection with antimicrobial-resistant strains (6). Deaths from *C. jejuni* infection are rare and occur primarily in infants, the elderly, and patients with underlying illnesses (2).

Sequelae to Infection

Guillain-Barré syndrome (GBS), a demyelating disorder resulting in acute neuromuscular paralysis, is a serious sequela of *Campylobacter* infection (7). An estimated one case of GBS occurs for every 1,000 cases of campylobacteriosis (7). Up to 40% of patients with the syndrome have evidence of recent *Campylobacter* infection (7). Approximately 20% of patients with GBS are left with some disability, and approximately 5% die despite advances in respiratory care. Campylobacteriosis is also associated with Reiter syndrome, a reactive arthropathy. In approximately 1% of patients with campylobacteriosis, the sterile postinfection process occurs 7 to 10 days after onset of diarrhea (8). Multiple joints can be affected, particularly the knee joint. Pain and incapacitation can last for months or become chronic.

Both GBS and Reiter syndrome are thought to be autoimmune responses stimulated by infection. Many patients with Reiter syndrome carry the HLA B27 antigenic marker (8). The pathogenesis of GBS (9) and Reiter syndrome is not completely understood.

Treatment of *C. jejuni* Infections

Supportive measures, particularly fluid and electrolyte replacement, are the principal therapies for most patients with campylobacteriosis (10). Severely dehydrated patients should receive rapid volume expansion with intravenous fluids. For most other patients, oral rehydration is indicated. Although *Campylobacter* infections are usually self limiting, antibiotic therapy may be prudent for patients who have high fever, bloody diarrhea, or more than eight stools in 24 hours; immunosuppressed patients, patients with bloodstream infections, and those whose symptoms worsen or persist for more than 1 week from the time of diagnosis. When indicated, antimicrobial therapy soon after the onset of symptoms can reduce the median duration of illness from approximately 10 days to 5 days. When treatment is delayed (e.g., until *C. jejuni* infection is confirmed by a medical laboratory), therapy may not be successful (10). Ease of administration, lack of serious toxicity, and high degree of efficacy make erythromycin the drug of choice for *C. jejuni* infection; however, other antimicrobial agents, particularly the quinolones and newer macrolides including azithromycin, are also used.

Antimicrobial Resistance

The increasing rate of human infections caused by antimicrobial-resistant strains of *C. jejuni* makes clinical management of cases of campylobacteriosis more difficult (11,12). Antimicrobial resistance can prolong illness and compromise treatment of patients with bacteremia. The rate of antimicrobial-resistant enteric infections is highest in the developing world, where the use of antimicrobial drugs in humans and animals is relatively unrestricted. A 1994 study found that most clinical isolates of *C. jejuni* from U.S. troops in Thailand were resistant to ciprofloxacin. Additionally, nearly one third of isolates from U.S. troops located in Hat Yai were resistant to azithromycin (11). In the industrialized world, the emergence of fluoroquinolone-resistant strains of *C. jejuni* illustrates the need for prudent antimicrobial use in food-animal production (12). Experimental evidence demonstrates that fluoroquinolone-susceptible *C. jejuni* readily become drug-resistant in chickens when these drugs are administered (13). After flouroquinolone use in poultry was approved in Europe, resistant *C. jejuni* strains emerged rapidly in humans during the early 1990s (12). Similarly, within 2 years of the 1995 approval of fluoroquinolone use for poultry in the United States, the number of domestically acquired human cases of ciprofloxacin-resistant campylobacteriosis doubled in Minnesota (14). In a 1997 study conducted in Minnesota, 12 (20%) of 60 *C. jejuni* isolates obtained from chicken purchased in grocery stores were ciprofloxacin-resistant (14).

Pathogenesis

The pathogenesis of *C. jejuni* infection involves both host- and pathogen-specific factors. The health and age of the host (2) and *C. jejuni*-specific humoral immunity from previous exposure (15) influence clinical outcome after infection. In a volunteer study, *C. jejuni* infection occurred after ingestion of as few as 800 organisms (16). Rates of infection increased with the ingested dose. Rates of illness appeared to increase when inocula were ingested in a suspension buffered to reduce gastric acidity (16).

Many pathogen-specific virulence determinants may contribute to the pathogenesis of *C. jejuni* infection, but none has a proven role (17). Suspected determinants of pathogenicity include chemotaxis, motility, and flagella, which are required for attachment and colonization of the gut epithelium (Figure 2) (17). Once colonization occurs, other possible virulence determinants are iron acquisition, host cell invasion, toxin production, inflammation and active secretion, and epithelial disruption with leakage of serosal fluid (17).

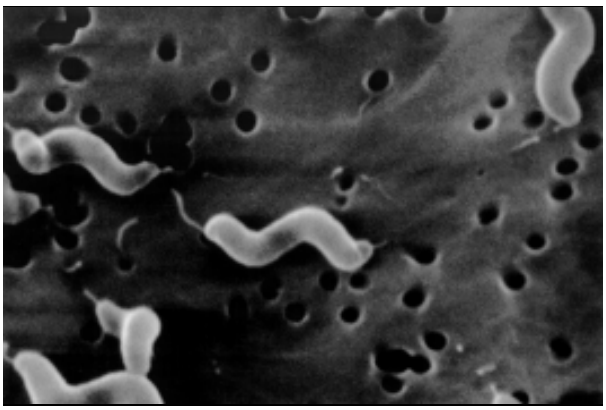

Figure 2. Scanning electron microscope image of *Campylobacter jejuni*, illustrating its corkscrew appearance and bipolar flagella. Source: Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia.

Survival in the Environment

Survival of *C. jejuni* outside the gut is poor, and replication does not occur readily (17). *C. jejuni* grows best at 37°C to 42°C (18), the approximate body temperature of the chicken (41°C to 42°C). *C. jejuni* grows best in a low oxygen or microaerophilic environment, such as an atmosphere of 5% O_2, 10% CO_2, and 85% N_2. The organism is sensitive to freezing, drying, acidic conditions (pH ≤ 5.0), and salinity.

Sample Collection and Transport

If possible, stool specimens should be chilled (not frozen) and submitted to a laboratory within 24 hours of collection. Storing specimens in deep, airtight containers minimizes exposure to oxygen and desiccation. If a specimen cannot be processed within 24 hours or is likely to contain small numbers of organisms, a rectal swab placed in a specimen transport medium (e.g., Cary-Blair) should be used. Individual laboratories can provide guidance on specimen handling procedures (18).

Numerous procedures are available for recovering *C. jejuni* from clinical specimens (18). Direct plating is cost-effective for testing large numbers of specimens; however, testing sensitivity may be reduced. Preenrichment (raising the temperature from 36°C to 42°C over several hours), filtration, or both are used in some laboratories to improve recovery of stressed *C. jejuni* organisms from specimens (e.g., stored foods or swabs exposed to oxygen) (19). Isolation can be facilitated by using selective media containing antimicrobial agents, oxygen quenching agents, or a low oxygen atmosphere, thus decreasing the number of colonies that must be screened (18,19).

Subtyping of Isolates

No standard subtyping technique has been established for *C. jejuni*. Soon after the organism was described, two serologic methods were developed, the heat-stable or somatic O antigen (20) and the heat-labile antigen schemes (21). These typing schemes are labor intensive, and their use is limited almost exclusively to reference laboratories. Many different DNA-based subtyping schemes have been developed, including pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) analysis (22). Various typing schemes have been developed on the basis of the sequence of *fla*A, encoding flagellin (23); however, recent evidence suggests that this locus may not be representative of the entire genome (24).

Transmission to Humans

Most cases of human campylobacteriosis are sporadic. Outbreaks have different epidemio-

logic characteristics from sporadic infections (2). Many outbreaks occur during the spring and autumn (2). Consumption of raw milk was implicated as the source of infection in 30 of the 80 outbreaks of human campylobacteriosis reported to CDC between 1973 and 1992. Outbreaks caused by drinking raw milk often involve farm visits (e.g., school field trips) during the temperate seasons. In contrast, sporadic *Campylobacter* isolates peak during the summer months (Figure 1). A series of case-control studies identified some risk factors for sporadic campylobacteriosis, particularly handling raw poultry (25,26) and eating undercooked poultry (27-31) (Table). Other risk factors accounting for a smaller proportion of sporadic illnesses include drinking untreated water (29); traveling abroad (25); eating barbequed pork (28) or sausage (27); drinking raw milk (29, 32) or milk from bird-pecked bottles (33); and contact with dogs (27) and cats (29,31), particularly juvenile pets or pets with diarrhea (25,34). Person-to-person transmission is uncommon (25,32). Overlap is reported between serotypes of *C. jejuni* found in humans, poultry, and cattle, indicating that foods of animal origin may play a major role in transmitting *C. jejuni* to humans (35).

In the United States, infants have the highest age-specific *Campylobacter* isolation rate, approximately 14 per 100,000 person years. As children get older, isolation rates decline to approximately 4 per 100,000 person years for young adolescents. A notable feature of the epidemiology of human campylobacteriosis is the high isolation rate among young adults, approximately 8 per 100,000 person years. Among middle-aged and older adults, the isolation rate is < 3 per 100,000 person years (2). The peak isolation rate in neonates and infants is attributed in part to susceptibility on first exposure and to the low threshold for seeking medical care for infants (2). The high rate of infection during early adulthood, which is pronounced among men, is thought to reflect poor food-handling practices in a population that, until recently, relied on others to prepare meals (2).

Reservoirs

The ecology of *C. jejuni* involves wildlife reservoirs, particularly wild birds. Species that carry *C. jejuni* include migratory birds—cranes, ducks, geese (36), and seagulls (37). The organism is also found in other wild and domestic bird species, as well as in rodents (38). Insects can carry the organism on their exoskeleton (39).

Table. Epidemiologic studies of laboratory-confirmed cases of sporadic campylobacteriosis

Number Cases	Number Controls	Date	Population	Location	Foods associated with illness	Animal contacts	Ref.
52	103	1989-1990	Residents of three counties	Norway	Poultry, sausage	Dogs	27
218	526	1982-1983	HMO patients	Washington State	Undercooked chicken	Animals with diarrhea	30,34
29	42	1990	Residents of Manchester	England	Bottled milk[a]		33
45	45	1983-1984	University students	Georgia	Chicken	Cats	31
53	106	1982-1983	Rural children	Iowa	Raw milk		32
40	80	1981	Residents of Denver, Ft. Collins	Colorado	Untreated water, raw milk, undercooked chicken	Cats	29
54	54	1982	Residents of Rotterdam	Netherlands	Chicken, pork, barbequed foods		28
10	15	1982	Residents of Larimer County	Colorado	Preparing chicken		26
55	14	1980	Residents of Göteborg	Sweden	Preparing chicken	Kitten, dog with diarrhea	25

[a]Bottle tops pecked by wild birds.

The intestines of poultry are easily colonized with *C. jejuni*. Day-old chicks can be colonized with as few as 35 organisms (40). Most chickens in commercial operations are colonized by 4 weeks (41,42). Vertical transmission (i.e., from breeder flocks to progeny) has been suggested in one study but is not widely accepted (43). Reservoirs in the poultry environment include beetles (39), unchlorinated drinking water (44), and farm workers (41,42,45). Feeds are an unlikely source of campylobacters since they are dry and campylobacters are sensitive to drying.

C. jejuni is a commensal organism of the intestinal tract of cattle (46). Young animals are more often colonized than older animals, and feedlot cattle are more likely than grazing animals to carry campylobacters (47). In one study, colonization of dairy herds was associated with drinking unchlorinated water (48).

Campylobacters are found in natural water sources throughout the year. The presence of campylobacters is not clearly correlated with indicator organisms for fecal contamination (e.g., *E. coli*)(49). In temperate regions, organism recovery rates are highest during the cold season (49,50). Survival in cold water is important in the life cycle of campylobacters. In one study, serotypes found in water were similar to those found in humans (50). When stressed, campylobacters enter a "viable but nonculturable state," characterized by uptake of amino acids and maintenance of an intact outer membrane but inability to grow on selective media; such organisms, however, can be transmitted to animals (51). Additionally, unchlorinated drinking water can introduce campylobacters into the farm environment (44,48).

Campylobacter in the Food Supply

C. jejuni is found in many foods of animal origin. Surveys of raw agricultural products support epidemiologic evidence implicating poultry, meat, and raw milk as sources of human infection. Most retail chicken is contaminated with *C. jejuni*; one study reported an isolation rate of 98% for retail chicken meat (52). *C. jejuni* counts often exceed 10^3 per 100 g. Skin and giblets have particularly high levels of contamination. In one study, 12% of raw milk samples from dairy farms in eastern Tennessee were contaminated with *C. jejuni* (53). Raw milk is presumed to be contaminated by bovine feces; however, direct contamination of milk as a consequence of mastitis also occurs (54). Campylobacters are also found in red meat. In one study, *C. jejuni* was present in 5% of raw ground beef and in 40% of veal specimens (55).

Control of *Campylobacter* Infection

On the Farm

Control of *Campylobacter* contamination on the farm may reduce contamination of carcasses, poultry, and red meat products at the retail level (27). Epidemiologic studies indicate that strict hygiene reduces intestinal carriage in food-producing animals (41,42,45). In field studies, poultry flocks that drank chlorinated water had lower intestinal colonization rates than poultry that drank unchlorinated water (42,44). Experimentally, treatment of chicks with commensal bacteria (56) and immunization of older birds (57) reduced *C. jejuni* colonization. Because intestinal colonization with campylobacters readily occurs in poultry flocks, even strict measures may not eliminate intestinal carriage by food-producing animals (39,41).

At Processing

Slaughter and processing provide opportunities for reducing *C. jejuni* counts on food-animal carcasses. Bacterial counts on carcasses can increase during slaughter and processing steps. In one study, up to a 1,000-fold increase in bacterial counts on carcasses was reported during transportation to slaughter (58). In studies of chickens (59) and turkeys (60) at slaughter, bacterial counts increased by approximately 10- to 100-fold during defeathering and reached the highest level after evisceration. However, bacterial counts on carcasses decline during other slaughter and processing steps. In one study, forced-air chilling of swine carcasses caused a 100-fold reduction in carcass contamination (61). In Texas turkey plants, scalding reduced carcass counts to near or below detectable levels (60). Adding sodium chloride or trisodium phosphate to the chiller water in the presence of an electrical current reduced *C. jejuni* contamination of chiller water by 2 $\log_{10}$ units (62). In a slaughter plant in England, use of chlorinated sprays and maintenance of clean working surfaces resulted in a 10- to 100-fold decrease in carcass

contamination (63). In another study, lactic acid spraying of swine carcasses reduced counts by at least 50% to often undetectable levels (64). A radiation dose of 2.5 KGy reduced *C. jejuni* levels on retail poultry by 10 $\log_{10}$ units (65).

Conclusions

C. jejuni, first identified as a human diarrheal pathogen in 1973, is the most frequently diagnosed bacterial cause of human gastroenteritis in the United States. Sequelae including GBS and reactive arthritis are increasingly recognized, adding to the human and economic cost of illness from human campylobacteriosis. The emergence of fluoroquinolone-resistant infections in Europe and the United States, temporally associated with the approval of fluoroquinolone use in veterinary medicine, is also a public health concern. The consumption of undercooked poultry and cross-contamination of other foods with drippings from raw poultry are leading risk factors for human campylobacteriosis. Reinforcing hygienic practices at each link in the food chain—from producer to consumer—is critical in preventing the disease.

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Comparative Genomics and Host Resistance against Infectious Diseases

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The large size and complexity of the human genome have limited the identification and functional characterization of components of the innate immune system that play a critical role in front-line defense against invading microorganisms. However, advances in genome analysis (including the development of comprehensive sets of informative genetic markers, improved physical mapping methods, and novel techniques for transcript identification) have reduced the obstacles to discovery of novel host resistance genes. Study of the genomic organization and content of widely divergent vertebrate species has shown a remarkable degree of evolutionary conservation and enables meaningful cross-species comparison and analysis of newly discovered genes. Application of comparative genomics to host resistance will rapidly expand our understanding of human immune defense by facilitating the translation of knowledge acquired through the study of model organisms. We review the rationale and resources for comparative genomic analysis and describe three examples of host resistance genes successfully identified by this approach.

Two major elements underlie a thorough understanding of the pathogenesis of virtually any infectious disease: identification and characterization of the virulence factors and in vivo survival mechanisms of the invading microorganism (e.g., surface attachment factors, exotoxins, or enzymes that disrupt cellular homeostasis [1]) and understanding of the components of the host response that lead to elimination of the invading pathogen and resolution of disease. (These include both nonspecific [or innate] immune defense mechanisms, such as the complement cascade, and adaptive elements, such as clonally derived lymphocytes capable of eliminating specific targets [2]). The traditional approach to human infectious diseases has been to focus research on the study of important pathogens. The outcome of investigation of relevant bacteria, viruses, fungi, and parasites has led to the production of protective vaccines, antimicrobial agents, and effective strategies for control and elimination of disease outbreaks. A principal advantage of microbiologic research is the relative ease with which the organisms may be obtained, manipulated, and analyzed in the laboratory. Because microbial genomes are smaller, complete cloning and DNA sequencing of several microorganisms have been achieved and have paved the way for comprehensive study of gene expression and genome organization (3,4). In contrast are relatively limited advances in our understanding of the molecular basis of host defense. The study of host immune defense in humans is inherently complex; obstacles to greater understanding include limited opportunities for controlled observation and experimental manipulation, a large genome, and until recently, a lack of molecular techniques capable of facilitating genomewide analysis.

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Genetic Analysis

One of the principal aims of the study of host response to infectious diseases is to uncover novel components of the host immune system critical to robust host defense. Identification of these components at a molecular level is the first step in understanding how the host deals with an infectious challenge and lays a foundation upon which rational therapies that augment host resistance may someday be designed. Despite this promise, the interaction between host and

pathogen that leads to infection is multidimensional, dynamic, and exceedingly complex. From a genomic perspective, a thorough understanding of the pathogenesis of a given infection would include a complete inventory of the spatial and temporal expression of the genes by both the host and pathogen from the time of exposure to the final resolution of the infection. Given the potentially large number of factors that contribute to host defense, precise gene identification is a formidable challenge. Nevertheless, researchers have recently made progress in dissecting and identifying the most important individual genetic elements that govern the host response to important pathogens—largely through the use of animal models of human disease (5). Of the model organisms amenable to genetic analysis, the mouse is by far the most well-developed and physiologically relevant system for study of human host defense (6,7). Identification of commercially available inbred strains of mice that show a differential response to a well-defined infectious challenge is the first requirement for study of genetically regulated host resistance factors. Once distinct phenotypes are identified, controlled breeding is carried out to determine the mode of inheritance of the phenotype (simple or complex). Correlation of the inheritance of susceptibility or resistance to a specific infectious challenge with one or more chromosomal regions is then performed by using linkage analysis. Finally, known genes within the genetic interval must be evaluated and novel genes must be positionally cloned to elucidate the underlying molecular basis of immune defense. Comparative genomic analysis is a logical extension of these principles (8). Knowledge of the genomic organization of human and mouse, for example, facilitates direct localization and identification of the human orthologues of susceptibility genes identified through experimental challenge. These genes can then be tested as candidates for human disease susceptibility through mutation analysis.

Genetic Linkage Maps

Genetic linkage maps provide an organizational framework for genes and phenotypes in the genome (9). Maps, by establishing the location, order, and relative distance of genes, anonymous DNA markers, and biologically important traits along a species' chromosomes, are critical tools in analyzing genetic contribution to a given disease state. Genetic maps can help precisely localize chromosomal region(s) linked to host resistance phenotypes and provide the starting point for identification of the causative gene(s). During the past decade, comprehensive genetic maps spanning the genomes of mouse and human, have been created largely through the initiative of the Human Genome Project (10).

Mapping the Human Genome

A great deal of effort has resulted in the creation of a whole genome human linkage map, consisting of 5,624 microsatellite markers located to 2,335 positions (11). The DNA markers in this map are highly informative and are densely distributed, with an average interval between markers of 1.6 centimorgans (cM) (1 cM = a 1% rate of recombination during meiosis, or approximately 1 million bp). Other comprehensive maps have been assembled on the basis of a collection of more than 16,000 distinct transcribed sequences (including known genes and gene fragments) or expressed sequence tags, which are estimated to represent at least 50% of all genes in the human genome (12). This human transcription map has been integrated with selected microsatellite markers from the Généthon collection, thus allowing the position of gene-based markers to be resolved to specific intervals measured in centimorgans. The map is available electronically (13). Work is also under way to generate comprehensive physical maps of the human genome in which the relative location of markers is defined by the actual length along the chromosome, rather than by recombination events (14,15).

Mapping the Mouse Genome

Among model organisms, genetic mapping is most well established in the mouse, having begun in 1915 with the discovery of the first linkage group (16). Controlled crosses of common laboratory strains segregating a small number of visible phenotypes such as coat color then became the mainstay of genetic mapping. In the past decade, two major breakthroughs have revolutionized the technique of mouse genetic mapping and paved the way for generation of high-resolution whole genome maps. The first was the development of the interspecific cross, involving a laboratory strain (*Mus musculus*) and a distantly related species *Mus spretus* (17),

allowing literally thousands of genes to be mapped within the same cross. The second advance was the development of abundant genetic markers rapidly typable by polymerase chain reaction (PCR) (termed microsatellites), which amplified polymorphisms in simple sequence length repeats such as [CA]n (Figure) (18). Several comprehensive genetic maps of the mouse (based on genes or microsatellites) have been developed, and in some cases, these are being integrated. At least three are publicly available, while the others are available for mapping in a collaborative arrangement (19). As of January 1997, more than 17,000 markers had been mapped in the mouse (one locus approximately every 200kb), including more than 5,000 genes and more than 10,000 (mostly microsatellite) DNA markers.

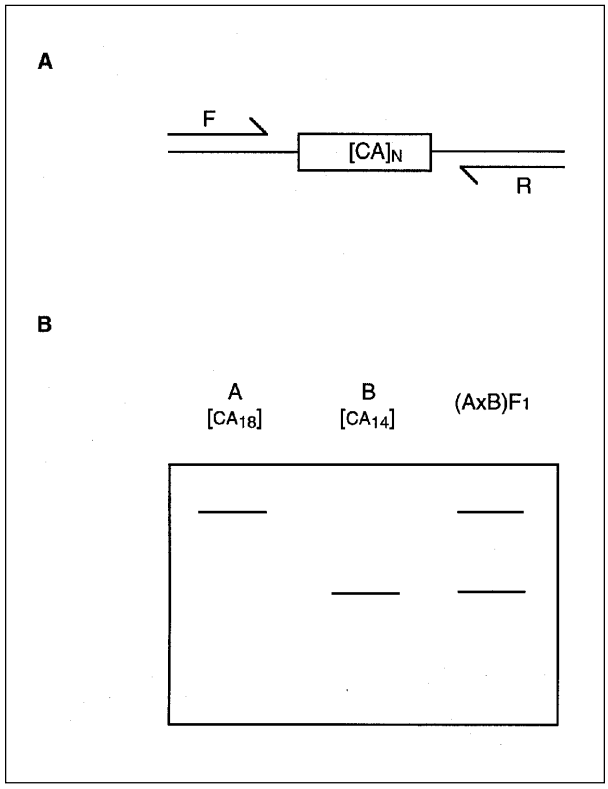

Figure. Schematic representation of microsatellite marker analysis in mice. A) Flanking forward (F) and reverse (R) oligonucleotides are designed to specifically amplify a simple sequence repeat by polymerase chain reaction (PCR) (in this case a CA dinucleotide). The length of the dinucleotide (N) varies among inbred mouse strains. B) Gel electrophoresis of a PCR-amplified microsatellite in homozygous parental strains A and B and heterozygous F_1 progeny. The larger microsatellite from strain A migrates more slowly than that of strain B. Inheritance of both parental alleles is shown in the F1.

Mapping in Other Species

Genetic mapping has been widely embraced by the scientific community; more than 30 vertebrate species are the subject of genetic mapping projects, and high-resolution maps of microsatellite markers have been developed for humans, mice, rats, cows, sheep, pigs, fish, and chickens (19). Two invertebrates, *Drosophila melanogaster* (a dipteran fly) and *Caenorhabditis elegans* (a nematode), also have complete genetic and physical maps; the complete nucleotide sequence of the latter is expected in the near future. The status of individual genetic mapping projects and resources has been summarized, along with a compilation of databases for species-specific or comparative mapping reference (19,20). Integrating the data from these species-specific projects in a form that allows relevant information from diverse organisms to be assembled is a major challenge to biologic information systems. The most extensive coverage of mammalian species homologies is the Mouse Genome Database of The Jackson Laboratory (21). Initially developed for the mouse, comparative mapping data for more than 55 species may be searched online, with links to related genomic resources, such as the Human Genome Database, Ratmap, SheepBase, and PigBase.

Comparative Genetic Mapping

Because of the density of genetic markers positioned along the chromosomes of both organisms, the comparative map of the mouse and human genomes is the most well developed of all species. In a comprehensive summary of mouse/human homology published in 1996, 1,416 loci were placed on both maps by using human physical mapping data and mouse genetic maps (22). This comparison defined 181 conserved linkage groups, approximately 90% of the mouse genome. Further comparative mapping with newly discovered genes and expressed sequence tags will refine the chromosomal relationships between mouse and human.

Integrating Maps and Aligning Genomes

The integration of existing genetic maps of different species is a formidable challenge.

Accurate, comprehensive comparisons of gene arrangements across different species will rapidly advance our understanding of all aspects of biology by allowing rapid information exchange across different model organisms and experimental systems. Several approaches have been used in developing universal mapping probes for diverse genomes (23-25). Of the two classes of loci used to construct gene maps, coding gene sequences (Type I markers), which show conservation among distantly related mammalian species, are most useful as landmarks for comparing linkage and syntenic association. Highly polymorphic sequences (Type II markers), such as microsatellites, are more abundant and are invaluable for mapping within a pedigree but are less useful for comparative purposes because they do not show adequate sequence conservation to recognize locus homology between mammalian orders. In 1993, a list of anchored reference loci for comparative genome mapping in mammals was proposed; it consists of 321 Type I markers equivalently spaced throughout the mammalian genome (26). This approach allowed the position of homologous loci in the maps of four species (human, mouse, cattle, and cat), which represent different mammalian orders, to be established. Interspecies comparison of conserved exon sequences of homologous genes has generated a new overlapping set of anchor loci called comparative anchor tagged sequences (25). Large-scale mapping of these sequences in several species may be an efficient way of developing high-resolution comparative maps with essentially complete genome coverage.

Alternative Techniques for Comparative Genomic Analysis

Mammalian genomes may be compared at several levels by using a variety of tools and strategies tailored to individual objectives. Although direct sequence comparison of whole genomes will provide the highest resolution for comparative study, this sophisticated form of analysis is at least several years away from being realized. At a cytologic level, species may be compared by fluorescence in situ hybridization (FISH) with single or multiple probes (single or multicolor Zoo-FISH), producing rapid, high-resolution chromosomal localization detectable by microscopy. Alternatively, libraries from microdissected or individual flow-sorted chromosomes may be constructed and used as fluorescence-labeled chromosome "paints" to probe the chromosomes of other species and identify homologous regions (27,28). The main advantage of chromosome painting is its rapid overall evaluation of the extent and character of genomic conservation among distantly related species, such as pig and cattle. In contrast to FISH, chromosome painting does not allow determination of gene order or high-resolution demarcation of chromosomal breakpoints. Radiation hybrid panels, another method for physical assignment of homologous loci (29,30), are generated by irradiation and subsequent fusion of a cell line containing a chromosome from one species, such as human, on another background, such as hamster. The donor DNA is fragmented at random, resulting in a series of lines retaining only fragments of the original chromosome. Conserved genes from other species may be mapped to the homologous region of the human genome by comparing the PCR pattern for each cell line to reference loci with well-established map positions.

Models of Human Disease

Identifying genetically regulated host immune responses might significantly advance our understanding of the molecular targets and immunologic mechanisms critical to robust defense against pathogenic microbes. To date the number of host defense genes that have been cloned remains small; comparative genomics has the potential to accelerate gene discovery by allowing available data for model organisms to be rapidly applied to the study of human disease. We summarize three examples of human host resistance genes in the following section; in each example, genetic analysis of mouse models of the human disease phenotype played a crucial role in the initial discovery of the human homologue or served as a means of validating the identity of the proposed human candidate disease gene.

Nramp 1 and NRAMP1

The Mouse *Nramp1* Gene

In classic inbred strains of mice, natural resistance to infection with *Mycobacterium bovis* (BCG), *M. lepraemurium*, *Salmonella* Typhimurium, and *Leishmania donovani* is controlled by the *Bcg* locus, also known as *Ity* and *Lsh* (31-33). The major effect of the *Bcg* gene is to

modulate the growth rate of these diverse pathogens in cells of the reticuloendothelial system of the mouse during the preimmune phase of the infection (33). Resistant and susceptible strains are distinguished by the kinetics of infection shown by pathogen counts (CFUs or Leishmania-forming units) in liver and spleen after infection. The susceptible phenotype is characterized by a higher net growth rate of BCG, Salmonella, or Leishmania in the reticuloendothelial system during the early phase of infection, followed by specific immune responses in BCG- and *L. donovani*–infected mice or by a rapidly lethal infection with the virulent pathogen *S.* Typhimurium. *Bcg* is inherited as a simple autosomal dominant Mendelian trait in crosses between classical strains of laboratory mice; it was localized to mouse chromosome 1 by linkage analysis (34). Using a positional cloning strategy, Vidal et al. (35) isolated the *Nramp1* (natural resistance-associated macrophage protein 1) gene as a strong candidate for the *Bcg* mutation based on its map location, its macrophage-restricted expression pattern and a nonconservative $Gly^{169}Asp$ substitution in the protein of all susceptible strains. Creation of a null allele at *Nramp1* then provided formal proof that a mutation within *Nramp1* is the cause of the mouse susceptibility to infection with *M. bovis*, *S.* Typhimurium, and *L. donovani* (36).

Nramp1, an integral membrane phosphoglycoprotein located in the late endosome/lysosome compartment of resting macrophages, is recruited to the maturing phagosomal membrane (37), consistent with its potential function in controlling the replication of intracellular parasites by altering the intravacuolar environment in which they reside. Nramp1 is part of an ancient family of proteins with highly conserved members in mammals (including humans, cows, rats, sheep), birds, invertebrates (*C. elegans*, *D. melanogaster*), plants *(Oryza sativa*, *Arabidopsis thaliana*), fungi (*Saccharomyces cerevisiae*), and even bacteria (*M. leprae* and *Escherichia coli*) (38,39). This family is characterized by a highly conserved hydrophobic core consisting of 10 transmembrane (TM) domains with a structural organization typical of families of ion transporters and channels. In addition, the most highly conserved segments of the Nramp family (TM8-TM9 intracellular loop) show impressive similarity with the highly conserved region of mammalian voltage-gated K^+ channels of the *shaker* type (40).

Several issues concerning the biochemical function of Nramp1 with respect to intracellular survival of taxonomically unrelated pathogens remain unresolved. Studies of the function of *Nramp1*-related sequences (*Nramp2* and *Smf1* in model organisms) provide insight into how Nramp1 confers resistance to microbial agents. *Nramp2* has been isolated in mouse and human and shows a high degree of similarity to *Nramp1* (77% overall similarity), with identical hydropathy profiles and predicted secondary structures (41,42). Mouse and human *Nramp2* mRNA are both widely expressed in contrast with the tissue-specific expression of *Nramp1* (41, 42). Recently, Nramp2 was shown to be a metal ion transporter with broad divalent cation specificity (including Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, and Pb^{2+}), driven by the proton electrochemical gradient in *Xenopus laevis* oocytes (43). Studies using the yeast double mutant *SMF1/SMF2* provided additional support concerning the function of Nramp2 as a divalent cation transporter. Inactivation of *SMF1* and *SMF2,* two yeast *Nramp* homologues encoding divalent cation transporters (44), is specifically complemented by Nramp2 (45). In vivo, Nramp2 plays an important role in normal iron transport. Mutation within Nramp2 causes microcytic anemia in *mk* mutant mice because of severe defects in intestinal iron uptake (46). Interestingly, the missense mutations in mutant *Nramp1* and *Nramp2* alleles introduce a charged amino acid in two adjacent positions of TM4, confirming the importance of this region of both proteins for normal function. It has been suggested that Nramp1 may also be a divalent cation transporter; its role in reticuloendothelial cells remains unexplored (40,44).

The Chicken *NRAMP1* Gene

The discovery of *Nramp1* allowed the study of its role in susceptibility to related infections in other species. Salmonellosis, one of the most common causes of food poisoning in humans, is frequently caused by ingestion of contaminated poultry products; efforts to identify salmonella resistance genes in poultry could lead to more efficient poultry control strategies, thereby reducing secondary human morbidity. Genetic regulation of chicken host resistance exists, as

inbred poultry lines differ in their susceptibility to infection with several strains of *Salmonella.* Segregation analysis with a combination of *Salmonella*-resistant and *Salmonella*-susceptible lines has shown that resistance to infection is fully dominant and is not sex-linked or associated with the major histocompatibility complex (47). The candidacy of the chicken *Nramp1* homologue was tested in the differential resistance of inbred chicken lines to infection with *S.* Typhimurium by using sequencing and linkage analyses (48). Through the use of a mouse cDNA, the chicken homologue *Nramp1* has been cloned and shown to share 68% identity with the mouse gene (49). As demonstrated in mice, the macrophage is a major site of NRAMP1 mRNA expression in chickens (49). *NRAMP1* mRNA transcripts from *S.* Typhimurium–resistant or –susceptible chickens were analyzed to identify amino acid sequence variants that could be associated with the disease phenotype. Eleven sequence variants in *Nramp1* mRNA were obtained from three *Salmonella*-resistant and three *Salmonella*-susceptible chicken lines; almost all (10) resulted in silent mutations or conservative changes (to amino acids with similar physical properties) that were detected both in resistant and susceptible chicken lines, while only one sequence variant resulted in a non-conservative substitution of a positively charged residue (Arg^{223} by a polar residue (Gln^{223}). This allelic variant was specific to the susceptible line C and was clearly associated with survival to infection (a resistance allele at *NRAMP1* improved survival rate from 13% to 27%) (48). Taken together, these data strongly suggest a direct role of NRAMP1 in susceptibility to infection in chickens.

The Human *NRAMP1* Gene

Work in inbred strains of mice has established unambiguously that *Nramp1* has an important role in determining resistance to mycobacterial infections and has encouraged several research groups to test the association of *NRAMP1* with corresponding human infections. Host genetic factors play a major role in determining the outcome of mycobacterial infections in humans, as shown by racial variation in susceptibility to infection and higher concordance of tuberculosis and leprosy among monozygotic twins compared with dizygotic twins and siblings (50,51). Segregation analysis in a population from Desirade Island (French West Indies) has demonstrated that susceptibility to leprosy (regardless of the clinically defined subtype) is controlled by a major gene not linked to the major histocompatibility complex (52). Through use of a candidate gene approach, population association studies, and linkage analysis, several genes (HLA-linked genes, tumor necrosis factor, collectin, vitamin D receptor, interferon gamma receptor) have each been associated with susceptibility to mycobacterial infections (53,54).

The chromosomal region surrounding *Nramp1* on mouse chromosome 1 has been conserved on the telomeric end of human chromosome 2q35 and contains the human *NRAMP1* orthologue (55). Sequence comparison of the mouse and human Nramp1/NRAMP1 proteins showed a high degree of conservation between the two species (85% identity, 92% similarity); the most conserved region was the intracellular loop containing the consensus sequence transport motif (56). In humans, the highest sites of *NRAMP1* expression are peripheral blood leukocytes and lungs (56). The high degree of sequence homology between mouse and human *NRAMP1*, the presence of similar regulatory elements within the promoter regions of the genes, and similar tissue expression patterns support the notion that the NRAMP1 protein exerts similar roles in vivo in both mouse and humans.

A number of polymorphic variants have been used to study the association of NRAMP1 and susceptibility to leprosy and tuberculosis (57-60). One study based on the segregation analysis of certain *NRAMP1* haplotypes in 20 multiplex families involving 168 individuals from South Vietnam clearly showed that *NRAMP1* was involved in predisposition to leprosy (61). Another large study measuring the association of *NRAMP1* with clinical tuberculosis in a population of Gambia (West Africa) demonstrated that polymorphic variations within the human *NRAMP1* gene affect susceptibility to the disease (62). Nevertheless, susceptibility to either leprosy or tuberculosis appears to be genetically heterogeneous since the role of *NRAMP1* was observed only in certain ethnic groups (63,64).

Identification of *Nramp1* illustrates the value of comparative genomics for identification and characterization of the biologic basis for

differences between susceptible and resistant hosts. Genetic dissection of the mouse model of *M. bovis* infection was crucial to the identification of similar mechanisms governing the human response to medically important pathogens such as tuberculosis and leprosy. Comparative genomics was also important in accelerating the identification of an important host resistance gene for salmonellosis in the chicken (a species of significant agricultural importance), where the available genetic tools are modest, relative to mice and humans.

Chediak-Higashi Syndrome (CHS)

CHS is a rare autosomal recessive disorder characterized by partial ocular and cutaneous albinism, a mild bleeding diathesis, and peripheral sensorimotor neuropathy. The most serious phenotype among CHS patients, however, is a marked increased in susceptibility to bacterial infection that may lead to death during the first 2 decades of life. These clinical features are attributable to dysfunctional granule-containing cells including melanocytes, platelets, Schwann cells, neurons, and granulocytes (65,66). On the basis of phenotypic similarity, the *beige* (*bg*) mutation in mice has long been regarded as a model for CHS (67). Several components of the immune system are affected in *Beige*/CHS. Neutrophils exhibit defective chemotaxis and reduced intracellular killing for up to 90 minutes after bacterial phagocytosis, and their granules lack the serine proteases cathepsin G and elastase because of a failure of normal protein sorting (68,69). Natural killer cell activity is defective, causing impaired cytolysis of tumors and virally infected cells; cytotoxic T-cell responses against allogeneic tumor cells are also abnormal (70,71). Mice with the *bg* mutation have increased susceptibility to a variety of pathogens, including cytomegalovirus, *Leishmania donovani*, *Candida albicans*, and a variety of pathogenic bacteria (*E. coli, Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus pneumoniae*) (72-74).

To identify the genetic basis of this host resistance defect, the *bg* gene was localized to a 0.24 cM interval of proximal mouse chromosome 13 by genetic mapping of three mouse backcrosses segregating this phenotype (75). A DNA contig of this region spanning 2,400 kb was constructed from large-capacity yeast artificial chromosomes and P1 bacteriophage clones (76). Using yeast artificial chromosome complementation and direct cDNA selection, two groups subsequently identified portions of a candidate gene for *bg*, named *Lyst* (lysosomal trafficking regulator) (77,78). *Lyst*, ubiquitously expressed in the mouse, has a maximum transcript size of approximately 12kb and possible complex alternative splicing. Several mutations predicted to severely truncate the Lyst polypeptide were identified within each transcript. Through the use of partial sequence data for mouse *Lyst*, 27 cDNAs corresponding to the human gene were identified and assembled into a complete human gene sequence of 13,499 bp, with an open reading frame of 11,403 bp (79). Comparison of the partial 3' mouse cDNA to the human sequence demonstrated 77.2% nucleotide identity and 87.9% amino acid identity, indicating that human and mouse genes are highly homologous, and sequence analysis of three CHS patients identified pathologic mutations in all.

Comparative genetic mapping between the region of mouse chromosome 13 with the *bg* mutation and the human genome indicates homology with distal chromosome 1q. Consistent with this alignment, genetic mapping of the human CHS locus in affected families localized it to 1q42-1q44 as part of a conserved linkage group shared with mouse chromosome 13 (80,81). Radiation hybrid mapping also assigned the human CHS candidate gene to 1q43, confirming that the *bg* phenotype in mouse and human CHS are both caused by mutations in orthologous genes (79). Database searches with the complete nucleotide sequence of the CHS gene showed significant homology to open reading frames from *S. cerevisiae* and *C. elegans*, as well as a human cell division control protein-4 (CDC4L) (82). The modular architecture of the CHS protein is similar to Vps15, a yeast serine/threonine kinase protein kinase thought to be part of a membrane-associated signal transduction complex regulating intracellular protein trafficking (83). To date, the function of the CHS gene remains unknown, although it may be similar to Vps15 and may be part of a novel gene family.

X-Linked Agammaglobulinemia (XLA)

XLA, one of the first primary immunodeficiency disorders described in humans, is the prototypic example of the protective role of humoral immunity against common bacterial pathogens (84). XLA is characterized by a

profound deficiency of B-lymphocyte development at two sequential stages of maturation within the bone marrow (85). This defect results in marked reductions in the serum levels of all three major classes of immunoglobulins and a profound decrease in the number of B lymphocytes in the peripheral blood as well as in the lymphoid follicles and germinal centers of lymph nodes. The clinical manifestations generally begin by the end of the first year of life, once the level of maternally derived antibodies has declined. Bacterial infections with organisms such as *S. pneumoniae, Haemophilus influenzae, S. aureus,* and *Pseudomonas* species are most common, with the respiratory tract being most frequently affected. Gastrointestinal infection with *Salmonella* or *Campylobacter* have also been reported, as have urogenital infections with *Mycoplasma* or *Chlamydia*. XLA patients have defective host resistance to enteroviruses, since neutralizing antibody is important in controlling these pathogens during their passage through the blood stream. Resistance to other infections for which intact T lymphocyte function is required (e.g., tuberculosis or histoplasmosis) remains intact.

Recognition of the familial occurence of this rare disorder and pedigree analysis demonstrated an X-linked recessive inheritance pattern of the trait (86). Carrier females could not be detected because they are phenotypically normal, with normal serum levels of immunoglobulin. Linkage studies of over 500 individuals from 60 families mapped the gene for XLA to the midportion (Xq22) of the X chromosome, cosegregating with the polymorphic genetic marker DXS178 (87,88). By using complementary strategies of positional cloning and low-stringency cDNA library screening, two groups identified a novel *src*-like cytoplasmic tyrosine kinase, named *Btk* (Bruton agammaglobulinemia tyrosine kinase) as a strong candidate gene for XLA (89, 90). *Btk* was mapped to the XLA locus by FISH and somatic cell hybrid analysis and was expressed in cell lines representing all stages of B cell development, myelomonocytic cell lines, and a macrophage cell line; it was not detectable in T lineage cell lines (90). In transformed B-cell lines from individuals affected with XLA, the expression level of *Btk* mRNA and protein, and consequently its kinase activity, was reduced or absent. Southern blot analysis of DNA from pedigrees with XLA cases showed restriction fragment length alterations that segregated in an X-linked recessive pattern; detailed analysis disclosed either genomic DNA deletions in the region encompassing *Btk* or missense point mutations resulting in nonconservative amino acid substitutions at important residues in the putative protein-tyrosine kinase domain (89). These findings provide strong evidence that the failure of normal B-cell growth and differentiation in XLA is caused by abnormal function of an intracellular protein tyrosine kinase.

The CBA/N inbred mouse strain's X-linked immunodeficiency (*xid*) has been regarded as an experimental model for human XLA since it was first described in 1972 (91). B lymphocytes from these mice exhibit pleiotropic defects in development and function. Normal numbers of pro-B, pre-B, and surface immunoglobulin-positive B cells exist in the bone marrow, while peripheral B-cell numbers are significantly reduced (30% of normal). The B lymphocytes that are present have an abnormal surface marker phenotype, and B-cell proliferation triggered through the surface immunoglobulin M (IgM) receptor or surface immunoglobulin cross-linking is impaired, as are responses to a number of other mitogenic stimuli including lipopolysaccharide, interleukins IL-5 and IL-10, CD38 receptors, and CD40 ligands. Consistent with these defects, CBA/N mice have reduced serum IgM and IgG3 antibody levels and cannot make antibody responses when challenged with type-2 thymus-independent antigens (e.g., polysaccharides and hapten-polysaccharide conjugates). As with human XLA, impaired humoral immunity can result in increased susceptibility to bacterial pathogens, including *S.* Typhimurium (92). Inheritance of the susceptibility trait was linked to the *xid* locus by using back-cross and F2 progeny derived from crosses of CBA/N and DBA/2N parental strains.

To determine whether XLA and *xid* were caused by mutations in homologous genes, two groups performed genetic mapping of *xid* and *Btk*. The *Btk* gene was closely linked to the *xid* locus in the distal region of the mouse X chromosome by using an interspecific back-cross mapping panel (93), and precise co-localization of *Btk* and *xid* was observed in 1,114 segregating back-cross progeny (94). Normal and mutant mouse strains did not differ in *Btk* expression or in vitro kinase activity. Sequence

analysis of the mouse *Btk* transcript in CBA/N and several immunocompetent mouse strains (including the CBA/CaHN progenitor) demonstrated a point mutation within the first coding exon that is predicted to convert a highly conserved arginine residue to cysteine. This amino acid substitution occurs within the pleckstrin homology domain in the amino-terminal region of the protein and is presumed to alter normal B-cell signaling by disrupting protein-protein interactions. To unequivocally confirm that mutations in *Btk* were responsible for the *xid* phenotype, targeted gene disruption (a gene knockout experiment) was performed in embryonic stem cells (95,96). Complete elimination of Btk protein production identically reproduced the *xid* phenotype, indicating that the naturally occurring point mutation produces a complete loss-of-function phenotype or results in a protein with dominant negative properties (presence of a single mutant allele is sufficient to block normal gene function). The severe early B-lymphocyte developmental arrest of human XLA was not observed, which suggests that *Btk* function in mice is accompanied by a compensatory mechanism operating during early B-cell development to rescue B-cell maturation.

On the basis of comparative mapping and sequence analysis, human XLA and the mouse *xid* phenotype are clearly homologous disorders caused by mutations in orthologous genes. Nevertheless, although the underlying genetic alteration in both species was successfully identified, a number of issues remain unresolved. First, the phenotypes observed in these two disorders are not identical; the more severe block of early lymphocyte development in XLA results in a greater deficiency of peripheral B cells relative to the CBA/N mouse strain, suggesting that the requirement for *Btk* in early murine B-cell development is less stringent than that for humans. Second, the range of pathogens to which humans are are highly susceptible appears more diverse than the range for mice. Finally, the exact role of *Btk* in normal B-cell physiology remains to be demonstrated. Thus far, identification of BTK has led to carrier detection and prenatal counselling; additional characterization of a mouse model with great similarity to the human condition could advance our understanding of the fundamental processes underlying B-lymphocyte development and function.

Conclusions

Complete understanding of infectious disease pathogenesis requires identification and characterization of host genes that regulate the response to virulent microorganisms. Through evolutionary selection, a series of innate immune defense mechanisms have evolved to protect the host against the constant threat of microbial injury and direct the development of specific adaptive immune responses. Genetic analysis of naturally occurring variation in the host response among model organisms has successfully identified novel genes such as *Nramp1*, *Lyst*, and *Btk*, thus providing new insights into the molecular nature of host resistance. Rapid advances are now being made in the creation and integration of dense genetic maps of model organisms and humans. Comparative genomics will play an increasingly important role in facilitating the transfer of new knowledge from experimental models to a more complete understanding of human host resistance.

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Cyclospora: An Enigma Worth Unraveling

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In part, *Cyclospora cayetanensis* owes its recognition as an emerging pathogen to the increased use of staining methods for detecting enteric parasites such as *Cryptosporidium*. First reported in patients in New Guinea in 1977 but thought to be a coccidian parasite of the genus *Isospora*, *C. cayetanensis* received little attention until it was again described in 1985 in New York and Peru. In the early 1990s, human infection associated with waterborne transmission of *C. cayetanensis* was suspected; foodborne transmission was likewise suggested in early studies. The parasite was associated with several disease outbreaks in the United States during 1996 and 1997. This article reviews current knowledge about *C. cayetanensis* (including its association with waterborne and foodborne transmission), unresolved issues, and research needs.

Cyclospora Overview

Cyclospora cayetanensis is a protozoan parasite (subphylum Apicomplexa, subclass Coccidiasina, order Eucoccidiorida, family Eimeriidae). The organism's link to the Eimeriidae extends to the genus level through use of molecular phylogenetic analysis techniques (1). Collected data link infection to a single host—humans. In 1993, asexual meronts were described from jejunal enterocytes of humans (2). In 1997, two types of meronts and sexual stages were observed in jejunal enterocytes of biopsy specimens from infected patients excreting oocysts, confirming that the entire life cycle could be completed within a single host (3); infected persons excrete unsporulated oocysts. In the laboratory, oocysts are induced to sporulate in potassium dichromate in a petri dish at ambient temperatures (25°C to 30°C). After 1 week and up to 2 weeks, approximately 40% of oocysts contain two sporocysts with two sporozoites in each (4). Excystation of sporulated oocysts occurs in vitro when oocysts are subjected to bile salts and sodium taurocholate and mechanical pressure from a glass tube mortar and pestle (5). These findings suggest that direct person-to-person transmission is unlikely.

Oocysts measure 8 μm to 10 μm in diameter and stain variably acid-fast. Without the use of an ocular micrometer, oocysts of *Cyclospora* might be easily confused with those of *Cryptosporidium* or other fecal artifacts that stain acid-fast positive, as was the case in a pseudo-outbreak of cyclosporiasis reported in Florida (6). *Cyclospora* oocysts are easily observed by phase contrast microscopy, and the algal-like morula appearance is evident in fresh stool specimens. A useful and distinguishing feature is oocyst autofluorescence, which appears blue by Epi-illumination and a 365-nm dichroic exciter filter and green by a 450-nm to 490-nm dichroic filter.

Susceptible humans are infected by ingesting sporulated oocysts. While unknown, the infectious dose is presumed to be low. Symptoms of infection may include watery diarrhea, mild to severe nausea, anorexia, abdominal cramping, fatigue, and weight loss. Diarrhea can be intermittent and protracted (3,7,8). Persons with no previous immunity as well as very young children in developing countries are likely to exhibit symptoms. Limited data suggest that in disease-endemic countries, frequent exposure may predispose to asymptomatic infection in children and absence of infection in adults (9). Symptomatic infections can be treated with trimethoprim-sulfamethoxazole (Bactrim) (9-11).

Cyclospora infections have been confirmed in North, Central, and South America, the Caribbean, England, eastern Europe, Africa, the Indian subcontinent, Southeast Asia, and Australia (12). In the United States, England, and Australia, most cases were first observed in

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travelers returning from the areas listed above (7,13,14). As more indigenous cases are reported from all areas, however, a cosmopolitan distribution of *Cyclospora* appears possible. A seasonal distribution of infection, coinciding with wet or warm months of the year, has also been suggested (15).

Association with Waterborne Transmission

While the organism causing *Cyclospora* infection was still being identified, an outbreak occurred in the staff of a Chicago hospital in 1990 (16). Infection was confirmed in 11 of 21 persons exhibiting diarrheal symptoms and lasted up to 9 weeks with alternating cycles of disease and remission. Epidemiologically, infections were associated with drinking tap water (in a resident's dormitory) possibly contaminated with stagnant water from a rooftop storage reservoir. In an isolated incident (also in Chicago), an 8-year-old child became ill and passed oocysts in the feces 1 week after swimming in Lake Michigan (7). In another isolated incident, a man from Utah became ill with severe watery diarrhea and passed oocysts after cleaning his basement, which had been flooded by sewage backup following heavy rains (8). The man's house was located near a dairy farm and much of the sewage backup was attributed to water runoff from this site. In yet another isolated incident in the United States, consumption of well water was implicated in the infection of one of three patients in Massachusetts (17).

Two outbreaks of *Cyclospora* infection in Nepal have also been linked to waterborne transmission (18,19). In the first outbreak in 1992, expatriates, who were more likely to drink untreated water or milk reconstituted with water, became ill with diarrhea and passed oocysts. The infections occurred during the summer, which coincided with annual epidemics among the expatriates. The second waterborne disease outbreak occurred in 12 of 14 British soldiers, despite chlorination of the water involved. In this outbreak, *Cyclospora* oocysts were demonstrated for the first time in drinking water, which consisted of a mixture of river and municipal water.

Though not directly connected with water-associated disease outbreaks, *Cyclospora* oocysts have been isolated from wastewater in sewage lagoons adjacent to an area of endemic disease in Lima, Peru (20); their presence was confirmed by microscopy and PCR. Water from these sewage lagoons is used to irrigate pasture land, corn fields, and trees. In other parts of Lima, water from such lagoons is used to irrigate vegetable crops.

Association with Foodborne Transmission

While the Nepal study conducted in 1992 strongly suggested waterborne transmission of *Cyclospora*, only 28% of infected patients reported drinking untreated water or milk possibly contaminated with untreated water (18). Therefore, other modes of transmission were likely, although none was identified. Foodborne transmission was suspected when consumption of raw or undercooked meat and poultry products was reported as part of case histories before the infectious organism was identified as *Cyclospora* (21,22). Foodborne transmission was first suggested in 1995 when the illness of an airline pilot was associated with food prepared in a Haitian kitchen and brought on board the airplane (23). *Cyclospora* is endemic in Haiti; this study underscored that this type of illness could be acquired from meals brought on board without visiting the country in which infection originated.

Foodborne transmission of *Cyclospora* in the United States, first reported in 1995, was widely reported in 1996 and 1997 (24-28). Some reports early in 1996 implicated strawberries, but as more epidemiologic information was gathered, attention shifted to raspberries. In 1996, a total of 1,465 cases of cyclosporiasis were reported from 20 states (predominantly east of the Rocky Mountains), the District of Columbia, and two Canadian provinces (24). Almost half (725 cases) were event associated; the remaining (740 cases) were sporadic (i.e., not epidemiologically linked to other cases); 978 (67%) cases were laboratory confirmed; 55 clusters of cases were associated with social events. A total of 3,035 persons attended these events; 1,339 (44.1%) were interviewed, and of these 735 (54.1%) were designated case-patients. *Cyclospora* infection was laboratory confirmed in 238 (32.8%) cases. Raspberries were definitely served at 50 events and possibly at four more. Even in the documented 740 sporadic cases in 1996, many patients recalled eating some type of berries. Of the 54 cluster events at which raspberries were or may have been served, well-documented

traceback data as to the source were uncovered for 29; of these, 21 were definitely traceable to raspberries imported from Guatemala, and an additional eight may have originated there. Twenty-five (86%) of the 29 well-documented events were traceable to one (versus more than one) exporter per event. Further tracings showed that as few as five Guatemalan farms could have accounted for the 25 events traceable to a single exporter per event. In part because of previous links with waterborne transmission, it was postulated that the berries were contaminated when sprayed with insecticides or fungicides mixed with water containing sporulated oocysts.

As of August 1997, 1,450 cases of cyclosporiasis (550 laboratory confirmed) were reported (28). Many cases were cluster-associated and involved raspberries linked to Guatemala. In addition, 25 confirmed and 20 possible clusters of cases of cyclosporiasis were associated with consumption of food that contained fresh basil. An additional two clusters of cases in Florida were linked with eating mesclun lettuce (28). In each situation, the outbreaks were linked to non-Guatemalan fresh produce.

Cyclospora oocysts have been isolated from vegetables from a disease-endemic area of Lima, Peru, and from Nepal (29,30). Although the number of oocysts recovered was small, encountered in only a few samples, and not associated with any known disease outbreak, the implication was clear: foodborne transmission by this route could occur. In addition, oocysts experimentally seeded on vegetables could not easily be removed by washing (30). Washing of vegetables, even though highly recommended as a means of reducing risk for infection, may therefore not totally eliminate the risk.

Unresolved Issues

Unresolved issues concerning *Cyclospora* fall into three broad categories: environmental survival, transmission to humans, and epidemiology. The boundaries of these categories frequently overlap.

Environmental Survival

The biggest issues of concern in this category are oocyst distribution in the environment, oocyst survival under changing conditions, and oocyst sporulation times under changing environmental conditions. All these factors affect transmission.

Because of technologic limitations, *Cyclospora* oocysts have only been recovered in very limited numbers from water sources and vegetables (19,20,29,30). A heavy reliance has been placed on techniques used for isolating *Cryptosporidium*, which are inadequate (31). Very little is known about conditions that may favor the survival of *Cyclospora*. Preliminary studies have shown that oocysts subjected to -20°C for 24 hours and exposure to 60°C for 1 hour cannot be induced to sporulate. Oocyst storage at 4°C or 37°C for 14 days retards sporulation (32). The most intriguing environmental issue is oocyst sporulation time. The report that confirmed the identity of *Cyclospora* indicates that the organism requires 1 to 2 weeks to completely sporulate and become infectious under ambient conditions of 25°C to 30°C (5). Oocysts maintained at 4°C can sporulate within 6 months (4). These periods are longer than those reported for most coccidia; therefore, direct person-to-person transmission is unlikely. Also, (if confirmed under changing conditions) a prolonged sporulation time would imply that oocysts favor a moist environment, ideally water. Early in the Guatemalan berry investigations, water used to irrigate plants was thought to play a role in contaminating raspberries with oocysts. This notion, which would likely apply only to berries grown with spray irrigation, however, has largely been discarded since direct contact exposure to excessive moisture promotes rapid fruit deterioration and most raspberries grown in Guatemala rely on drip irrigation. The exact method of contamination is not known, and even though use of insecticides and fungicides made with oocyst-contaminated water has been hypothesized, its role has yet to be confirmed. If this hypothesis is true, how these agents might affect oocyst viability is also not known. Another unresolved issue is how the water might have become contaminated.

Transmission to Humans

The primary issues concerning transmission of *Cyclospora* to humans are infectious dose and species specificity. For most coccidia that infect humans and animals (e.g., *Cryptosporidium* [33]), the infectious dose is presumed to be low (34). What we know about the waterborne transmission of *Cryptosporidium* and how few of

its oocysts are usually isolated from water is likely true for *Cyclospora* (35). However, only two foodborne outbreaks of cryptosporidiosis have been reported (one involved fresh pressed cider and the other chicken salad) (36,37). *Cryptosporidium* is immediately infectious upon passage from an infected person, and oocysts are usually passed in large numbers if the person is symptomatic. Unlike what has been reported for *Cyclospora* to date, *Cryptosporidium* oocysts are ubiquitous in the environment and could easily contaminate foods, especially vegetables. In one study, *Cryptosporidium* oocysts were recovered more frequently from vegetables than *Cyclospora* oocysts (30). In addition, *Cryptosporidium* infectious to humans has many known animal hosts (38).

The issue of potential animal hosts for *Cyclospora* has not been resolved. *Cyclospora*-like organisms have been recovered from ducks, chickens, dogs, and primates (39-41). Only in primates has there been any concrete evidence identifying the agent as a species of the genus *Cyclospora*, and whether it is the same as *C. cayetanensis* is not known (41). For the other animal species mentioned, recovered oocysts, if they were oocysts of *Cyclospora*, may have been passing through these hosts. Attempts at finding animal hosts infected with *Cyclospora*-like organisms in human disease-endemic areas have largely failed, as have preliminary attempts at infecting conventionally used laboratory animals. Some researchers have convincingly shown on the basis of molecular data that *Cyclospora* and *Eimeria* are closely related (1): others have even suggested that *Cyclospora* should be considered a mammalian *Eimeria* species (42). To clarify the taxonomic issue, small subunit rRNA sequences from *Isospora* should be compared with those of *C. cayetanensis* and with *Cyclospora* isolates from nonhuman primates. In addition, conventional and molecular taxonomists should name the species on the basis of combined phenotypic and genotypic characteristics.

Epidemiology

Even though epidemiologic investigations of *Cyclospora* have been thorough and convincing, they raise environmental and transmission issues that require further investigation. The two areas we will consider are the relative geographic restriction of cases and attendant traceback issues associated with clusters of cyclosporiasis cases and potential indigenous infections within the United States and elsewhere.

Unraveling the first issue involves tracing imported fruits or vegetables in a forward direction (possible distribution sites) as well as tracing them back (to their originating sites). In the raspberry-associated outbreaks of 1996, good traceback data were obtainable for 29 of 55 clusters. All sites (except one) were east of the Rocky Mountains. For the 25 events traceable to one (versus more than one) exporter per event, 33 (85%) of 36 shipments entered through Miami, Florida (24). If berries were also being distributed in large quantities to other, largely western regions of the country during this period, would we not expect more infections in western regions? This point, along with the attendant epidemiologic investigations, helped dissociate strawberries from reported *Cyclospora* infections. California strawberry growers were as likely or more likely to ship strawberries within their own region of the United States as they were to ship them elsewhere, yet most infections occurred in eastern regions of the country. In addition, Guatemalan raspberries are imported into the United States in large quantities twice a year, yet no outbreaks occurred during the winter months when this importation occurs, which indicates that the epidemiology of this infection in countries such as Guatemala where the berries are grown needs further study.

The issue of indigenous U.S. infections should be investigated. Waterborne and sporadic cases have occurred in which no association could be made to raspberry consumption (7,8,16-19,24,29). Preliminary data (in one region of the United States) have linked *Cyclospora* infection to gardening and working with soil (43).

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Using Monoclonal Antibodies to Prevent Mucosal Transmission of Epidemic Infectious Diseases

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Passive immunization with antibodies has been shown to prevent a wide variety of diseases. Recent advances in monoclonal antibody technology are enabling the development of new methods for passive immunization of mucosal surfaces. Human monoclonal antibodies, produced rapidly, inexpensively, and in large quantities, may help prevent respiratory, diarrheal, and sexually transmitted diseases on a public health scale.

In 1975, Köhler and Milstein noted that monoclonal antibodies (MAbs) "...could be valuable for medical and industrial use" (1). Since then, the use of MAbs has become routine in the research and diagnostic laboratory, but antibodies have yet to be used to their maximum potential in medical and public health applications. Two recent reviews of the therapeutic use of antibodies suggest that systemically administered antibodies may play an important role in treating infections by drug-resistant pathogens as well as pathogens for which no antimicrobial drugs are available (2,3). However, the greatest potential for MAbs probably lies in prevention since antibodies are in general more effective for prophylaxis than for therapy (3,4). From a public health perspective, prevention is especially important (5). In particular, direct application of MAbs to mucosal surfaces blocks the entry of pathogens into the body.

We review here the evidence of antibody efficacy in preventing disease and recent advances that have facilitated the development of MAbs for mucosal applications in humans. Finally, we consider the public health potential of topical delivery of MAbs for preventing mucosal transmission of infections.

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Immunologic Strategies for Preventing Mucosal Transmission

Vaccines that stimulate systemic immunity can prevent systemic disease, but generally fail to prevent mucosal disease. Vaccines that stimulate active mucosal immunity have demonstrated good efficacy in animal models, but with few exceptions (polio and influenza vaccines), have not been as effective as they could be in humans. Some of the discrepancies between study results in animals and humans are probably due to a failure of studies in animals to model immune evasion strategies of pathogens (6) that occur in humans. These strategies include rapid evolution of variable strains (7), pathogens that coat themselves with host antigens (8), and pathogens that are transmitted to a new host by hiding inside cells shed by the infected host (cell vectors) (9). Furthermore, most vaccines successful in stimulating mucosal immunity in animals contain irritating adjuvants or attenuated pathogens, which are generally considered unacceptable for use in humans; vaccines with human-safe adjuvants have not generated high concentrations of protective antibody in the mucosa. Current research is investigating improved immunogens, delivery vehicles, and adjuvants, as well as exploring the best inductive sites for generating a protective mucosal immune response at a specific mucosal surface (10).

In contrast to vaccines, passive immunizations can deliver protective levels of antibodies immediately and directly to the susceptible

mucosal surface (Figure 1-top). Also, with passive mucosal immunization, it may be possible to defeat some key immune evasion strategies by using antibodies directed against host cell vectors, host antigens that coat the pathogen, or receptors used by pathogens to enter target cells (11). In addition, new methods for the sustained release of antibodies offer the possibility of long-term protection (12).

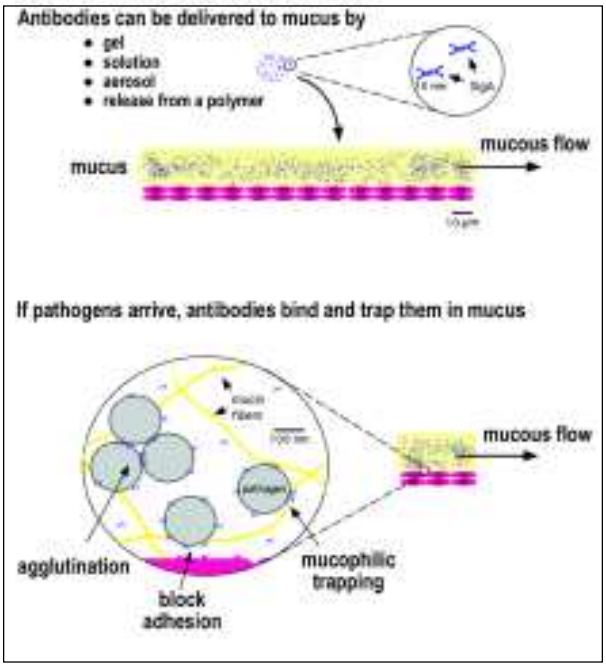

Figure 1. Topical delivery of pathogen-specific MAbs can protect the mucosal epithelium. (Top) Protective MAbs (in this figure, secretory immunoglobulin A; SIgA) can be topically applied to the mucosa in various ways. (Bottom) In mucus, MAbs are believed to act by a number of mechanisms to prevent penetration of the mucous layer and subsequent infection of target cells (62). MAbs can trap pathogens in the mucous gel by forming low affinity bonds with mucin fibers and can agglutinate pathogens into clusters too large to diffuse through the mucous gel.

Efficacy of Antibodies in Preventing Disease

The first use of immune serum for preventing disease by passive immunization was reported more than 100 years ago by von Behring and Kitasato (13). Subsequently, systemic passive immunization with antibodies has been proven effective in preventing many diseases. By binding to a pathogen, systemically delivered antibodies can inhibit attachment to and fusion with target cells, inhibit internalization by target cells, inhibit uncoating inside a cell, aggregate pathogens thereby preventing them from reaching target cells, interact with complement to lyse the pathogen, induce phagocytosis of the pathogen, and cause killer cells to lyse the pathogen by antibody-dependent cellular cytotoxicity (14). Table 1 lists the highest efficacy reported for systemically delivered antibodies in preventing disease in mammalian species and against a wide range of pathogens that infect humans. No antiviral treatments are available for most viruses listed in the table, yet antibodies can prevent the diseases caused by all of these viruses.

Although less studied than systemic passive immunization, the prophylactic use of mucosal antibodies predates the therapeutic use of immune sera. Antibodies delivered in mother's milk have been protecting the gastrointestinal tract of nursing infants since the mammary gland first evolved approximately 50 million years ago. Most infections begin in mucosal surfaces (approximately 400 m^2 in an adult human); supplementing the antibody repertoire in a mucous secretion (Figure 1-top) thus offers an effective method for protecting a mucosal surface against pathogens to which the host has not been exposed or become immune. In addition to the protective mechanisms described above, antibodies delivered to mucosal surfaces can trap pathogens in the mucous gel, make them mucophilic, and prevent their diffusion and motility (Figure 1-bottom); as a result, pathogens trapped in mucus are shed from the body with the normal flow of mucous secretions or are digested if these secretions enter the digestive tract (61-63). Topical passive immunization of mucosa can block transmission of bacteria, viruses, fungi, and parasites that infect humans (Table 2).

The predominant (and perhaps the most appropriate for mucosal delivery) antibody isotype on most human mucosal surfaces is secretory immunoglobulin A (SIgA); efficient methods for producing SIgA have been reported (82,83). SIgA, a tetravalent dimer of monomeric IgA associated with two polypeptides (joining chain and secretory component), is especially stable and well suited to function in the

Table 1: Examples of highly effective systemic passive immunization

Pathogen	Species[a]	Antibody[b]	Prevention (%)	DRS[c]	Ref.
Viruses					
Chikungunya	mou	p	100		(15)
Cytomegalovirus	hum	p	50	X	(16)
Dengue	mou	p	100		(17)
Ebola	bab	p	80		(18)
Hantavirus	mou	m	100		(19)
Herpes simplex (genital)	mou	m	100	X	(20)
(ocular)	mou	m	100		(21)
HIV	mou	m	100	X	(22)
Hepatitis A	hum	p	90		(23)
Hepatitis B	hum	p	92		(24)
Influenza	mou	m	100		(25)
Lassa	mon	p	100		(26)
Measles	mou	m	100		(27)
Polio	hum	p	58		(28)
Rabies	mou	m	100		(29)
Reovirus	mou	m	100		(30)
Rift Valley fever	ham	p	100		(31)
Respiratory syncytial	hum	m	100		(32)
		p	40		(33)
Rubella	hum	p	57		(34)
Varicella zoster	hum	p	100		(35)
Venezuelan equine encephalomyelitis	mou	p	100		(36)
Bacteria					
Borrelia burgdorferi	ham	p	100		(37)
Bordetella pertussis	mou	m	100	X	(38)
Chlamydia pneumoniae	mou	p	100		(39)
Chl. trachomatis	mou	m	90		(40)
Escherichia coli	rat	m	100	X	(41)
Francisella tularensis	mou	p	100		(42)
Group B *Streptococcus*	mou	m	100	X	(43)
Haemophilus influenzae	rat	p	100	X	(44)
Mycoplasma pneumoniae	ham	p	80		(45)
Neisseria meningitis	mou	m	90	X	(46)
Proteus mirabilis	mou	m	100	X	(47)
Pseudomonas aeruginosa	mou	p	100	X	(48)
Salmonella Typhimurium	mou	p	100	X	(49)
Shigella flexneri	rab	p	100	X	(50)
Staphylococcus aureus	rab	m	100	X	(51)
Streptococcus pneumoniae	mou	p	90	X	(52)
Treponema pallidum	ham	p	100		(53)
Yersinia pestis	mou	p	100		(54)
		m	NR[d]		(55)
Fungi					
Candida albicans	mou	p	> 67	X	(56)
Cryptococcus neoformans	mou	m	70	X	(57)
Parasites					
Plasmodium falciparum	mon	p	75	X	(58)
Toxoplasma gondii	mou	m	100		(59)

[a]Species: mou=mouse; hum=human; bab=baboon; mon=monkey; ham=hamster; rat= rat; rab=rabbit.
[b]Antibody: m=monoclonal; p=polyclonal.
[c]DRS=Drug-resistant strains reported (from Ref. 60).
[d]NR = not reported

Table 2: Examples of highly effective topical passive immunization of mucosa

Pathogen	Species[a]	Route[b]	Antibody[c]	Prevention	Ref.
Viruses					
Herpes simplex	mou	v	m	100%	(64,65)
		r	m	100%	(66)
Influenza	fer	o	p	100%	(67)
	mou	n	p	> 4[d]	(68)
Rotavirus	hum	o	p	100%	(69, 70)
Respiratory syncytial	mon	n	m	3-4[d]	(71)
Bacteria					
Chlamydia trachomatis	mou	v	m	90%	(72)
Clostridium difficule	ham	o	p	100%	(73)
Escherichia coli	hum	o	p	100%	(74)
Porphyromonas gingivalis	hum	o	m	100%	(75)
Shigella flexneri	hum	o	p	100%	(76)
Staphylococcus aureus	mou	n	p	3-4[e]	(77)
Streptococcus mutans	hum	o	m	100%	(78)
Vibrio cholerae	mou	o	m	100%	(79)
Fungi					
Candida albicans	mou	v	p	>50[f]	(80)
Parasites					
Cryptosporidium parvum	mou	o	m	77[g]	(81)

[a]Species tested in: mou=mouse; fer=ferret; hum=human; mon=monkey; ham=hamster.
[b]Delivery route of pathogen and antibody: v=vaginal; r=rectal; o=oral; n=nasal.
[c]Antibody: m=monoclonal; p=polyclonal.
[d] $\log_{10}$ reduction in virus titer.
[e] $\log_{10}$ reduction in cfu.
[f] % reduction in cfu.
[g] % reduction in number of parasites.

enzymatically hostile environment that prevails at mucosal surfaces (84). SIgA, the least phlogistic class of antibody (84), is the least likely to induce inflammatory responses that can make it easier for toxins and pathogens to breach the mucosal surface. Immune exclusion of antigens, enzymes, and toxins has been repeatedly demonstrated in vivo, and protection generally correlates with levels of SIgA antibodies in the relevant mucous secretions. Finally, the protective role of SIgA has been demonstrated in many systems (85).

Recent Advances in mAb Technology

Generating High-Affinity Human MAbs

Since the advent of cloning of human antibodies from combinatorial libraries constructed from seropositive persons (86,87), generation of fully human MAbs against human pathogens has become routine (Figure 2) (88). For example, from a single bone marrow donor, human MAbs were prepared against HIV, respiratory syncytial virus (RSV), cytomegalovirus, herpes simplex virus types 1 and 2, varicella zoster virus, and rubella virus (88). MAbs can even be obtained from naive libraries prepared from unexposed persons (if the library has a large enough repertoire) (89); therefore, antibodies against pathogens lethal to humans can be generated. Alternatively, human MAbs can be generated by traditional immunization of commercially available mice that have been genetically engineered to contain human immunoglobulin loci in their germline (Figure 2) (90,91).

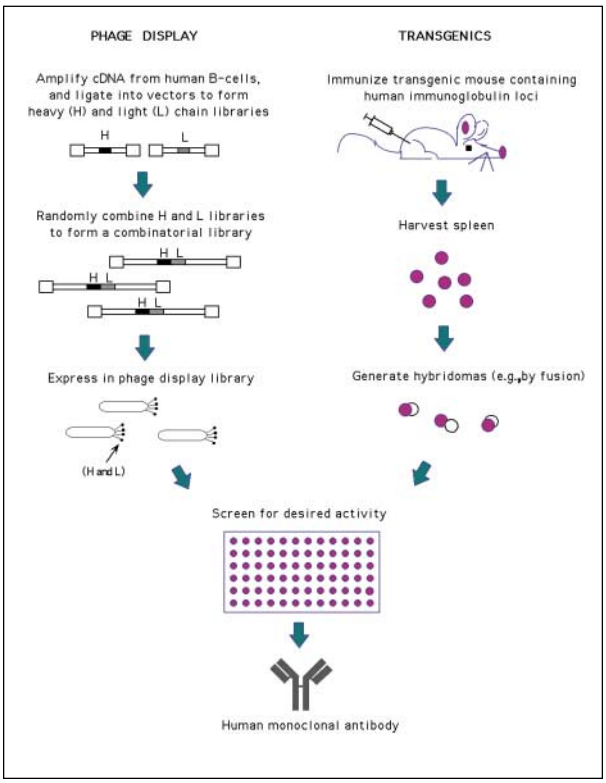

Figure 2. Generation of human monoclonal antibodies. (Phage display) Heavy and light chain cDNA isolated from human B-cells is used to generate a combinatorial library in which random heavy (H) and light chain (L) pairings are expressed on the surface of phage. These phage can then be screened for antigen binding by traditional techniques (e.g., ELISA). Since only the antigen binding region is used in the phage display process, the selected clone is then placed into an appropriate expression vector to produce a full antibody molecule.(Transgenics) Genetically manipulated mice have been produced with inactivated endogenous immunoglobulin genes, and with unrearranged human immunoglobulin gene segments introduced (90,91). These mice are then immunized with antigen, and hybridomas are produced by traditional routes. (See refs. 88, 89 for more technical information on these two methods and refs. 92, 93 for comparisons of these two methods).

Dramatic enhancement of the affinity of an mAb has been demonstrated by molecular biologic techniques in which mutants of an antibody are generated and then screened for higher affinity or higher neutralization activity (93-95). For example, the affinity of one anti-HIV mAb has been enhanced 420-fold, and this matured antibody neutralizes more HIV strains than the original mAb (94). Furthermore, expressing a mAb as a multivalent isotype, such as SIgA or IgM, can dramatically enhance the potency of an antibody by increasing the avidity (96) or agglutination activity (14). For example, an anti-*Escherichia coli* IgM was 1,000-fold more effective in protecting neonatal rats than its class-switched IgG (both in vitro and in vivo)(41). From a commercial standpoint, a 1,000-fold increase in avidity could translate into a 1,000-fold decrease in dose and subsequent cost. Also, a large dose of a highly potent mAb can substantially increase the duration of protection (97).

Production Systems

MAbs have traditionally been produced in cell culture and have been prohibitively expensive for most preventive uses. Over the years, however, the cost has continually dropped; MAbs are now being produced in cell culture for $200 to $1,000 per gram (98,99). Production of MAbs has recently been reported in both transgenic plants and animals (82,100,101). Both of these systems are expected to lower costs dramatically. Indeed, transgenic plants can be scaled up in agricultural fields to produce tons of "plantibody," and plant-produced antibody is predicted to cost less than U.S. $1/g (102). The actual cost, however, will remain unknown until large-scale batches are produced, purified, and formulated in accordance with Good Manufacturing Practices.

Safety and Regulatory Status

More than 80 MAbs are now in clinical trials (most for cancer imaging or therapy) and more than one quarter of these are in phase III trials (103). Few safety problems have been reported for systemic applications; antibodies are now considered "biotechnology-derived pharmaceuticals" by the U.S. Food and Drug Administration (FDA)—enabling a more straightforward regulatory process than in the past (92,104). Even though MAbs have often been evaluated for systemic applications, only recently have they been evaluated in humans for mucosal applications. This new interest in mucosal antibodies may be partially due to the increasing recognition of the importance of mucosal immunity. Only two clinical trials have evaluated topically delivered MAbs: intranasally delivered anti-RSV in infants at high risk (105) and orally delivered anti-*Streptococcus mutans* in adults (106); no major adverse effects were reported in these studies.

Safety concerns, such as peptide and glycosylation immunogenicity, are important when MAbs are delivered systemically but are likely to be of less concern when MAbs are applied to the mucosa, a surface that has evolved to interact with the external environment. Indeed, antibodies delivered to the lumen of a mucosal surface have minimal interaction with circulating immune cells. Although proteins, and even antibodies, can be absorbed through mucosal surfaces (107,108), generally only small quantities are absorbed (109,110). The inability of SIgA to activate complement by the classic pathway is likely involved in maintaining the integrity of mucosal surfaces (63); therefore, SIgA may be preferable to IgG or IgM for many mucosal applications.

The FDA "Points to Consider" for characterization of antibodies produced in cell-culture and transgenic animals (111) are better defined than for characterization of antibodies produced in transgenic plants; however, plant-derived antibodies are free of animal viruses and may therefore not require rigorous viral inactivation processing steps. In addition, although glycosylation patterns of MAbs produced in mammalian cell-culture and transgenic animals are closer phylogenetically to humans than glycosylation patterns in plants, given our repeated exposure to plant sugars in food and personal care products, it is unlikely that any of these patterns are novel to human immune systems (112). In fact, in a recently completed clinical trial with repeated applications of plant-produced antibody for the prevention of oral colonization by *S. mutans*, no safety problems were encountered, nor were there any detectable human anti-plant antibody responses (113).

Selection for resistant organisms by widespread and repeated use of antibiotics is a serious health concern (60). Drug-resistant strains of a wide variety of pathogens have already been reported (Table 1). Antibiotic or antiviral treatment of infected persons in which pathogens are actively replicating provides a strong evolutionary selection process for developing drug-resistant pathogens. In contrast, MAbs are less likely to create resistant organisms when used in a preventive context at a mucosal surface against a pathogen that is not yet actively replicating. Even if a systemic infection does occur during topical use of MAbs, resistant organisms will likely not be created since the pathogen will not be replicating and evolving in the presence of the mAb applied to the mucosal surface. This is in marked contrast to the settings in which antibiotics and antiviral drugs select for resistant strains (60). If MAbs are used frequently on a population level, the risk of selecting for resistant organisms may increase. When the emergence of resistant strains is of particular concern, the tendency to select mAb-resistant organisms could be minimized by using cocktails of mucosal antibodies directed at multiple antigenic targets (2,114). Because new MAbs can be produced with a rapid turnaround time (discussed below), the emergence of an antibody-resistant strain could be countered by producing a new mAb directed toward the mutated epitope or another antigenic target of the resistant strain. Indeed, the flexibility of the antibody structure to create a virtually inexhaustible repertoire of antigen binding specificities suggests that immunoglobulins evolved in part as a means to cope rapidly with new pathogens.

Turnaround Time for Developing a New mAb

Since human MAbs can be identified quickly by cloning variable regions from specific antigen-binding human lymphocytes (115) or panning combinatorial libraries (87), antibodies could be used as a rapidly developed method for defending against new pathogens. The time required for

collecting lymphocytes from a seropositive person, screening for an appropriate antibody, cloning, and expressing the antibody in culture in a well-equipped laboratory is 1 to 3 months; quantities sufficient for protecting persons at high risk or those at the focal point of an outbreak could be available in fewer than 6 months. High-capacity production in quantities sufficient for broad public health application could be available in several years, assuming that the safety of antibodies as a class of molecules is established and an infrastructure is in place for producing these antibodies. While in rare instances vaccines can be developed this quickly (e.g., the 1976 influenza vaccine [5]), new vaccines, antibiotics, and antiviral therapies usually take considerably longer to develop. Moreover, even though passive immunization may require repeated applications, MAbs delivered to a mucosal surface can provide immediate protection against infection.

Potential Preventive Uses for Topically Delivered MAbs

From a public health perspective, MAbs are most promising for preventing gastrointestinal, respiratory, and reproductive tract infections. These infections cause almost 11 million deaths annually worldwide, accounting for more than 50% of the deaths caused by communicable diseases and 22% of deaths by all causes (116). Sexually transmitted diseases (STDs) accounted for 87% of all cases reported among the top ten most frequently reported diseases in 1995 in the United States; more than 12 million Americans are infected with STDs each year at an estimated annual cost of more than $12 billion (117).

If a track record of safety and efficacy can be achieved, mucosal antibodies will probably be most useful as over-the-counter products that could reach populations not well integrated into the health-care system. The condom, a nonmedical over-the-counter personal protection product, has played an important preventive role in the HIV epidemic. Personal protection provided by over-the-counter antibody-based technology could play a similar role in future emerging disease epidemics.

Diarrheal Disease

Studies in animal models have demonstrated that orally delivered antibodies were 100% effective in preventing rotavirus (70) and cholera (79) infections. In humans, orally delivered bovine antibodies were 100% effective in preventing rotavirus (118), enterogenic *E. coli* (74), *Shigella* infection (76), and necrotizing enterocolitis (119).

For orally delivered MAbs, digestive degradation is a potential concern. However, significant levels of functional antibody survive treatment with pepsin at pH 2 or with a pool of pancreatic enzymes at pH 7.5 in vitro (120). In addition, most ingested IgA in milk survives passage through the gastrointestinal tract of infants (121); intact antibody delivered orally with an antacid survived passage through the gastrointestinal tract of adults (74,76). Assuming that a 10-mg dose of antibody is protective (i.e., assuming that the mAb is only 100-fold more potent than polyclonal preparations [118]), the production costs for the amount of plantibody needed for 100 days of protection could be approximately one cent (102).

Since diarrheal diseases are most prevalent in developing countries, preventive strategies must be extremely inexpensive; therefore, MAbs produced in plants or in the milk of animals are likely most suitable for these countries. Because of the speed with which MAbs pass through the gastrointestinal tract, antibodies delivered orally will need to be delivered frequently, perhaps more than once a day. In endemic-disease regions, MAbs could be delivered orally as a supplement with food or water.

Respiratory Disease

Animal studies have demonstrated the efficacy of nasal delivery of antibodies for the prevention of RSV infection (71) and influenza (68). In one study, topical application was approximately 100 times more effective than systemic delivery (122). Another study found an anti-RSV mAb (MEDI-493) to be approximately 100 times more effective than an equal quantity of a polyclonal preparation (32). These results suggest that 10,000 times less anti-RSV mAb would be required for topical applications than for systemically delivered polyclonal preparations. Protective systemic doses of MEDI-493 are approximately 100 mg (15 mg/kg) (32), so <1 mg might suffice for protection if this mAb were applied topically. Intranasally applied mAb has a residence half-time of a little under one day in the monkey (71), suggesting that once-a-day applications that deliver several-fold more than

a protective dose can provide continuous protection. MAbs for protecting the respiratory tract could be delivered in nose drops or by aerosol once a day to those at particular risk (e.g., infants and the elderly during influenza season) or to everyone living near the epicenter of an epidemic.

STDs

With the exception of hepatitis B, no vaccines are available for the prevention of STDs (Table 3). Until effective and safe vaccines are developed, vaginal delivery of a cocktail of anti-STD pathogen MAbs might make an effective new method for broad spectrum protection against STDs (11). In animal models, MAbs have been shown to protect against transmission of *C. albicans*, *C. trachomatis*, HSV, HIV, and syphilis (Tables 1, 2) (11). Antibodies have been delivered experimentally to the vagina in solution, gels, and more recently, by sustained release devices for long-term delivery of protective MAbs (123,124). Antibodies were found to be stable when stored in seminal fluid or cervical mucus for 48 hours at 37°C (125); no significant inactivation occurred over the pH range of the human vagina (pH 4 to 7) for at least 24 hours at 37°C (Zeitlin et al., unpub. obs.). Since the effective half-life of antibodies applied topically depends on the turnover time of mucus, a single vaginal application may thus provide protection for at least 1 day, and probably several days (97). If so, passive immunization of the vagina may extend protection to the occasional days when the user forgets to apply the mAb. Considering there are an estimated 5 billion acts of sexual intercourse per year in the United States (11), large-scale production of MAbs in plants may offer the best system for the low costs needed for such a public health initiative. In addition, because the most common class of infection in the first month of life is primarily caused by STD pathogens present in the birth canal (126), the same mucosal antibodies could be used in a predelivery cervicovaginal lavage or applied to newborns' eyes for studies in the prevention of ophthalmia neonatorum. Indeed, in some cultures the mother's colostrum, a fluid rich in SIgA, is applied to the newborns' eyes (127).

Table 3: Preventive vaccines or cures for major sexually transmitted disease pathogens

Pathogen	Vaccine	Cure	DRS[a]
Chlamydia trachomatis	no	yes	
Haemophilus ducreyi	no	yes	X
Hepatitis B	yes	no	
Herpes simplex 1 and 2	no	no	X
HIV-1 and 2	no	no	X
Human papilloma virus (HPV)	no	yes[b]	
Neisseria gonorrhoeae	no	yes	X
Treponema pallidum	no	yes	
Trichomonas vaginalis	no	yes	X

[a]Drug-resistant strains reported.
[b]Surgical removal of HPV-infected tissue is performed. HPV-related cervical cancer identified early has a high cure rate; however, in the United States, for every three new cases, there is approximately one death (117).

Conclusions

In animal models and human studies, antibodies have been shown to prevent a wide variety of infectious human diseases. Recent advances allow development of a new era of mucosal mAb-based products. These advances include the development of combinatorial libraries for rapid selection of human MAbs, the ability to increase dramatically the potency of a specific mAb, and the marked reduction in the cost of cell-culture–produced MAbs as well as the ability to produce MAbs inexpensively and at high capacity in transgenic animals and plants. In addition, since MAbs can be developed considerably more rapidly than most vaccines and antimicrobial drugs, MAbs may prove useful for combating emerging pathogens. Mucosal infections account for a large percentage of infectious disease-related illness and deaths; hence topical passive immunization with MAbs may offer a new opportunity for improving public health. Finally, many of the remaining safety issues regarding the human use of mucosal MAbs are likely to be addressed by clinical trials now under way.

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Dual and Recombinant Infections: An Integral Part of the HIV-1 Epidemic in Brazil

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We systematically evaluated multiple and recombinant infections in an HIV-infected population selected for vaccine trials. Seventy-nine HIV-1 infected persons in a clinical cohort study in Rio de Janeiro, Brazil, were evaluated for 1 year. A combination of molecular screening assays and DNA sequencing showed 3 dual infections (3.8%), 6 recombinant infections (7.6%), and 70 (88.6%) infections involving single viral subtypes. In the three dual infections, we identified HIV-1 subtypes F and B, F and D, and B and D; in contrast, the single and recombinant infections involved only HIV-1 subtypes B and F. The recombinants had five distinct B/F mosaic patterns: $B^{gag\text{-}p17}/B^{gag\text{-}p24}/F^{pol}/B^{env}$, $F^{gag\text{-}p17}/B^{gag\text{-}p24}/F^{pol}/F^{env}$, $B^{gag\text{-}p17}/B\text{-}F^{gag\text{-}p24}/F^{pol}/F^{env}$, $B^{gag\text{-}p17}/B\text{-}F^{gag\text{-}p24}/F^{pol}/B^{env}$, and $F^{gag\text{-}p17}/B\text{-}F^{gag\text{-}p24}/F^{pol}/F^{env}$. No association was found between dual or recombinant infections and demographic or clinical variables. These findings indicate that dual and recombinant infections are emerging as an integral part of the HIV/AIDS epidemic in Brazil and emphasize the heterogenous character of epidemics emerging in countries where multiple viral subtypes coexist.

Our understanding of the global molecular epidemiology of HIV-1 infections has improved substantially in recent years. Remarkable antigenic diversity, especially in the viral envelope, has emerged among HIV isolates worldwide (1). As the HIV/AIDS pandemic grows, viral strains are becoming more geographically dispersed, and the simultaneous presence of multiple subtypes in a given region is now common (2). Mixed infections and recombinants involving sequences of distinct HIV-1 subtypes (mosaics) are being recognized, but their prevalence and effect on the pandemic have not been fully evaluated (3). Consequently, the distribution of dual infections and mosaic viruses among different populations and the changes of this distribution over time are still relatively unknown. In addition, scientists are increasingly interested in possible differences in the transmission, epidemiologic patterns, and natural history of HIV-1 infections caused by more than one viral subtype and recombinant genomes. Finally, the efficacy of HIV-1 vaccines, primarily developed against subtype B viruses, may differ against more divergent recombinant variants and mixed infections of distinct HIV-1 subtypes. Such knowledge is vital to understanding the relevant role of mixed infections as a prerequisite for recombination and could be applied immediately in molecular epidemiology and immunotherapy.

Studies using convenience samples first documented mixed infections caused by viruses of subtypes B and E in Thailand (4). Subsequently, such studies have identified cases of dual infections with subtypes B, F, C, and D in Brazil (5-7), B and F in Puerto Rico (8), A and C in Rwanda (9), and triple HIV-1 infection with groups O and M of different clades in a single Cameroonian AIDS patient (10). In addition, potential dual infections have been detected by molecular screening assays among HIV-1 infected populations in Uganda and Kenya, where subtypes A and D coexist (11). The consequences of HIV-1 mixed infections may profoundly influence the

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dynamic of the pandemic through altered patterns of viral transmission and pathogenesis. Moreover, the resulting genetic variation may lead to the emergence of new HIV variants, including those with altered antigenicity and reduced sensitivity to detection by current diagnostic assays. The global emergence of such variants is exemplified in the impact of two HIV-1 recombinants of subtypes A/E and G/A on epidemics in Thailand and in certain parts of Central Africa, respectively (1,12-14). Interestingly, the presumptive parental subtypes E and G have not been identified.

Because Brazil has been selected as a World Health Organization field site for HIV-1 vaccine evaluation programs, priority has been given to extensive molecular examination of the prevalence and genetic diversity of HIV-1 strains circulating in the country. By November 1997, 116,277 AIDS cases had been reported to the Brazilian AIDS Control Program of the Ministry of Health (15). The 293 HIV-1 strains that have been molecularly characterized document that, although four HIV-1 subtypes—B, F, C, and D—are circulating in Brazil, only subtypes B and F are common (16-21). Moreover, five HIV-1 dual infections were identified in 21 HIV-infected patients during testing of molecular techniques that would discriminate between HIV-1 infections caused by single and multiple subtypes (5-7). Also, two cases of B/F recombinants have been found accidentally through sequence analysis of the *env* region (22). These data only indicate the potential for dual and recombinant infections in Brazil, where multiple subtypes circulate; they cannot, however, be used to assess the frequency of these infections among the HIV-infected population. In this study, we evaluated the proportion of HIV-1 dual and recombinant infections among 79 patients enrolled in a prospective clinical cohort study in Rio de Janeiro (an area where HIV-1 subtypes B and F are common).

The Study

Population

Part of an ongoing prospective clinical cohort study established in 1991 by the AIDS program of the Federal University of Rio de Janeiro, the HIV-1-infected patients have been continuously enrolled in the cohort for consultation, treatment, evaluation of different clinical parameters during the progression of the disease, and assessment of the genetic variation of HIV-1 strains (23). With informed consent, blood samples were obtained from the 79 patients, who consecutively attended the clinic between October and December 1994. For the purposes of this study, samples collected in 1994 were compared as needed, and follow-up specimens were collected 1 year later. The clinical profile of patients was based on the medical evaluation at the time of blood collection. Because the patients were randomly selected, the findings presented in this article likely reflect the trends in the HIV/AIDS epidemic in the Rio de Janeiro area. Epi Info, Version 6 program (CDC, Atlanta, GA, USA) was used to calculate frequencies, means, and analyses of variance of demographic information, clinical stages, and laboratory parameters.

Design

To investigate the proportion of mixed infections, we must distinguish between those involving two or more distinct HIV-1 strains and infection with a single intersubtype recombinant strain. By definition, the mixed infection occurs when multiple phylogenetically distinct copies of the same gene representing different viral genomes are present within one patient (24). In contrast, in the mosaic strain, different viral regions within the same genome are classified by phylogenetic analysis into different subtypes (3). Potential HIV-1 mixed infections involving distinct HIV-1 variants were segregated from single infections caused by only one subtype by using a restriction fragment length polymorphism (RFLP) screening assay of the *prt* gene (5,7). The restriction map profiles of the viral *prt* allow the segregation of HIV-1 strains to subtypes A, B, C, D, and F (Figure 1A). AluI digestion patterns separate subtypes A, C, and F from subtypes B and D. Sequential restriction analysis of *prt* using HinfI, BclI, and ScaI restriction enzymes further differentiates among these two subtype groups. The simultaneous occurrence of more than one digestion pattern indicates a potential mixed infection (Figure 1B). We selected recombinants among RFLP-subtyped single infections by additionally subtyping the C2-V3 *env* region with the heteroduplex mobility assay (25). Subtype discrepancies between *prt* and *env* regions were considered potential HIV-1 recombinants. All potential multiple infections

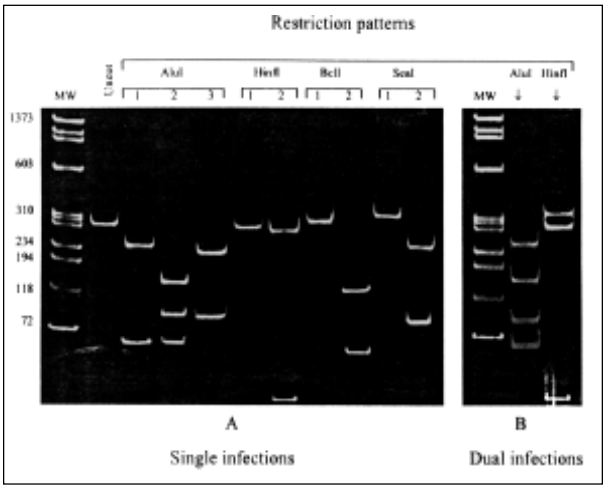

Figure 1. Differentiation between single (A) and dual (B) HIV-1 infections by the restriction fragment length polymorphism analysis of the polymerase chain reaction–amplified *prt*. A: Three AluI digestion patterns represent subtypes A, C, and F (pattern 1) and subtypes B and D (patterns 2 and 3); two HinfI patterns represent subtypes D (pattern 1) and B (pattern 2); two BclI patterns represent subtypes F (patterns 1) and A and C (pattern 2); two ScaI patterns represent subtypes A (pattern 1) and C (pattern 2). B: Two AluI digestion patterns (1 and 2) in the dually infected patient with HIV-1 subtypes F and B; two HinfI patterns (1 and 2) in the patient infected with subtypes B and D viruses.

and recombinants were analyzed by sequence analysis. The search for recombinants was further expanded by sequence analysis of the entire p17 *gag* or a 311-bp of the p24 *gag* fragment or both among preselected *prt-env* recombinants, all *prt-env* subtype F, and some *prt-env* subtype B viruses.

Polymerase Chain Reaction (PCR)

Uncultured or cultured peripheral blood mononuclear cells from patients were used for nested-PCR of the entire HIV-1 protease gene (*prt*, 297-bp), p24 *gag* fragment (311-bp), and C2-V3 domain of *env* (565-bp) (6,7). The outer primers for amplification of a 717-bp gag fragment spanning the entire 17 *gag* region and a 311-bp of p24 *gag* were LTRF: 5'GGGCTAATTTGGTCAAAAAGAAG; nucleotide position: 6-28, HIV-1_{MN}, and P24RA: 5'ATGTCACTTCCCCTTGGTTCT; nucleotide position: 1482-1502. The inner primers were P17F: 5'GCAAGAGGCGAGGGGCAGCAGCCG; nucleotide position: 716-739, and P17R: 5'CCCATTCTGCAGCTTCATTGA; nucleotide position: 1413-1433. The outer primers for amplification of a 1,444-bp fragment from p24 *gag* to the reverse transcriptase region were P24F1: 5'ATAGAGGAAGAGCAAAACAAAA; nucleotide position: 1,099 to 1,120, and MOPR1: 5'AAAATTGGAGTATTGTATGGATT; nucleotide position: 2,724 to 2,746. The inner primers were P24FA: 5'CAAAATTACCCTATAGTGCA; nucleotide position: 1,177 to 1,196, and MOPR2: 5'GGTCCATCCATTCCTGGTTT; nucleotide position: 2,601 to 2,620. PCR conditions were the same for amplification of all viral regions (7).

Sensitivity of Detection of Dual Infections

To determine the sensitivity of detection of dual infections by the RFLP assay, we mixed 5, 10, 25, 50, and 100 copies of subtypes B and F cloned proviral DNA in equal proportions or in ratios 1:2, 1:4, 1:10, and 1:20 for nested PCR amplification of *prt* (Table 1). For each

Table 1. Sensitivity of detection of HIV-1 dual infections caused by viruses of subtypes B and F

No. of viral subtypes		Ratio	No. of experi-	AluI digestion patterns of prt		
F	B	F:B	ments	B	F	B&F
100	100	1:1	1	-	-	+
50	50	1:1	4	-	-	+
25	25	1:1	4	-	+ (1)	+ (3)
10	10	1:1	4	-	-	+
5	5	1:1	4	-	+ (3)	+ (1)
100	5	20:1	2	-	+ (1)	+ (1)
100	10	10:1	2	-	+ (1)	+ (1)
100	25	4:1	2	-	+	-
100	50	2:1	2	-	-	+
5	100	1:20	1	-	-	+
10	100	1:10	1	-	-	+
25	100	1:4	1	-	-	+
50	100	1:2	1	-	-	+
100	0		3	-	+	-
50	0		3	-	+	-
25	0		3	-	+	-
10	0		3	-	+	-
5	0		2	-	+	-
0	100		3	+	-	-
0	50		2	+	-	-
0	25		3	+	-	-
0	10		3	+	-	-
0	5		3	+	-	-

Absence (-) and presence (+) of AluI digestion pattern of *prt* characteristic for subtype B, subtype F, and combination of subtypes B and F (Fig. 1). The cloned proviral DNA of HIV-1 subtypes B and F spanning a 1444-bp fragment from p24 *gag* to *rt* was used for the nested PCR amplification of *prt*. Amplified products were digested with AluI restriction enzyme, and the presence of two digestion patterns was analyzed on a 10% polyacrylamide gel by ethidium-bromide staining.

combination, one to four independent PCR reactions were run. The simultaneous amplification of the viral *prt* of subtypes B and F was evaluated by the RFLP assay. PCR controls included DNA templates of single HIV-1 subtypes.

Cloning, Sequencing, and Phylogenetic Analysis

The PCR-amplified proviral *prt* sequences from potential dual infections were cloned by using the Original TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). DNA from 30 clones of each specimen was screened for distinct HIV-1 sequences by the RFLP assay (7). Double-stranded viral DNA from selected clones or from direct PCR-amplified *prt*, p17 and p24 *gag*, and C2-V3 *env* products was cycle-sequenced in both directions with fluorescent dye–labeled sequencing terminators (26). Sequencing reactions were run in an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were aligned by the CLUSTAL multiple sequence alignment program (27). After gaps were eliminated, the aligned sequences were analyzed by the maximum likelihood method, with the fastDNAml program, which uses randomized data input and global rearrangement (28). Additionally, the neighbor joining method (PHYLIP package version 3.5c [29]) was used, with or without bootstrapping. The stability of the tree's topology was tested by pruning (removing one sequence from the alignment and rerunning the phylogenetic analysis). The SIV-cpz sequences (GenBank accession no. X52154) were used as outgroups. HIV-1 sequences generated in this study have been submitted to GenBank.

Results

Detection of Mixed Infections

RFLP analysis of the *prt* gene showed the simultaneous presence of two different digestion patterns in specimens from 10 of 79 patients. A complex pattern composed of elements of AluI patterns #1 (subtypes A, C, or F) and #2 (subtypes B or D) was identified in nine patients, and a combination of two HinfI digestion patterns (subtypes B and D) was found in one patient (Figure 1B). These data suggest that each of 10 patients could be infected with multiple distinct HIV-1 subtypes. The remaining 69 samples were classified as single infections caused by viruses of *prt* subtypes B (59) and F (10).

The PCR-amplified viral *prt* products from these 10 patients were cloned and sequenced to confirm the presence of mixed infections. Sequence analysis showed the simultaneous presence of two distinct HIV-1 variants in only three patients: BR45, BR62, and BR83. The nucleotide divergence between the two *prt* sequences within the patients was 6.8% for BR45, 7.4% for BR62, and 7.1% for BR83, indicating two distinct HIV-1 variants in each of these patients (6). Phylogenetic analysis confirmed these findings and demonstrated that the divergent HIV-1 *prt* sequences segregated into subtypes B and F (BR45), subtypes D and F (BR62), and subtypes B and D (BR83) (Figure 2a). In the remaining seven specimens, the observed RFLP results were consequences of either point mutations in AluI restriction site (five cases) or G A hypermutation (two cases), which occurred across one of the sequences within each specimen and destroyed the defining AluI sites. These changes in AluI restriction sites gave rise to genetically distinct quasispecies within the patients but did not represent distinct subtypes, as further confirmed by phylogenetic analysis (e.g., BR55-1 and BR55-2, and BR99-1 and BR99-2 in Figure 2a). Thus, despite the presence of mixed AluI digestion patterns in these seven specimens, they were classified as single infections of subtype B variants.

To address the issue of potential laboratory contamination, we collected repeat blood samples from dually infected patients approximately 1 year later and processed them on separate occasions. (Blood was unlikely to be contaminated during collection because a disposable vacutainer system was used to obtain each blood sample.) The sequence data from the first and second blood samples of each person showed a 98% to 99% similarity. Also, the viral sequences from these patients were distinct from those of laboratory strains (Figure 2a; 855M, 8,986, and 9,001) commonly used as standards.

Sensitivity of Detection of Dual Infections

To investigate the sensitivity of the RFLP screening method, we performed reconstruction experiments, in which two distinct viral DNA templates of subtypes B and F were analyzed in the same reaction mixture (Table 1). When equal proportions of 10 to 100 HIV-1 DNA template copies were used for *prt* amplification, two viral subtypes could be simultaneously detected in all

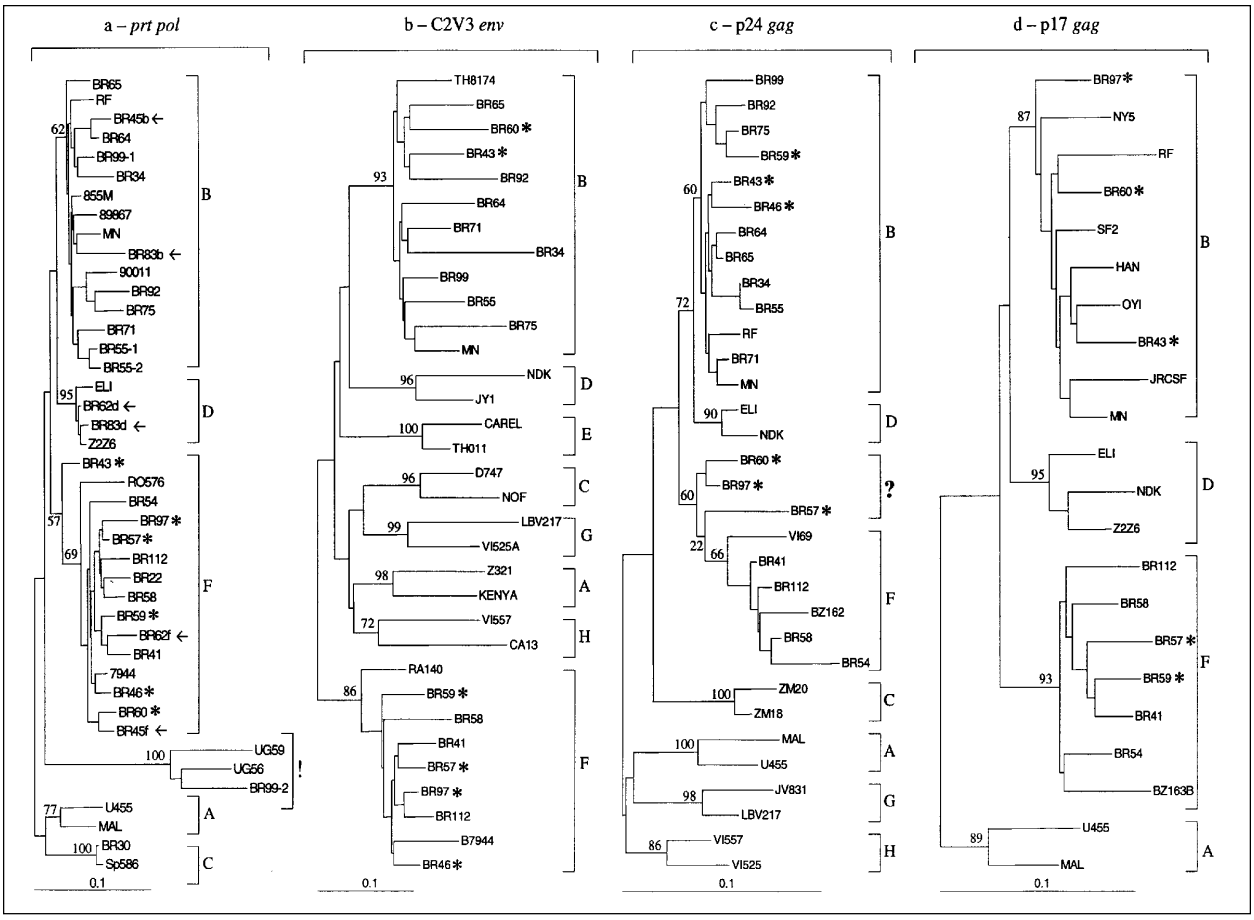

Figure 2. Phylogenetic classification of HIV-1 sequences from Brazilian patients (denoted with BR prefix). The trees were constructed on the basis of DNA sequences of *prt* (a), *env* (b), *gag*-p24 (c), and *gag*-p17 (d) by the neighbor-joining method. Numbers at the branch nodes connected with subtypes indicate bootstrap values. An arrow indicates dually infected specimens; an asterisk shows viral sequences, which clustered into different lineages depending on which parts of viral genome were analyzed; ! represents hypermutated sequences; ? represents unclassified subtype of p24 *gag* sequences. The distinct HIV-1 subtypes are delineated. The scale bar indicates an evolutionary distance of 0.10 nucleotides per position in the sequence. Vertical distances are for clarity only. GenBank accession numbers: *prt* [AF099155-99171;AF079986-79989;AF079991; and AF079994-79996]; *env* [AF113560-113576]; p17*gag* [AF115443-115451]; p24*gag* [AF115780-115797].

but one of 19 experiments). However, when five or fewer copies of each viral subtype were used, two subtypes were identified in only one of four assays; in the remaining three assays either subtype B or subtype F amplicons, but not both, were found. Similarly, in experiments containing varying proportions of subtypes B and F DNA templates, the simultaneous presence of two viral subtypes could be detected in only 8 (66%) of 12 experiments. In comparison, 5 to 100 copies of a single viral subtype (B or F) were routinely amplified and identified in all control reactions.

Detection of Intersubtype HIV-1 Recombinants

Parallel RFLP/heteroduplex mobility assay screening for HIV-1 subtypes in the *prt* and *env* regions identified two potential recombinants among 76 single infections. Subtype F *prt* and subtype B *env* were found in both specimens BR43 and BR60 during this initial screening. The remaining specimens were classified into subtypes F (n = 8) and B (n = 66) in both *prt* and *env* regions. These *prt-env* potential recombinants, all subtype F specimens, and eight selected subtype B samples were further evaluated by sequence analysis, which confirmed the results of the screening assays (Figure 2a, 2b).

The search for the HIV-1 recombinant genome in these 18 samples was further expanded to the *gag* region. Mosaic sequences of subtypes B and F were found within the p17 or p24 *gag* (Table 2, Figure 2, discussion below) in four *prt-env* subtype F variants (BR46, BR57, BR59, and BR97). Similarly, in one of two *prt-env* recombinants (BR60), the *gag* sequences had a mosaic pattern. In contrast, the p24 *gag* sequences of eight *prt-env* subtype B specimens were homogeneous and also classified as subtype B (Figure 2). Taken together, the comparative molecular analysis of *gag*, *pol*, and *env* regions allowed the identification of six specimens that carried HIV-1 recombinant genomes, representing five distinct mosaic structures: $B^{gag\text{-}p17}/B^{gag\text{-}p24}/F^{pol}/B^{env}$, $F^{gag\text{-}p17}/B^{gag\text{-}p24}/F^{pol}/F^{env}$, $B^{gag\text{-}p17}/B\text{-}F^{gag\text{-}p24}/F^{pol}/F^{env}$, $B^{gag\text{-}p17}/B\text{-}F^{gag\text{-}p24}/F^{pol}/B^{env}$, and $F^{gag\text{-}p17}/B\text{-}F^{gag\text{-}p24}/F^{pol}/F^{env}$.

The potential crossover breakpoints within 717-bp of the p17-p24 *gag* mosaic sequences were examined by comparison with nucleotide signature patterns characteristic for subtypes B and F viruses (Figure 3). We performed comparative analyses with aligned DNA sequences of recombinants (BR57, BR59, BR60, and BR97), subtype B (MN and BR43), and subtype F variants (BR41, BR54, BR58, and BR112). This analysis confirmed an intragene recombination within p24 *gag* in specimens BR57, BR60, and BR97—a finding consistent with our failure to phylogenetically assign these *gag* sequences to any known subtype (Figure 2c).

The putative breakpoints within the intragene recombinant sequences were located between nucleotides 97 and 137 in sample BR57 and between nucleotides 173 and 213 in BR60 and BR97 (Figure 3). The exact breakpoint position could not be determined because of extensive sequence homology between subtypes B and F in this viral region. This analysis also revealed putative crossover breakpoints for variants BR57 and BR59 in proximity to the coding region for the p17-p24 protein-processing site. The potential breakpoints between the second half of *gag* and the beginning of *pol* region in variants BR43, BR46, and BR59 are being investigated.

To examine the possibility of in vitro recombination during PCR amplification (30), we performed PCR amplification of the long fragments covering the *gag* and *prt* area in the endpoint- diluted lysates (31). To ensure that endpoint PCR products were amplified from single copy templates, we used samples only from dilutions at which 1 of 10 PCR amplifications were productive for further sequence analysis. The comparative analysis of the entire p17 *gag*, p24 *gag*, and *prt* sequences demonstrated 98% homology between the undiluted and diluted lysates—strong evidence that the recombinant sequences were not a result of the PCR amplification process.

Table 2. p17/p24 *gag*, *prt*, and C2-V3 genetic subtyping of HIV-1 DNA sequences[a] from peripheral blood mononuclear cells collected from 18 patients in Rio de Janeiro

		Genotypes			
Specimen	No.	*gag* p17	*gag* p24	*prt*	C2-V3
Group 1[b]	8	ND[c]	B	B	B
Group 2[d]	4	F	F	F	F
(BR46)		(NA[e])	(B)	(F)	(F)
(BR59)		(F)	(B)	(F)	(F)
(BR57)		(F)	(B/F)	(F)	(F)
(BR97)		(B)	(B/F)	(F)	(F)
(BR60)		(B)	(B/F)	(F)	(B)
(BR43)		(B)	(B)	(F)	(B)

[a]Recombinant specimens are shown in parentheses; B/F – mosaic structure within a 311-bp of the p24 *gag* fragment consisting of subtypes B and F sequences.
[b]Group 1: BR34, BR52, BR55, BR64, BR65, BR71, BR75, and BR92.
[c]ND=not done.
[d]Group 2: BR41, BR54, BR58, and BR112.
[e]NA = not available due to negative PCR.

Epidemiologic and Clinical Characteristics

The mean ages of patients infected with HIV-1 of single subtype B or F were not different from those with dual or recombinant infections ($p = 0.77$) (Table 3). In addition, the patients did not differ significantly by gender, risk group, and clinical stage of disease ($p = 0.44$ to $p = 0.48$). The patients infected with HIV-1 subtype B (403) and subtype F (854) did, however, differ ($p = 0.04$) by mean CD4 counts. Although dates of seroconversion were not known for all patients, the earliest HIV-positive results were reported among patients infected with subtype B (in agreement with previous observations that the spread of subtype B viruses occurred earlier than other HIV-1 subtypes in Brazil [16,17]), which might explain the difference in CD4 counts.

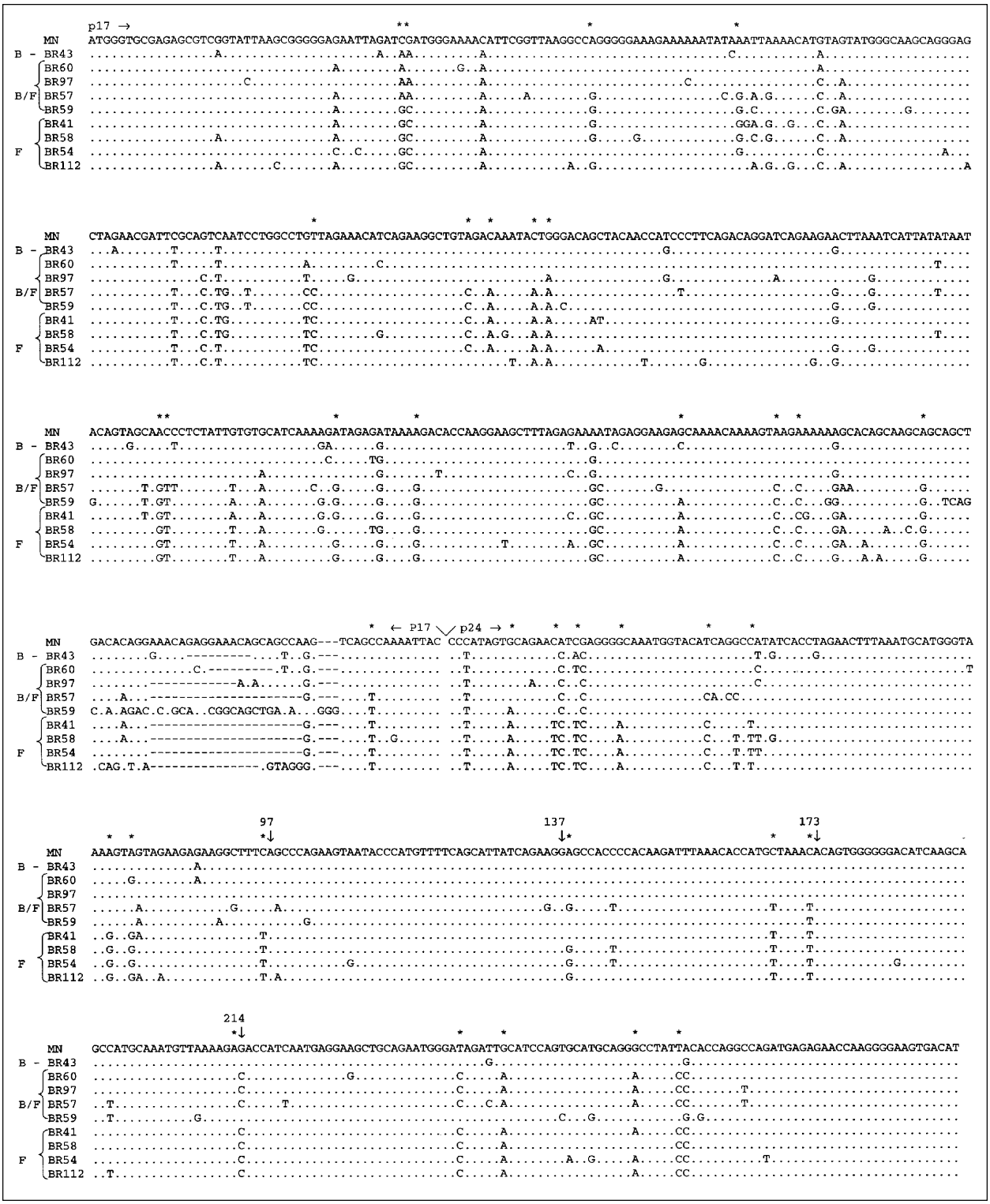

Figure 3. Analysis of the putative recombination within the *gag* region. The aligned sequences were classified into subtype B, subtype F, and recombinant subtype B/F on the basis of linearity of subtype assignment for the p17-p24 *gag* region. Asterisks show characteristic nucleotide patterns for subtypes B and F sequences; dots represent nucleotides homologous to the MN *gag* sequence; dashes indicate gaps introduced to maintain the alignment; and arrows indicate the potential recombination regions within the p24 *gag* fragment. The nucleotide position is marked.

Table 3. Characteristics of the study population

Characteristic	Dual infection n=3	Recombinant infection n=6	Subtype B n=66	Subtype F n=4
Gender (Female/male)	1/2	3/3	24/42	3/1
Mean age in years (range)	36 (30-44)	34 (25-45)	37 (23-60)	34 (26-43)
Clinical stage[a]				
1	3	5	29	4
2	-	1	17	-
3	-	-	14	-
4	-	-	6	-
Mean CD4 cells (range)	527 (404-743)	484 (190-821)	403 (59-1281)[b]	823 (570-1270)
Years of 1st serologic tests				
1985-1987	-	-	2	-
1988-1991	2	2	30	2
1992-1994	1	4	34	2
Heterosexual	2	5	27	4
Homosexual	1	-	19	-
Bisexual	-	-	8	-
Blood transfusion recipient	-	-	3	-
Intravenous drug user	-	-	1	-
Multiple factors	-	1	2	-
Unknown	-	-	6	-

[a]WHO staging system [32].
[b]Based on data available for 54 patients.

Conclusions

HIV-1 infections caused by dual and intersubtype-recombinant genome may be relatively common among HIV-1-infected Brazilians. Using both heteroduplex mobility assay and RFLP screening methods, as well as sequencing, we identified three dual (3.8%) and six recombinant (7.6%) infections involving distinct viral subtypes among 79 HIV-1-infected persons from Rio de Janeiro.

We chose viral *prt* to screen for dual infections because this highly conserved region provides the best opportunity for simultaneous amplification of distinct HIV-1 variants in the same PCR reaction. Proviral *prt* sequences can be routinely amplified from approximately 95% of all analyzed seropositive samples collected from the Americas, Asia, Africa, and Europe (data not shown). Moreover, the RFLP assay of the *prt* gene is convenient for screening a large number of samples (5). The detection of B/F, B/D, and F/D dual infections is in agreement with our 1993 study among patients from the same Rio de Janeiro cohort, which showed five dual HIV-1 infections involving subtypes F and B (one case), F and D (one case), and B and C (three cases of familial clustering) among 21 HIV-infected persons (5-7). Interestingly, dual infections involving HIV-1 subtype D continue to be detected in patients from Rio de Janeiro, an area with a high predominance of HIV-1 subtypes B and F, but rare subtype D, single infections. Because retrospective specimens (peripheral blood mononuclear cells) were not available for these patients, we could not confirm whether the acquisition of mixed strains was sequential (superinfection) or simultaneous. However, combination of subtypes F or B with rare subtype D viruses among some dual infections may suggest that in these cases two viral strains might be acquired through cotransmission rather than through superinfection.

All naturally occurring HIV-1 dual infections are likely not detected by current methods. First, quantitative differences in two distinct viral DNA templates in the sample can lead to selective PCR amplification of only one subtype. Second, despite targeting conserved genes such as *prt*, some divergent viral strains may escape PCR amplification because of primer mismatches. Finally, a single nucleotide mutation in the endonuclease restriction site can abrogate the recognition pattern and distort detection of dual infections in the RFLP analysis. These observations suggest that the rate of HIV-1 mixed infections within this Brazilian cohort might be even higher than 4%. Our previous findings of five dual infections among 21 patients from the same cohort support this assumption. If

we take into account these five cases, the percentage of mixed infections caused by viruses of distinct subtypes circulating between 1993 and 1994 among 100 patients analyzed from this Rio de Janeiro cohort would increase to 8%.

The potential underestimate of mixed infections is highlighted by the additional detection of 6 (7.6%) distinct recombinants within the cohort. This finding is consistent with the estimated 5% to 10% intersubtype mosaics among HIV-1 genomes in the Los Alamos database (3). Interestingly, all recombinant or mosaic genomes described in this report involved only subtypes B and F viral regions, although dual infections caused by other subtypes were also circulating in this cohort. Moreover, our results indicate that recombination between *gag* and *pol* (*prt*) regions is more frequent than between *pol* and *env* and lead to speculation that such stable B/F mosaics have selective advantage. Such observations support the assumption that recombination within the *gag* gene occurs more often than within other viral regions (33) and emphasize the need for rapid subtyping methods specific to the *gag* region.

To investigate the potential impact of dual and recombinant infections on the clinical status of patients, we compared the clinical and demographic characteristics of these patients with those of patients infected with one nonrecombinant viral subtype. Epidemiologic information for all 79 patients was available only at the first draw of blood. Although the results did not show significant differences between the two groups, the possibility of differences exists.

Our findings provide the first baseline measure of the range of HIV variability in Rio de Janeiro from 1993 to 1994 and the proportion of dual and recombinant infections among the HIV-infected Brazilian population. Future systematic molecular epidemiologic surveys of HIV heterogeneity in Rio de Janeiro may show the potential changes in the molecular profile of the HIV/AIDS epidemic over time; the laboratory tools we used to identify single, dual, and recombinant infections may be useful in such investigations. Nevertheless, our study on genetic variation of HIV-1 subtypes among blood donors from the state of Rio de Janeiro documented the presence of mosaic viruses of subtypes B and F and subtypes B and D in blood units collected in 1996 (34). Moreover, recent genetic analysis of viral strains collected in 1997 from HIV-1-infected patients living in Manaus (a city in Brazil's Amazon region) showed the presence of dual infections and recombinants caused by subtypes B and F viruses (A. Tanuri, pers. comm.). Therefore, these data indicate that the heterogenic pattern of HIV-1 infections, first observed in Rio de Janeiro, also exists in other regions of Brazil.

Our findings indicate that mixed infections and mosaic viruses may be more common in worldwide epidemics than previously thought and, therefore, may provide a basis for developing AIDS vaccines and predicting the global evolution of HIV.

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Artur Ramos is a graduate fellow at the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. His laboratory research focuses on HIV-1 genetic variability worldwide. His research interests include application of molecular biology techniques to epidemiology of HIV.

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Genetic Diversity and Distribution of *Peromyscus*-Borne Hantaviruses in North America

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The 1993 outbreak of hantavirus pulmonary syndrome (HPS) in the southwestern United States was associated with Sin Nombre virus, a rodent-borne hantavirus; The virus' primary reservoir is the deer mouse (*Peromyscus maniculatus*). Hantavirus-infected rodents were identified in various regions of North America. An extensive nucleotide sequence database of an 139 bp fragment amplified from virus M genomic segments was generated. Phylogenetic analysis confirmed that SNV-like hantaviruses are widely distributed in *Peromyscus* species rodents throughout North America. Classic SNV is the major cause of HPS in North America, but other Peromyscine-borne hantaviruses, e.g., New York and Monongahela viruses, are also associated with HPS cases. Although genetically diverse, SNV-like viruses have slowly coevolved with their rodent hosts. We show that the genetic relationships of hantaviruses in the Americas are complex, most likely as a result of the rapid radiation and speciation of New World sigmodontine rodents and occasional virus-host switching events.

Hantaviruses, rodent-borne RNA viruses, can be found worldwide. The Old World hantaviruses, such as Hantaan, Seoul, and Puumala, long known to be associated with human disease, cause hemorrhagic fever with renal syndrome of varying degrees of severity (1). After hantavirus pulmonary syndrome (HPS) was discovered in the southwestern United States in 1993 (2-4), intensive efforts were begun to detect and characterize hantaviruses in North America and determine their public health importance (5). As of January 1999, 205 HPS cases had been confirmed in 30 states of the United States, and 30 cases had been confirmed in three provinces of Canada; most cases occurred in the western regions of both countries. While Sin Nombre virus (SNV) has been identified as the cause of most HPS cases in North America, an increasingly complex array of additional hantaviruses has appeared (Table 1).

Surveys of rodents for hantavirus antibody have shown hantavirus-infected rodents in most areas of North America (3;6-9; Ksiazek et al., unpub. data; Artsob et al., unpub data). Serologic evidence of hantavirus infection has been found in North American rodents of the family *Muridae*. Most North American hantaviruses are associated with the subfamily *Sigmodontinae*; only a small number are associated with the subfamilies *Arvicolinae* or *Murinae*. To determine the number and distribution of hantaviruses in North America, we conducted a nucleotide sequence analysis of a polymerase chain reaction (PCR) fragment amplified from a large number of representative HPS patient and hantavirus-infected rodent samples from throughout the region. We focused on the North American viruses (particularly those associated with *Peromyscus* species rodents), although the nucleotide sequences of many hantaviruses from

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Table 1. Hantaviruses in the New World

Virus[a]	Disease[b]	Known or suspected host	Location	Virus isolate
Sigmodontinae associated				
Sin Nombre	HPS	*Peromyscus maniculatus* (grassland form)	West & Central U.S. and Canada	Y
Monongahela	HPS	*P. maniculatus* (forest form)	Eastern U.S. and Canada	N
New York	HPS	*P. leucopus* (eastern haplotype)	Eastern U.S.	Y
Blue River		*P. leucopus* (SW/NW haplotypes)	Central U.S.	N
Bayou	HPS	*Oryzomys palustris*	Southwestern U.S.	Y
Black Creek Canal	HPS	*Sigmodon hispidus* (eastern form)	Florida	Y
Muleshoe		*S. hispidus* (western form)	Southern U.S.	N
Caño Delgadito		*S. alstoni*	Venezuela	Y
Andes	HPS	*Oligoryzomys longicaudatus*	Argentina and Chile	Y
Oran	HPS	*O. longicaudatus*	Northwestern Argentina	N
Lechiguanas	HPS	*O. flavescens*	Central Argentina	N
Bermejo		*O. chacoensis*	Northwestern Argentina	N
Hu39694	HPS	Unknown	Central Argentina	N
Pergamino		*Akadon azarae*	Central Argentina	N
Maciel		*Bolomys obscurus*	Central Argentina	N
Laguna Negra	HPS	*Calomys laucha*	Paraguay and Bolivia	Y
Juquitiba	HPS	Unknown	Brazil	N
Rio Mamore		*O. microtis*	Bolivia and Peru	Y
El Moro Canyon		*Reithrodontomys megalotis*	Western U.S. and Mexico	N
Rio Segundo		*R. mexicanus*	Costa Rica	N
Arvicolinae associated				
Prospect Hill		*Microtus pennsylvanicus*	N. America	Y
Bloodland Lake		*M. ochrogaster*	N. America	N
Prospect Hill-like		*M. pennsyl./montanus/ochrogaster*	N. America	N
Isla Vista		*M. californicus*	Western U.S. and Mexico	N
Murinae associated				
Seoul	HFRS	*Rattus norvegicus*	Worldwide	Y

[a]Major virus types or species are in bold and indented below the rodent subfamilies with which they are associated; related genetically distinct virus lineages that may represent additional species or subspecies are indented below virus types and species.
[b]HPS = hantavirus pulmonary syndrome; HFRS = hemorrhagic fever with renal syndrome.

South America and elsewhere were included as outgroups to increase the resolution of the analysis.

Genetic Detection and Phylogenetic Analysis of New World Hantaviruses

The nucleotide sequences of 139 bp fragments of the G2 encoding region of virus M segments amplified by reverse transcriptase-PCR (RT-PCR) from 288 hantavirus-infected rodent and human samples were compiled from Genbank sources or from data reported here. Details of the specimen selection and methods of genomic analysis are provided in the Appendix. The Genbank accession numbers of those sequences published earlier (bigtree.xls) can be accessed from this article published on the journal home page (http://www.cdc.gov/eid). The entire aligned dataset (bigtree.nex), including 130 newly presented sample sequences, is also available on line. These sequences include those derived from 229 SNV-like viruses associated with *Peromyscus* species rodents from throughout North America. Maximum parsimony analysis of the aligned sequences was conducted with PAUP (12; Appendix), which resulted in a reasonably well-defined tree topology with several distinct lineages of SNV-like viruses and other clearly discernable hantaviruses (Figures 1, 2). Bootstrap analysis showed that while several of the major nodes of the tree were not well supported (values of 50% or less), many others were robust (values of 70% or higher) (Figures 1, 2). In most phylogenetic analyses, bootstrap values provide highly conservative estimates of the probability of correctly inferring

Figure 1

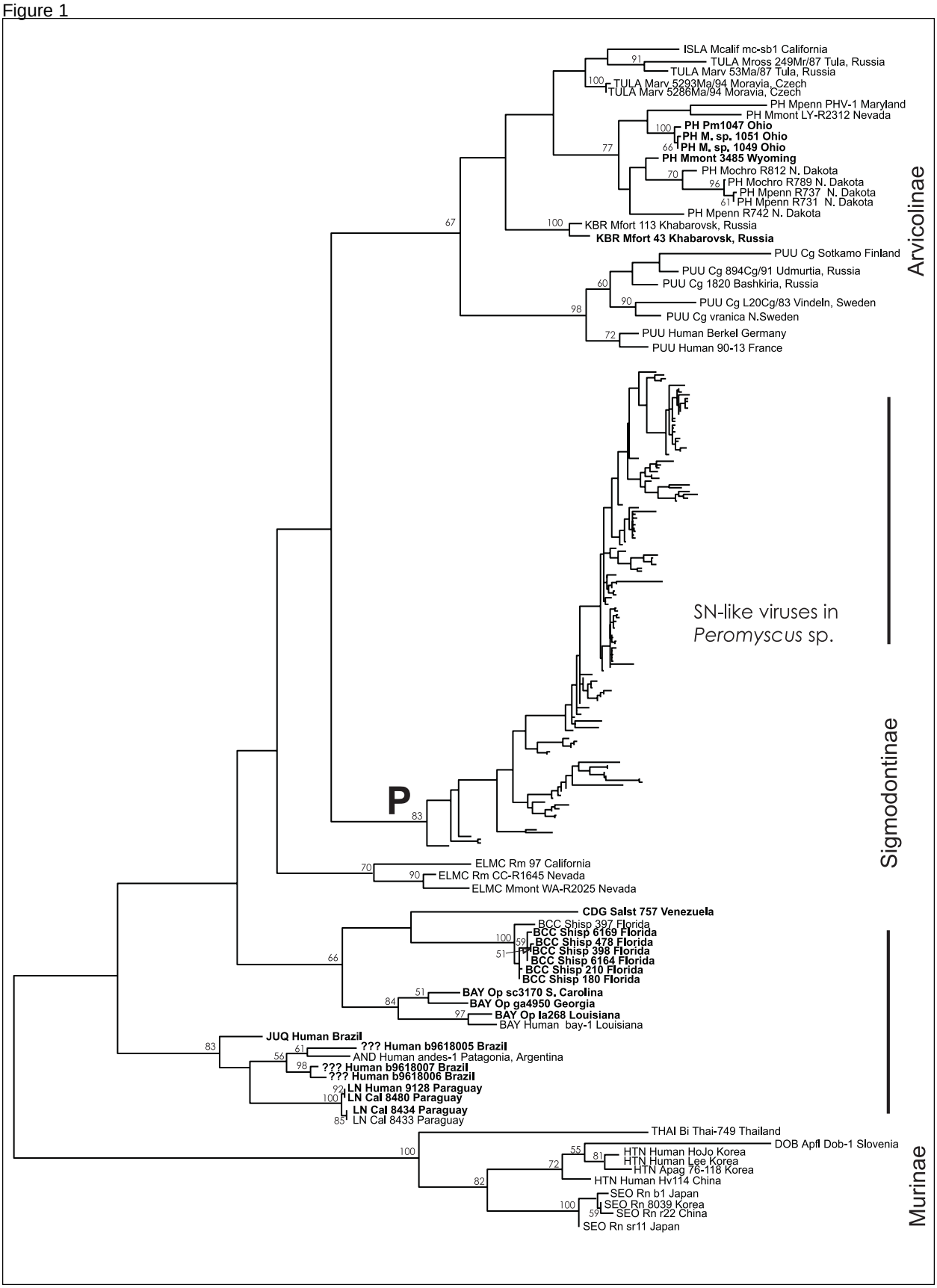
ISLA Mcalif mc-sb1 California
TULA Mross 249Mr/87 Tula, Russia
TULA Marv 53Ma/87 Tula, Russia
TULA Marv 5293Ma/94 Moravia, Czech
TULA Marv 5286Ma/94 Moravia, Czech
PH Mpenn PHV-1 Maryland
PH Mmont LY-R2312 Nevada
PH Pm1047 Ohio
PH M. sp. 1051 Ohio
PH M. sp. 1049 Ohio
PH Mmont 3485 Wyoming
PH Mochro R812 N. Dakota
PH Mochro R789 N. Dakota
PH Mpenn R737 N. Dakota
PH Mpenn R731 N. Dakota
PH Mpenn R742 N. Dakota
KBR Mfort 113 Khabarovsk, Russia
KBR Mfort 43 Khabarovsk, Russia
PUU Cg Sotkamo Finland
PUU Cg 894Cg/91 Udmurtia, Russia
PUU Cg 1820 Bashkiria, Russia
PUU Cg L20Cg/83 Vindeln, Sweden
PUU Cg vranica N.Sweden
PUU Human Berkel Germany
PUU Human 90-13 France
Arvicolinae
SN-like viruses in
Peromyscus sp.
P
Sigmodontinae
ELMC Rm 97 California
ELMC Rm CC-R1645 Nevada
ELMC Mmont WA-R2025 Nevada
CDG Salst 757 Venezuela
BCC Shisp 397 Florida
BCC Shisp 6169 Florida
BCC Shisp 478 Florida
BCC Shisp 398 Florida
BCC Shisp 6164 Florida
BCC Shisp 210 Florida
BCC Shisp 180 Florida
BAY Op sc3170 S. Carolina
BAY Op ga4950 Georgia
BAY Op la268 Louisiana
BAY Human bay-1 Louisiana
JUQ Human Brazil
??? Human b9618005 Brazil
AND Human andes-1 Patagonia, Argentina
??? Human b9618007 Brazil
??? Human b9618006 Brazil
LN Human 9128 Paraguay
LN Cal 8480 Paraguay
LN Cal 8434 Paraguay
LN Cal 8433 Paraguay
THAI Bi Thai-749 Thailand
DOB Apfl Dob-1 Slovenia
HTN Human HoJo Korea
HTN Human Lee Korea
HTN Apag 76-118 Korea
HTN Human Hv114 China
SEO Rn b1 Japan
SEO Rn 8039 Korea
SEO Rn r22 China
SEO Rn sr11 Japan
Murinae

Figure 2

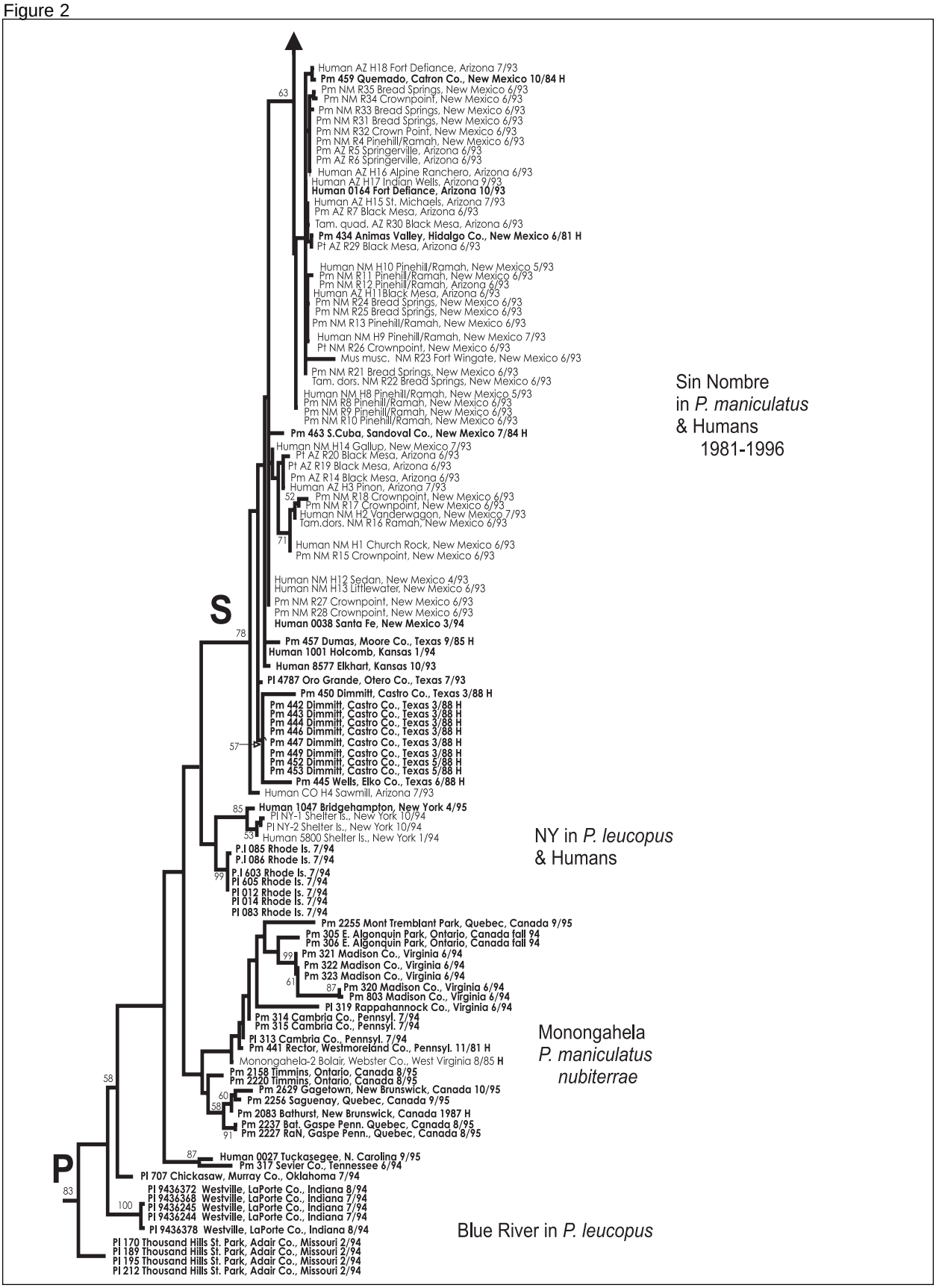
Human AZ H18 Fort Defiance, Arizona 7/93
Pm 459 Quemado, Catron Co., New Mexico 10/84 H
Pm NM R35 Bread Springs, New Mexico 6/93
Pm NM R34 Crownpoint, New Mexico 6/93
Pm NM R33 Bread Springs, New Mexico 6/93
Pm NM R31 Bread Springs, New Mexico 6/93
Pm NM R32 Crown Point, New Mexico 6/93
Pm NM R4 Pinehill/Ramah, New Mexico 6/93
Pm AZ R5 Springerville, Arizona 6/93
Pm AZ R6 Springerville, Arizona 6/93
Human AZ H16 Alpine Ranchero, Arizona 6/93
Human AZ H17 Indian Wells, Arizona 9/93
Human 0164 Fort Defiance, Arizona 10/93
Human AZ H15 St. Michaels, Arizona 7/93
Pm AZ R7 Black Mesa, Arizona 6/93
Tam. quad. AZ R30 Black Mesa, Arizona 6/93
Pm 434 Animas Valley, Hidalgo Co., New Mexico 6/81 H
Pt AZ R29 Black Mesa, Arizona 6/93
Human NM H10 Pinehill/Ramah, New Mexico 5/93
Pm NM R11 Pinehill/Ramah, New Mexico 6/93
Pm NM R12 Pinehill/Ramah, Arizona 6/93
Human AZ H11 Black Mesa, Arizona 6/93
Pm NM R24 Bread Springs, New Mexico 6/93
Pm NM R25 Bread Springs, New Mexico 6/93
Pm NM R13 Pinehill/Ramah, New Mexico 6/93
Human NM H9 Pinehill/Ramah, New Mexico 7/93
Pt NM R26 Crownpoint, New Mexico 6/93
Mus musc. NM R23 Fort Wingate, New Mexico 6/93
Pm NM R21 Bread Springs, New Mexico 6/93
Tam. dors. NM R22 Bread Springs, New Mexico 6/93
Human NM H8 Pinehill/Ramah, New Mexico 5/93
Pm NM R8 Pinehill/Ramah, New Mexico 6/93
Pm NM R9 Pinehill/Ramah, New Mexico 6/93
Pm NM R10 Pinehill/Ramah, New Mexico 6/93
Pm 463 S.Cuba, Sandoval Co., New Mexico 7/84 H
Human NM H14 Gallup, New Mexico 7/93
Pt AZ R20 Black Mesa, Arizona 6/93
Pt AZ R19 Black Mesa, Arizona 6/93
Pm AZ R14 Black Mesa, Arizona 6/93
Human AZ H3 Pinon, Arizona 7/93
Pm NM R18 Crownpoint, New Mexico 6/93
Pm NM R17 Crownpoint, New Mexico 6/93
Human NM H2 Vanderwagon, New Mexico 7/93
Tam.dors. NM R16 Ramah, New Mexico 6/93
Human NM H1 Church Rock, New Mexico 6/93
Pm NM R15 Crownpoint, New Mexico 6/93
Human NM H12 Sedan, New Mexico 4/93
Human NM H13 Littlewater, New Mexico 6/93
Pm NM R27 Crownpoint, New Mexico 6/93
Pm NM R28 Crownpoint, New Mexico 6/93
Human 0038 Santa Fe, New Mexico 3/94
S
Pm 457 Dumas, Moore Co., Texas 9/85 H
Human 1001 Holcomb, Kansas 1/94
Human 8577 Elkhart, Kansas 10/93
PI 4787 Oro Grande, Otero Co., Texas 7/93
Pm 450 Dimmitt, Castro Co., Texas 3/88 H
Pm 442 Dimmitt, Castro Co., Texas 3/88 H
Pm 443 Dimmitt, Castro Co., Texas 3/88 H
Pm 444 Dimmitt, Castro Co., Texas 3/88 H
Pm 446 Dimmitt, Castro Co., Texas 3/88 H
Pm 447 Dimmitt, Castro Co., Texas 3/88 H
Pm 449 Dimmitt, Castro Co., Texas 3/88 H
Pm 452 Dimmitt, Castro Co., Texas 5/88 H
Pm 453 Dimmitt, Castro Co., Texas 5/88 H
Pm 445 Wells, Elko Co., Texas 6/88 H
Human CO H4 Sawmill, Arizona 7/93
Human 1047 Bridgehampton, New York 4/95
PI NY-1 Shelter Is., New York 10/94
PI NY-2 Shelter Is., New York 10/94
Human 5800 Shelter Is., New York 1/94
P.I 085 Rhode Is. 7/94
P.I 086 Rhode Is. 7/94
P.I 603 Rhode Is. 7/94
PI 605 Rhode Is. 7/94
PI 012 Rhode Is. 7/94
PI 014 Rhode Is. 7/94
PI 083 Rhode Is. 7/94
Pm 2255 Mont Tremblant Park, Quebec, Canada 9/95
Pm 305 E. Algonquin Park, Ontario, Canada fall 94
Pm 306 E. Algonquin Park, Ontario, Canada fall 94
Pm 321 Madison Co., Virginia 6/94
Pm 322 Madison Co., Virginia 6/94
Pm 323 Madison Co., Virginia 6/94
Pm 320 Madison Co., Virginia 6/94
Pm 803 Madison Co., Virginia 6/94
PI 319 Rappahannock Co., Virginia 6/94
Pm 314 Cambria Co., Pennsyl. 7/94
Pm 315 Cambria Co., Pennsyl. 7/94
PI 313 Cambria Co., Pennsyl. 7/94
Pm 441 Rector, Westmoreland Co., Pennsyl. 11/81 H
Monongahela-2 Bolair, Webster Co., West Virginia 8/85 H
Pm 2158 Timmins, Ontario, Canada 8/95
Pm 2220 Timmins, Ontario, Canada 8/95
Pm 2629 Gagetown, New Brunswick, Canada 10/95
Pm 2256 Saguenay, Quebec, Canada 9/95
Pm 2083 Bathurst, New Brunswick, Canada 1987 H
Pm 2237 Bat. Gaspe Penn. Quebec, Canada 8/95
Pm 2227 RaN, Gaspe Penn., Quebec, Canada 8/95
Human 0027 Tuckasegee, N. Caroling 9/95
Pm 317 Sevier Co., Tennessee 6/94
P
PI 707 Chickasaw, Murray Co., Oklahoma 7/94
PI 9436372 Westville, LaPorte Co., Indiana 8/94
PI 9436368 Westville, LaPorte Co., Indiana 7/94
PI 9436245 Westville, LaPorte Co., Indiana 7/94
PI 9436244 Westville, LaPorte Co., Indiana 7/94
PI 9436378 Westville, LaPorte Co., Indiana 8/94
PI 170 Thousand Hills St. Park, Adair Co., Missouri 2/94
PI 189 Thousand Hills St. Park, Adair Co., Missouri 2/94
PI 195 Thousand Hills St. Park, Adair Co., Missouri 2/94
PI 212 Thousand Hills St. Park, Adair Co., Missouri 2/94
Sin Nombre in P. maniculatus & Humans 1981-1996
NY in P. leucopus & Humans
Monongahela P. maniculatus nubiterrae
Blue River in P. leucopus

Figure 2, continued

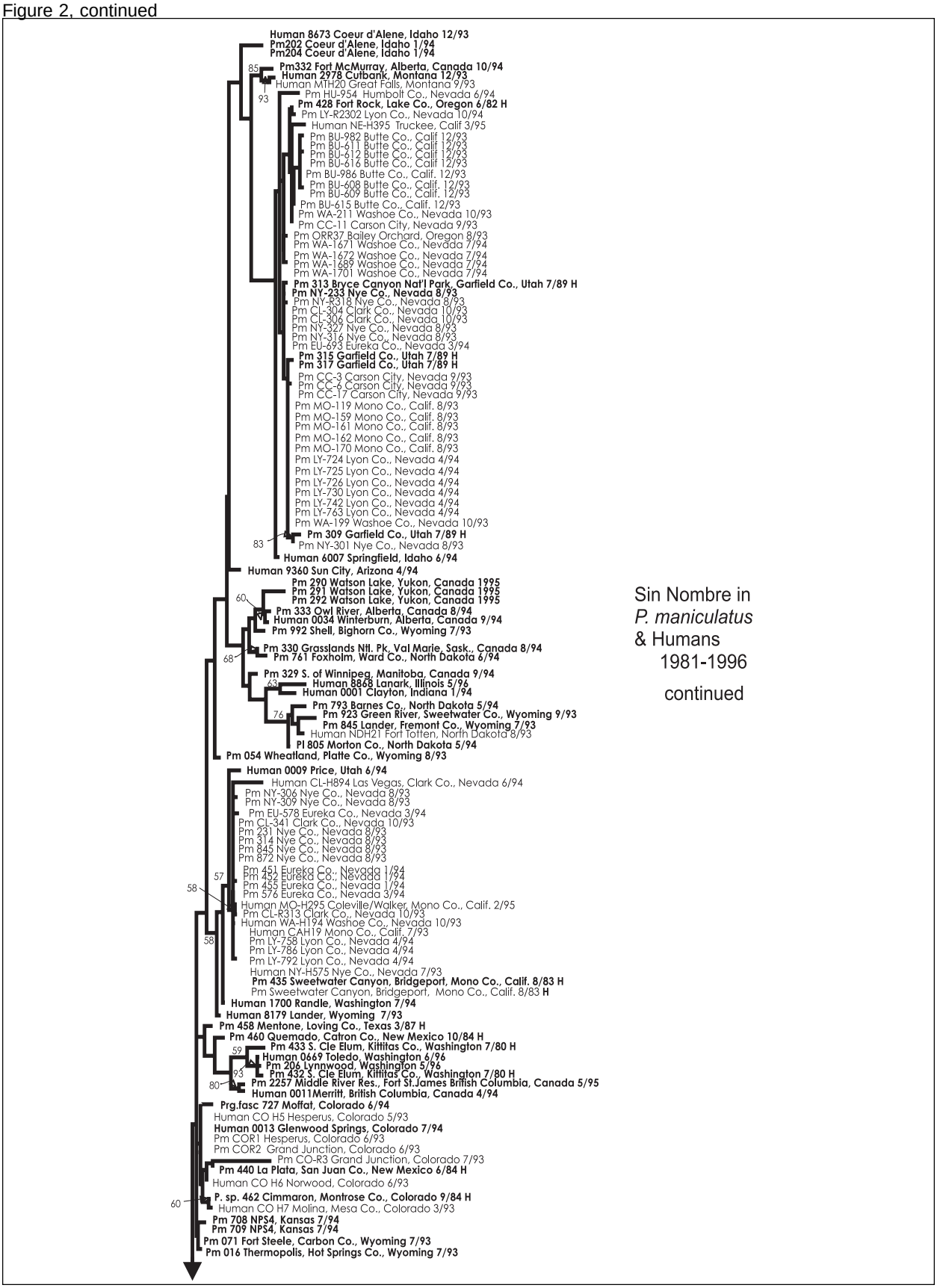

Figure 1. Overall hantavirus phylogenetic tree based on analysis of a 139 nucleotide fragment of the G2 coding region of the virus M segment. All newly reported sequences are shown bolded. The three virus groups corresponding to the hantaviruses carried by rodents of the subfamilies *Murinae*, *Sigmodontinae* and *Arvicolinae* are indicated. **P** indicates the clade containing the Sin Nombre-like viruses found in *Peromyscus* species rodents, the details of which are shown in figure 2. Horizontal branch lengths are proportional to the nucleotide step differences between taxa and predicted nodes. No scale bar is indicated as the actual number values are arbitary due to the weighting used in the successive approximations method (see appendix for details). Bootstrap values greater than 50% are indicated above branches. Virus labels include the virus or virus lineage name (ISLA, Isla Vista; TULA, Tula; PH, Prospect Hill or Prospect Hill-like; KBR, Khabarovsk; PUU, Puumala; SN, Sin Nombre; ELMC, El Moro Canyon; CDG, Caño Delgadito; BCC, Black Creek Canal; BAY, Bayou; JUQ, Juquitiba; AND, Andes; LN, Laguna Negra; THAI, Thailand; DOB, Dobrava; HTN, Hantaan; SEO, Seoul), species source of material (Mcalif, *Microtus californicus*; Mross, *Microtus rossiaemeridionalis*; Marv, *Microtus arvalis*; Mpenn, *Microtus pennsylvanicus*; Mmont, *Microtus montanus,* Mochro, *Microtus ochrogaster*; Mfort, *Microtus fortis*; Cg, *Clethrionomys glareolus*; Rm, *Reithrodontomys megalotis*; Salst, *Sigmodon alstoni*; Shisp, *Sigmodon hispidus*; Op, *Oryzomys palustris*; Cal, *Calomys laucha*; Bi, *Bandicota indica*; Apfl, *Apodemus flavicollis*; Apag, *Apodemus agrarius*; Rn *Rattus norvegicus*), identifier, and state, region or country of origin. A spreadsheet containing the details of all samples is available at http://www.cdc.gov/eid.

Figure 2. Phylogenetic tree of hantaviruses associated with *Peromyscus* species rodents. Figure provides a detailed view of clade **P** in figure 1. **S** indicates clade containing classical SN virus samples detected in humans or *P. maniculatus*. See figure 1 legend for overall tree description. Additional species source of material abbreviations include: Pm, *Peromyscus maniculatus*; Pl, *Peromyscus leucopus*, Prg.fasc, *Perognathus fasciatus;* Tam.quad, *Tamias quadrimaculatus*; Pt, *Peromyscus truei*; Mus musc., *Mus musculus*, and Tam.dors., *Tamias dorsalis*. Samples from historic materials are followed by an **H**.

the corresponding clades (13). Bootstrap values of 70% or higher corresponded to a probability of 95% or higher that the corresponding clade was correctly identified. Values of 50% or lower corresponded to a probability of 65% or lower that the clade was correctly identified (13).

Diversity of New World Hantaviruses

As expected on the basis of earlier nucleotide sequence analysis of a limited number of complete S or M hantavirus genome segments or virus genome fragments (5), the evolutionary relationships among hantaviruses were closely correlated with those of their known or suspected primary rodent reservoirs (Figure 1; Table 1). Hantaviruses associated with subfamily *Murinae* rodents (Hantaan, Dobrava, Seoul, and Thailand viruses) are clearly separated from those associated with *Arvicolinae* and *Sigmodontinae* rodents. The *Arvicolinae*-associated viruses (Puumala, Khabarovsk, Tula, Isla Vista, Prospect Hill [PH], and PH-like viruses]) form a reasonably well-supported clade, but the phylogenetic position of this group relative to the *Murinae*- and *Sigmodontinae*-associated viruses is not well resolved.

The New World hantaviruses of the *Arvicolinae* group, primarily associated with *Microtus* species voles, include not only the classic PH virus (labeled PHV-1), originally isolated from *M. pennsylvanicus* in Maryland (14,15), and two other distinct PH-like virus lineages recently found in this vole species in North Dakota (R737 and R731; R742), but also Isla Vista virus in *M. californicus*, PH-like hantavirus lineages in *M. ochrogaster* in North Dakota (R812 and R789), and *M. montanus* in Wyoming and Nevada (3485; LY-R2312) (16,17). Virus phylogenetic placement is not clearly correlated with *Microtus* species of origin, indicating that either spill-over infection or host switching may occur with these viruses. An apparent example exists in the Ohio rodent samples of spill-over of a PH-like virus infection from *Microtus* species rodents to a deer mouse *Peromyscus maniculatus* (Pm1047). These viruses have not been associated with HPS cases.

The viruses associated with the subfamily *Sigmodontinae* rodents are highly diverse and are made up of several distinct viruses and lineages in North and South America. All viruses associated with *Peromyscus* species rodents form a well-supported distinct monophyletic clade

(labeled P in Figure 1); these viruses constitute the major cause of HPS cases in North America. Other HPS-associated viruses in this group include Black Creek Canal virus, associated with *Sigmodon hispidus*. This virus, the cause of a single HPS case, has been genetically detected in cotton rats throughout southern Florida but, so far, nowhere else in the United States. Another genetically distinct virus, Muleshoe virus, has been identified in *S. hispidus* from the western part of its range (18), but sequences were not available for comparison at the time of our analysis. Caño Delgadito virus, found in *S. alstoni* in Venezuela (19), appears to be monophyletic with Black Creek Canal viruses. However, bootstrap support for this relationship is low (lower than 50%). Reasonable support is found for the clade containing both these *Sigmodon* sp.-associated viruses and the Bayou viruses, present in *Oryzomys palustris* throughout the southeastern United States from the Atlantic coast to Texas (20-22). Bayou viruses have been associated with three HPS cases (20,22). El Moro Canyon virus has been found in numerous harvest mice (*Reithrodontomys megalotis*) throughout the southwestern United States but has also been found in other rodents (e.g., WA-R2025, in *M. montanus*), presumably indicating spill-over infections (16,18,23,24). So far, these viruses have not been associated with human disease. The current phylogenetic analysis places these viruses in a distinct supported clade.

We analyzed hantaviruses that are also associated with HPS cases in South America and form a well-supported clade that encompasses viruses from Brazil, Argentina, and Paraguay, including the original Juquitiba virus detected in a human autopsy sample from an HPS patient in Brazil in 1993 (25-27). The rodent host for this virus is unknown. Two additional hantavirus lineages have been detected in more recent Brazilian HPS cases (Johnson and Nichol, unpub. data), suggesting that at least three genetically distinct hantaviruses are associated with HPS cases in Brazil. One of these lineages (b9618005) is phylogenetically closer to the Andes virus found in Argentina (28). Andes virus has recently been associated with several HPS cases in Patagonia; its likely host is *Oligoryzomys longicaudatus* (5,28,29). Finally, Laguna Negra viruses form a well-supported monophyletic lineage. This virus, associated with a large HPS outbreak in the Chaco region of Paraguay, is found in *Calomys laucha* rodents (10,30).

SNV-Like Viruses of *Peromyscus* Species Rodents

We analyzed 229 SNV-like viruses associated with *Peromyscus* species rodents; they form a well-supported (83%) clade (labeled P in Figure 1; details shown in Figure 2) and are distinct from other *Sigmodontinae*-associated hantaviruses. These SNV-like viruses include many classic SNVs, which are the major causes of HPS cases throughout the western and central United States and Canada, and are primarily associated with *P. maniculatus*. These viruses form a distinct, well-supported (78%) clade (labeled S in Figure 2), separate from other SNV-like viruses (Figure 2). Classic SNV 139 bp G2 fragments show up to an 18% nucleotide sequence divergence. Despite a number of exceptions, different genetic variants of SNV are grouped, generally speaking, by geography—an approximate geographic progression is apparent from the north and west toward the south and east, from the top of the tree down toward the node connecting these SNVs (labeled S in Figure 2). For instance, all samples from western Canada, including the Yukon, British Columbia, Alberta, Saskatchewan, and Manitoba are in the upper portion of this clade; two major lineages in California and Nevada (16,31) are also in this clade region. The lower part of the clade is dominated by viruses associated with the original Four Corners outbreak (New Mexico, Colorado, Utah, and Arizona) and other viruses from the Southwest, such as Kansas and Texas. Human HPS cases are represented throughout the SNV clade, indicating that these SNV variants can be associated with HPS illness.

In addition to recent samples, 30 SNV-like virus samples from the 1980s were included in the analysis to examine stability of the various SNV genetic lineages and their distribution (labeled H in Figure 2). Only small numbers of nucleotide differences, if any, were observed between old and recent virus sequences from the same geographic areas. The most striking example is the detection of identical viral G2 fragment sequences in rodents captured 12 years apart in New Mexico (Pm434) and Arizona (Pt AZ R29). Similarly, identical viral G2 sequences were found in rodents captured in eastern California in 1983 (our Pm435 and the previously

published Sweetwater Canyon sequence [32]) and in human and rodent materials from eastern California and western Nevada sampled 10 or more years later (e.g., Humans CAH19 and NY-H575, and Pm LY-758, 786, and 792). Other examples include 1 of 139 and 2 of 139 nucleotide sequence differences between Washington rodent Pm432 (captured in 1980) and Pm206 and HPS case 0669 (sampled 16 years later), respectively; only 2 of 139 nucleotides are different between Pm428 from southern Oregon and Pm LY-R2302 from northern Nevada, despite capture 12 years apart. These and other data (6,7,32,33) suggest that SNV has been present in North America for a considerable time and has been relatively stably maintained in rodent populations.

The next most closely related viruses are those detected in the northeastern United States, referred to as New York virus (34). These viruses have been detected in two human HPS cases and in *P. leucopus* in New York and Rhode Island (Figure 2). The 139 nucleotide fragments of these viruses have up to 10.1% nucleotide variation, and they differ from classic SNVs by at least 11.5% at the nucleotide level. The next closest group contains viruses associated with several "forest form" subspecies of *P. maniculatus* throughout the eastern United States and Canada, including the cloudland deer mouse (*P. maniculatus nubiterrae*), which inhabits the Appalachian mountain region (35). These viruses can also be found in some *P. leucopus* in this region (e.g., rodent Pl 313 from Pennsylvania). Up to 17.3% nucleotide variation can be seen among the 139 nucleotide fragments of these viruses. The name Monongahela has been suggested for this virus lineage (36), which differs from New York and SN viruses by at least 8.6% and 10.8% nucleotide differences, respectively. Another distinct hantavirus lineage can be seen in *P. maniculatus* in Tennessee and has been associated with an HPS case (0027) in eastern North Carolina. These viruses are 7.9% different from one another at the nucleotide level for the 139 nucleotide fragment analyzed, and at least 12.2%, 14.4%, and 15.8% different from New York, Monongahela, and SN virus lineages, respectively. Additional distinct virus lineages, recently referred to as Blue River virus (37), can be detected in *P. leucopus* in Oklahoma (Pl 707), Indiana (e.g., Pl 9436372 and Pl 9436378), and Missouri (e.g., Pl 170). The Oklahoma lineage virus is 10.1%, 10.8%, 15.8% different from the viruses in the Missouri, Indiana, and Tennessee lineages.

In addition to identifying the distinct SNV-like viruses and virus genetic lineages throughout North America, our study provides data suggesting the likely site of infection and minimum incubation time for some HPS cases. As reported earlier (2), the HPS case labeled CO H5 was originally described as an Arizona case because the person was residing near Springerville, Arizona, when the illness began. However, the person had been living in Hesperus, Colorado, 11 days before disease onset. The PCR fragment amplified from the case autopsy specimen and from the *P. maniculatus* trapped at the household in Hesperus matched exactly and differed from those amplified from *P. maniculatus* in the Arizona location (Figure 2). Similarly, a patient (labeled human 0038) whose symptoms began in Los Angeles, California, had been in the Santa Fe, New Mexico, area 28 to 35 days before illness onset. Analysis of PCR fragments linked the source of infection to New Mexico, rather than to California (Figure 2).

Virus and Host Genetic Relationships and Evolution

The genetic data we present indicate a broad spectrum of genetic variants of SNV-like viruses throughout North America, associated primarily with *Peromyscus* rodents. Recent analysis of rodent mitochondrial DNA sequence differences suggests that the different SNV-like virus lineages are primarily associated with different *Peromyscus* species, and in some cases, with phylogenetically distinct subspecies or mitochondrial DNA haplotypes (Morzunov and Nichol, unpub. data; 37). For instance, the classic SNV and the Monongahela virus lineages are found associated with the "grassland form" and "forest form" of *P. maniculatus*, respectively (they represent different subspecies and appear phylogenetically distinct with respect to their mitochondrial DNA [Morzunov and Nichol, unpub. data]). The New York virus, and the Blue River virus lineages found in Indiana and Oklahoma, appear associated with genetically distinct *P. leucopus* populations (37). This pattern likely reflects microadaptation of the virus to the rodent host and not just geographic isolation of the virus variants. This view is supported by the observation that even in areas

such as the eastern United States (particularly the Appalachian Mountain region), where *P. maniculatus* (forest form) and *P. leucopus* (eastern form) are sympatric and share microhabitat, extensive virus mixing between species is not seen; the Monongahela virus lineage is found predominantly in *P. maniculatus,* and the New York virus lineage in *P. leucopus*. Such data suggest that the broad correlation clearly evident between virus evolutionary relationships and those of their primary rodent reservoirs likely exists even at the finer level of closely related species and subspecies. However, the fact that the *P. leucopus*–associated New York virus appears phylogenetically closer to the *P. maniculatus*–associated viruses (SN and Monongahela) than to other *P. leucopus*-associated viruses (Blue River) suggests that this coevolutionary relationship is not absolute and that some species jumping (host-switching) may also have occurred. While the exact phylogenetic relationship of the SNV lineages to Monongahela, New York, and the other *P. leucopus* virus lineages is not well resolved by using the 139-bp G2 fragment we analyzed, analysis of more complete sequence data strongly supports a similar topology, placing New York virus firmly within the clade of *P. maniculatus*-borne viruses (37). This evidence, together with significant spill-over infection that sometimes occurs between sympatric rodents, illustrates the complexity of the hantavirus-host interactions.

This observation leads into another area of complexity, namely, the definition of distinct hantavirus serotypes or species. In the past, a newly identified arbovirus would be considered a distinct virus or virus serotype if a fourfold or greater two-way difference between this virus and previously recognized closely related viruses was obtained in virus neutralization assays. Despite the obvious biologic limitation (a single amino acid change can allow virus to escape from neutralization), this traditional criterion correlates remarkably well with more recent molecular data. One problem is that hantaviruses are generally difficult to isolate in tissue culture and are frequently noncytopathic, often making plaque assay analysis impractical (Table 1).

An attempt to define distinct virus species by using more widely used general criteria for the definition of biologic species is under way. Most defined species could be described as the lowest taxonomic unit that is geographically and ecologically contained, reproductively isolated, phylogenetically distinct, and self-sufficient. The apparent long-term maintenance and coevolution of phylogenetically distinct hantaviruses with different primary rodent reservoir species provides a foundation on which to build a hantavirus species definition. That is, if little host switching has occurred and if instead hantaviruses are associated with specific primary rodent reservoir species for many thousands of years, identification of a hantavirus in a unique primary rodent reservoir species would strongly suggest that in further analyses (e.g., nucleotide and amino acid sequence, cross-neutralization), it will be found to represent a new virus species. Hantaviruses maintained in rodent hosts from different genera (e.g., SNV in *Peromyscus* species rodents compared with Black Creek Canal virus in *Sigmodon* species rodents) will clearly meet the broad criteria for separate species status. This view is reinforced by recent data showing that stable reassortant viruses of different SNV genetic lineages can be readily detected in nature (31,38) and in tissue-culture mixed infections (39), but not in virus pairs such as SNV and Black Creek Canal virus (39). Difficulty can arise when trying to determine the species status of viruses maintained within rodent hosts of the same genera or species. So far, SN, New York, Monongahela, and Blue River viruses have been suggested as distinct hantaviruses with independent species names (5,36,37). The genetic analysis we present suggests that, as more hantavirus-infected *Peromyscus* species samples are analyzed, it is increasingly difficult to draw clear lines separating these virus species. The decision regarding whether to lump these viruses together as SNV-like viruses or to split them into separate species status will require the availability of neutralization data for several representatives of each virus, more detailed identification of the virus-host relationships, and more complete genetic characterization of both viruses and their hosts.

Appendix

Rodent and HPS Case Materials

The newly described nucleotide sequences were derived from rodent materials collected as part of a nationwide survey of rodents for hantavirus antibodies (Ksiazek et al., unpub. data). Most of the

human HPS-case blood and tissue autopsy samples were obtained and examined during the original investigation of an HPS outbreak in the Four Corners area of the southwestern United States in 1993 and as part of national surveillance for hantavirus disease throughout the United States from 1993 to 1997. Canadian rodent and HPS case materials were provided by the Laboratory Centre for Disease Control, Canada. Historic rodent samples were obtained from the Division of Biological Materials of the Museum of Southwestern Biology (Albuquerque, NM), University of New Mexico.

RNA Extraction, RT-PCR Amplification and Sequencing

Total RNA was extracted from human and rodent tissues, blood, or serum (2,10). Because of the hazardous nature of the virus, homogenization of rodent and human autopsy materials and extraction of RNA were performed in a certified class IIb laminar flow biosafety hood in Biosafety Level 3 containment. RNA was extracted from tissue or blood products by using acid guanidinium thiocyanate and phenol-chloroform and purified by using the RNaid Kit (Bio 101, La Jolla, CA). Nested RT-PCR assays were used to amplify DNA products containing a small fragment of the G2 coding region of M segment (2,10). Rodent and human samples were amplified separately, and all manipulations that might result in possible cross-contamination of samples were avoided. PCR products of correct size were sequenced with the same primers used for second-round PCR amplification in conjunction with various generations of sequencing kits available from Applied Biosystems, Inc. (Perkin Elmer, Foster City, CA). Sequences 139 nucleotides in length determined from each PCR product were used in phylogenetic analysis.

Oligonucleotide Primer Design

Oligonucleotide primers were used to generate DNA fragments from the G2 region of hantavirus M RNA (Table 2). In the initial phase of this project, amplification of hantavirus sequences from autopsy tissues of fatal HPS cases and hantavirus antibody-positive rodents in the southwestern United States used primers designed on the basis of nucleotide sequences conserved among PH and Puumala viruses (2). On the basis of SNV nucleotide sequences derived from these materials, new primers were designed and optimized for detection of SNV-like viruses associated with *P. maniculatus* (11). As more sequence data became available, additional generations of primers were refined that would detect hantaviruses from other geographic regions of the United States. The development of broadly reactive primers designed to detect hantaviruses associated with subfamily *Sigmodontinae* rodents (10) has eliminated the effort of amplifying RNA samples with many sets of primers.

Nucleotide Sequence and Phylogenetic Analyses

Compilation, alignment, and comparative nucleotide sequence analysis was carried out by using the Wisconsin Sequence Analysis Package, version 8.1 (Genetics Computer Group, Inc., Madison, WI) on a DEC 3000-500X AXP workstation (Digital Equipment Corp., Maynard, MA). Phylogenetic analysis was performed by maximum parsimony analysis using PAUP version 4.0 d52 (12) on a Power PC 9500. The size and complexity of the dataset prevented the

Table 2. PCR and sequencing primers

1st-round primers (5' to 3')	2nd-round primers (5' to 3')	Basis of primer design (ref.)	Amplicon size
TTTAAGCAATGGTG(C/T)ACTAC(T/A)AC CCATAACACAT(A/T)GCAGC	AGAAAGAAATGTGCATTTGC CCTGAACCCCATGC(A/T/C)CCATC	Puumala/Prospect Hill/ *Arvicolinae* (2)	278
TTTAAGCAATGGTG(C/T)ACTAC(T/A)AC CCATAACACAT(A/T)GCAGC	AAGGTAACACAGT(G/C)TCTGGATTC GGTTATCACTTAGATC(C/T)TGAAAGG	Sin Nombre/Western U.S. 1st generation (2)	185
AGAAAGATCTGTGGGTTTGC CCTGAACCCCAGGCCCCGT	AAGGTAACACAGT(G/C)TCTGGATTC GGTTATCACTTAGATC(C/T)TGAAAGG	Sin Nombre/Western U.S. 2nd generation (11)	185
TGTGTGTTTGGAGACCCTGG TC(A/G)ATAGATTGTGTATGCA	ATGTCAACAAC(A/G)AGTGGGATG CATGGGTTATCACTTAG(G/A)TC	Sin Nombre Nevada/ E. California (31)	185
CAGAAAGATCTGCGGGTTTGC CCCGAGCCCCATGCACCAT	CAAGGGAATACTGTCTCTGGATTT GATTGTCACTCAGATCTTGAAATG	Bayou virus/LA/ East Coast/ S. American (19,20)	185
TGTGAITATCAAGGIAAIAC ACIG(A/T)IGCICCATAICACAT	TGTGAITATCAAGGIAAIAC CCCCAIGCICCITCAAT	General *Sigmodontinae* (10)	242

use of branch and bound search method and weighting of the data matrix based on transition:transversion bias. Maximum parsimony analysis of the hantavirus G2 fragment nucleotide sequences was carried out by using the heuristic search option. The initial unweighted analysis showed considerable homoplasy within the dataset. A successive approximations method was used in which characters were weighted by using the maximum value of their rescaled consistency index (12), and the heuristic search repeated. Bootstrap analysis was carried out by 500 replicates of the heuristic search with random resampling of the data. The analysis required several months of computer time; thus, it was not possible to include some recently published additional hantaviruses sequences. The nucleotide sequence dataset (bigtree.nex) we used is available in NEXUS format (compatible with most phylogenetic analysis software packages) accessible through the journal website (http://www.cdc.gov/eid). A brief description of all samples analyzed (bigtree.exl in EXCEL 2.1 spreadsheet format), and the phylogenetic tree of Figures 1 and 2 can also be found at the same location on the website.

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Climatic and Environmental Patterns Associated with Hantavirus Pulmonary Syndrome, Four Corners Region, United States

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To investigate climatic, spatial, temporal, and environmental patterns associated with hantavirus pulmonary syndrome (HPS) cases in the Four Corners region, we collected exposure site data for HPS cases that occurred in 1993 to 1995. Cases clustered seasonally and temporally by biome type and geographic location, and exposure sites were most often found in pinyon-juniper woodlands, grasslands, and Great Basin desert scrub lands, at elevations of 1,800 m to 2,500 m. Environmental factors (e.g., the dramatic increase in precipitation associated with the 1992 to 1993 El Niño) may indirectly increase the risk for Sin Nombre virus exposure and therefore may be of value in designing disease prevention campaigns.

Relationships between environmental characteristics (i.e., climate, biome, elevation, habitat structure, and microhabitat), time, and hantavirus pulmonary syndrome (HPS) cases in the United States have not been systematically evaluated. We describe environmental factors associated with probable exposure sites for all diagnosed HPS cases in the Four Corners region (Arizona, New Mexico, Colorado, Utah) of the United States before 1996 and evaluate the strength of the associations between climate, time, and HPS cases.

Case and Exposure Site Identification

All cases met the surveillance case definition for HPS, which requires the presence of clinically compatible symptoms and laboratory confirmation (1).

Fifty-nine sites in Arizona, New Mexico, Colorado, and Utah were identified as probable exposure sites for HPS cases (sites at which HPS patients were most likely infected with Sin Nombre virus (SNV), according to previously collected data from environmental assessments and individual patient questionnaires [2]) occurring from 1985 to 1995. Rodent trapping and testing data were also used to determine exposure sites, including those identified by linking hantavirus genome sequences between patients and rodents (3,4). Our inability to determine precisely where persons were exposed to HPS may have created a selection bias.

Climate Investigations

Climate analyses were limited to 52 probable exposure sites of HPS cases (n = 53 cases) (two case-patients were exposed to SNV at the same

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site); onset of illness was between 1993 and 1995. Climate data for probable exposure sites were collected from the nearest weather station monitored by the National Oceanic and Atmospheric Administration's Western Regional Climate Center in Reno, Nevada. Ten years (1986 to 1995) of monthly precipitation sums and monthly averages of daily ambient temperature were obtained for each station. To be included, sites had to be located within a 30-km radius and a 300-m elevation range from their closest corresponding weather station and without a mountain range between the exposure site and the identified weather station. Twelve sites did not meet these inclusion criteria, six were removed from precipitation analyses, and five were removed from temperature analyses because of missing weather data. Data from 24 weather stations were used for the precipitation (n = 34 exposure sites) and temperature analyses (n = 35 exposure sites). Four weather stations represented multiple exposure sites because they were the closest available weather stations. Twenty-two (85%) exposure sites included in the analyses were within 15 km (mean = 13.2 km, standard deviation [SD] = 7.9 km) and 150 m of elevation (mean = 78.0 m, SD = 79.2 m) of their corresponding weather station. Data were analyzed with the SPSS and Epi-Info statistical software programs (5,6).

The use of climatic data collected from weather stations as far as 30 km away from probable exposure sites, with as much as a 300-m difference in elevation, may have created an ascertainment bias. Weather patterns, however, generally cover large regions. In addition, a withdrawal bias may have been introduced by eliminating exposure sites and weather stations that did not meet the inclusion criteria.

Precipitation Analysis

Precipitation totals from the 24 identified weather stations for each of the 48 months during 1992 through 1995 were compared with the corresponding calendar month's mean precipitation total for 1986 to 1991. Each month's mean precipitation difference was plotted against the number of HPS cases with onset of symptoms during that month for cases between 1993 and 1995. The Wilcoxon matched-pairs signed rank test was used to test the statistical significance of precipitation during two periods of substantial departure from normal precipitation patterns (i.e., 6-year mean sum precipitation data for the same calendar months). These weather stations, representing the 1993 to 1995 cases, reported above average ($p < 0.01$) precipitation totals during December 1992 through March 1993 and below average ($p < 0.01$) precipitation totals during June 1993 through July 1993 (Figure 1).

By using a Spearman's correlation, we found a negative correlation between the number of cases per month (onset date) and the number of months after the 1992 to 1993 El Niño weather pattern ($r_s = -0.70$; $p < 0.01$) (Figure 2). For this portion of the precipitation analysis, the months of onset were used for all 53 cases between 1993 and 1995. The end of the 1992 to 1993 El Niño was described as the month (March 1993) in which the abnormally high precipitation totals for November 1992 through March 1993 returned to normal, on the basis of the data in Figure 1.

The 1993 outbreak of HPS in the Four Corners region followed a dramatic increase in precipitation associated with the 1992 to 1993 El Niño phenomenon and peaked in the middle of a drought. Because the 1992 to 1993 El Niño resulted in an abundance of rodent food resources (e.g., vegetation and insects) and a 20-fold rodent population increase over the previous year at the Sevilleta National Wildlife Refuge in central New Mexico, increased rainfall from El Niño was associated with the 1993 HPS outbreak (7). A similar pattern of above average rainfall followed by drought was observed preceding an outbreak of HPS in western Paraguay in 1995 to 1996 (8). Our study shows that the number of HPS cases per month in the Four Corners region during 1993 to 1995 was negatively correlated with the number of months after the 1992 to 1993 El Niño. The data suggest that the association between the 1992 to 1993 El Niño and the number of HPS cases in the Southwest may have lasted for as long as 2 years.

The association between El Niño and the HPS outbreak is probably complex. The above average precipitation during the winter and spring of 1992 to 1993 may have increased rodent populations and thereby increased the likelihood of more rodent-to-rodent contact, rodent-to-human contact, and viral transmission, thereby resulting in the large number of cases in 1993 and 1994. In addition, as rodent populations surpassed the carrying capacity of their local

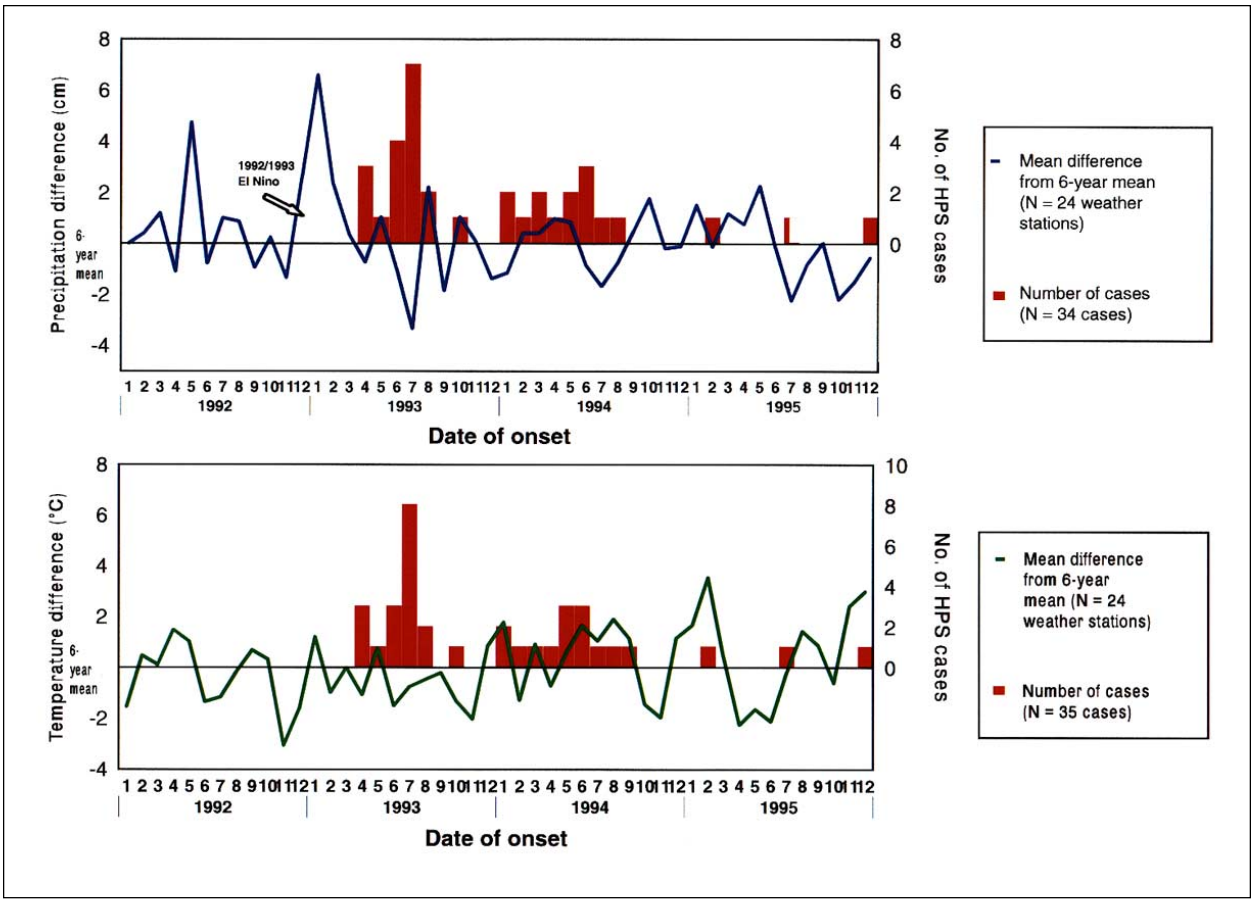

Figure 1. Mean difference in monthly precipitation and temperature between month of interest and 6-year mean (1986–1991) at the study sites and number of cases by month, 1993–1995.

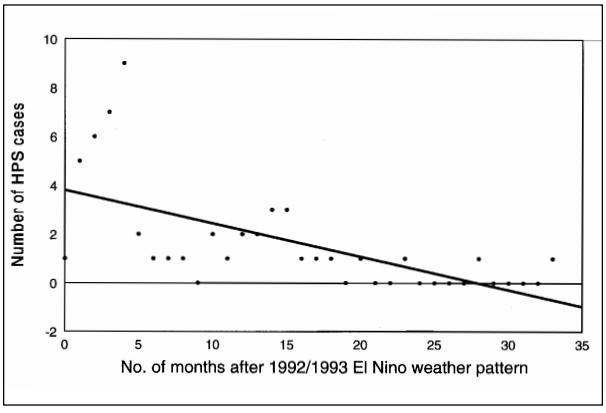

Figure 2. Correlation of hantavirus pulmonary syndrome (HPS) cases per month and number of months from end of El Niño period (n = 53 cases).

environments and precipitation plummeted, available food sources may have been depleted, resulting in rodent population stress. Increased stress likely increased rodent-to-rodent contact, as rodents competed for food and water, and increased rodent-to-human contact, as rodents moved into new, potentially less stressful environments, such as homes and peridomestic structures.

During 1995, no cases occurred in the original outbreak area near the Arizona-New Mexico border and in western Colorado, possibly because the effect of the 1992 to 1993 El Niño had dissipated. Preliminary data from longitudinal trapping studies in the Southwest suggest that the relative rodent densities decreased to normal levels in 1995 (J. Mills, T. Yates, pers. comm.). Hantavirus infection rates in rodents dramatically decreased at case sites in Arizona and Colorado 3 years after the outbreak (9).

Temperature Analysis

Monthly ambient temperature means from the 24 identified weather stations for each of the 48 months during 1992 through 1995 were compared to the corresponding calendar month's ambient temperature mean for 1986 to 1991. The mean difference was plotted against the number of HPS case onsets per month for cases occurring between 1993 and 1995. With all 24 weather stations included, the Wilcoxon matched-pairs signed rank test was used to test the statistical significance of two periods with substantial departures from normal temperature patterns (i.e., 6-year mean daily temperature averages for the same calendar months): 1) November 1992 through December 1992 and 2) January 1993. The 24 weather stations reported below average ($p < 0.01$) temperatures during November 1992 through December 1992, which corresponded with the onset of El Niño (Figure 1). Conversely, the mean temperature for January 1993 was above average ($p < 0.01$).

While interpreting variations in precipitation totals is fairly straightforward, interpreting variations in ambient temperature is far more difficult. Whether the lower-than-average ambient temperature during November 1992 through December 1992 affected rodent population dynamics is unknown; however, the higher-than-average temperatures during January 1993 may have promoted rodent survival during what is normally the coldest month of the year. (Figure 1 indicates that the 24 weather stations reported the most substantial mean temperature extremes in 1995.)

To examine the relationship between ambient temperature and month of exposure for HPS cases, we compared the distribution of daily ambient temperatures of probable month (defined as the month in which onset date minus 14 days [hypothesized mean incubation period] occurred) of exposure to daily ambient temperatures during the other 11 months of the same calendar year. Exposure months had higher ambient temperatures and a smaller temperature range than the other months in the same calendar year. The mean temperature for the probable exposure months was 15°C (SD = 7°C; range = 0°C - 24°C) (Figure 3), and the mean throughout the rest of the year was 11°C (SD = 9°C; range = -7°C - 36°C).

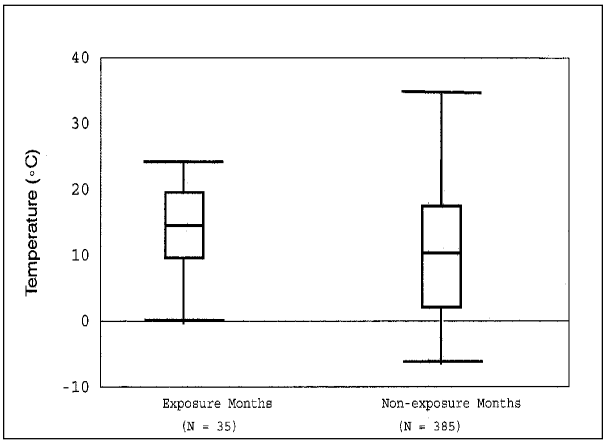

Figure 3. Distribution of monthly ambient temperatures for months estimated as the month SNV exposure occurred and the 11 nonexposure months for the same calendar year (n = 35 cases and 24 weather stations).

Spatial, Temporal, and Environmental Investigations

Spatial and environmental data were collected for 59 of the 64 known HPS cases that occurred before 1996 in the Four Corners region: Arizona (20 sites), New Mexico (25 sites), Colorado (7 sites), and Utah (7 sites). For five cases (in 1959, 1975, 1984, 1985, and 1993), the exposure site was not known. Habitat data (e.g., dominant biome type as described by Brown [10] within 200 m) were collected at each of the 59 probable exposure sites. Elevations were taken from U.S. Geological Service quadrant maps. All analyses were conducted with the SPSS and Epi-Info statistical software programs (5,6).

Spatial Analysis

Figures 4, 5, and 6 display the spatial and temporal distribution of probable hantavirus exposure sites in the Four Corners region, from 1993 through 1995 (n = 52). In 1993, most case-patients were exposed in northeastern Arizona and northwestern New Mexico; in 1994, HPS case-patients were exposed in all four states, including two new areas, central Utah and southern Arizona; and in 1995, HPS case-patients were exposed only in a small geographic area near the border of southcentral Colorado and northcentral New Mexico (on the eastern slope of the Rocky Mountains).

From 1993 through 1995, HPS cases in the Four Corners region shifted geographically. The

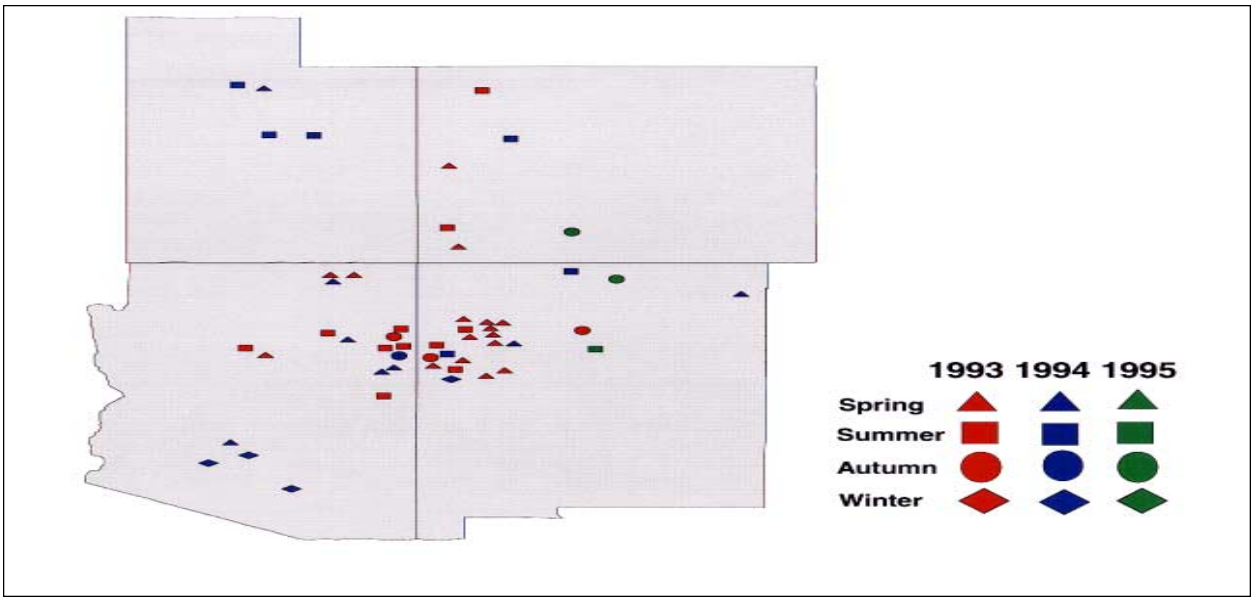

Figure 4. Hantavirus pulmonary syndrome cases in the Four Corners region, by probable exposure site location, 1993–1995 (n = 53 cases and 52 exposure sites).

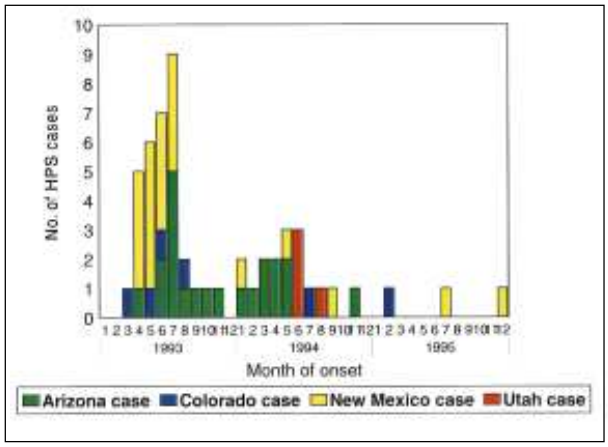

Figure 5. Hantavirus pulmonary syndrome cases in the Four Corners region by state, 1993–1995 (n = 53 cases and 52 exposure sites).

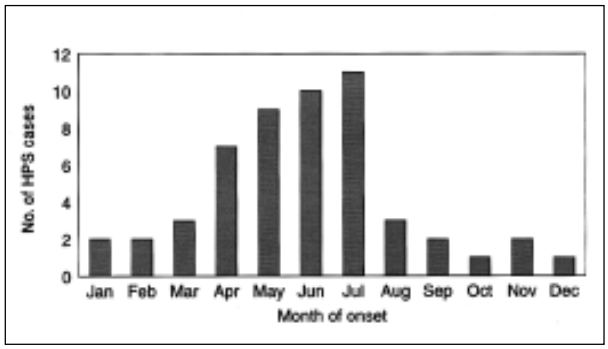

Figure 6. Hantavirus pulmonary cases in the Four Corners region, by month of onset, 1993–1995 (n = 53 cases and 52 exposure sites).

apparent spatial and temporal movement of HPS foci in this region mirrors the "focal nidality" exhibited by hemorrhagic fever with renal syndrome (HFRS), the Eurasian hantaviral manifestation (11-15). HFRS cases can occur sporadically throughout the year, but outbreaks occur seasonally (12,16). Seasonal prevalence varies by locality and meteorologic and climatic conditions that favor activity of rodents associated with viruses causing HFRS (12). Niklasson et al. (17) hypothesize that large outbreaks of HFRS occur in geographic "hot spots," which may depend on certain ecologic characteristics correlating with rodent populations, e.g., rodent habitats and environmental or meteorologic conditions (17). In the Four Corners states, seroprevalence of hantaviruses in rodents displays a similar focality (18). Our data suggest that HPS cases may have occurred in similar hot spots in this region between 1993 and 1995.

Temporal Analysis

The 1993 to 1995 HPS onset months were unevenly distributed (Kolmogrov-Smirnov test, $p = 0.01$), displaying a spring-summer seasonality, as has been reported previously (2,3,15) (Figure 6). While cases occurred throughout all seasons, probable exposures occurred during the time of year when the monthly mean ambient temperature was 0°C to 24°C.

The seasonality of HPS cases varies by location, elevation, and biome (Figures 4, 5, and 7).

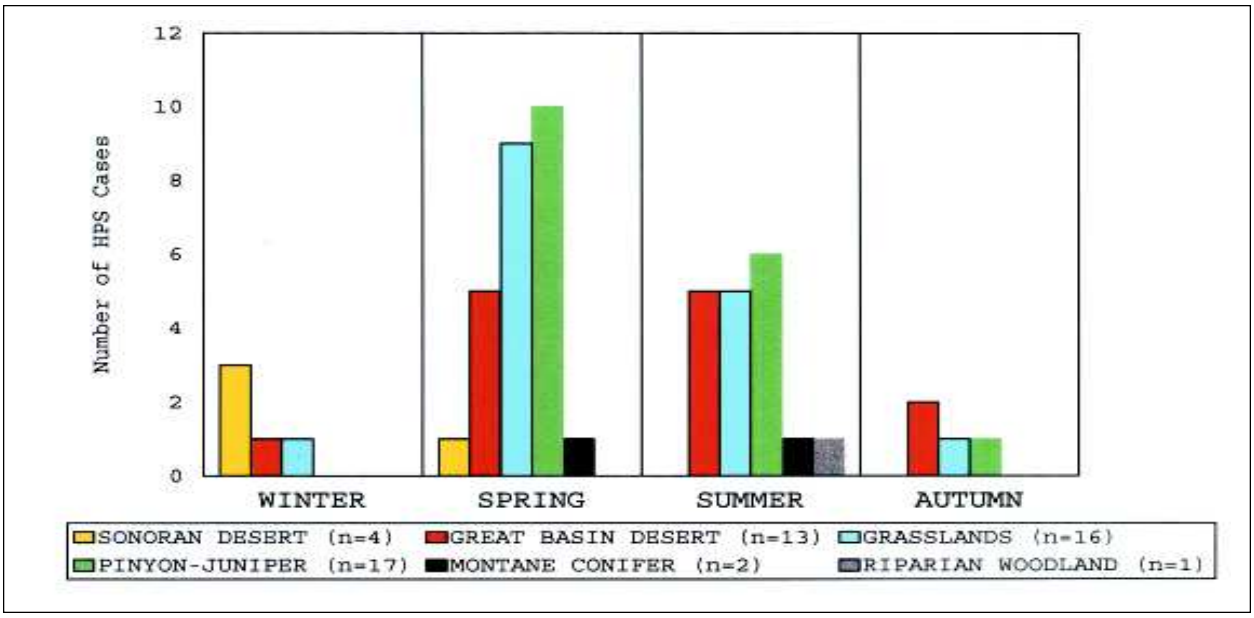

Figure 7. Number of hantavirus pulmonary syndrome cases occurring in specific biomes by season of onset (1993–1995) (n = 53 cases).

The clustering of all four Sonoran Desert HPS cases during the late winter and early spring and the two montane conifer cases during the late spring and summer, when the mean temperatures for both biomes are mild (12°C - 21°C), are the best examples of this trend. Most grassland, Great Basin scrub, pinyon-juniper, and montane conifer cases occurred during spring and summer (Figure 7).

Environmental Analysis

Probable exposure sites occurred in seven biomes, most often in pinyon-juniper woodland, grassland, and Great Basin desert scrub biomes (33.9%, 28.8%, and 23.7%, respectively) (Table). Approximately 66% (39/59) of case-patients were exposed at elevations of 1,800 m to 2,500 m; none was exposed at elevations higher than 2,500 m (Table). This description may reflect the typical biomes in which the 1993 outbreak took place but also holds for cases in Utah and Colorado. Mills et al. (18) reported the same habitat and elevation for sites with the highest *Peromyscus* densities (as well as the highest rodent SNV antibody prevalence) in the Four Corners states. Unpublished case-control data (J. Cheek and R. Bryan) show that significantly more HPS cases occurred in Great Basin pinyon-juniper than in any other biome on the Navajo reservation in the Southwest; the data also show that HPS cases are more likely at higher elevations. Environmental conditions surrounding HPS exposure sites appear similar to those surrounding plague exposure sites on the Navajo reservation (K. Gage, R. Enscore, unpub. data).

Table. Frequency of probable Sin Nombre virus exposure sites in selected biomes and elevation ranges

Biome type	Frequency (n = 59)	%
Sonoran Desert	4	6.8
Chihuahuan Desert	1	1.7
Great Basin Desert scrub	14	23.7
Great Basin and plains grassland	17	28.8
Great Basin pinyon-juniper	20	33.9
Riparian woodland	1	1.7
Montane conifer forest	2	3.4
Elevation range		
0 - 619	3	5.1
620 - 1,239	1	1.7
1,240 - 1,859	13	22.0
1,860 - 2,480	42	71.2

Probable exposure sites were most frequently found in rural areas, with five or fewer homes within a 1-km radius (64.4%); most exposure sites (88.1%) were found in areas with 25 or fewer homes. An almost equal number of exposure sites (44.1%) was more than 2 km away from a permanent water source as exposure sites (42.4%) within 1 km of a permanent water source. Overall, 104 plant species belonging to 72

genera were identified at exposure sites. The three most common dominant herbaceous species were *Artemisia tridentata* (big sagebrush), *Gutierrezia sarothrae* (snakeweed), and *Bromus tectorum* (downy chess grass); and the three most common dominant overstory and understory species were *Juniperus monosperma* (one-seed juniper), *J. osteosperma* (Utah juniper), and *Pinus edulis* (two-needle pinyon pine). For 21 of 59 sites with 5% slope or greater, more than twice as many had a northward aspect (9 of 21) than any other aspect (south = 4 of 21; east = 4 of 21; and west = 4 of 21).

While the habitat may have changed in minor ways between the time of exposure and data collection, no major habitat changes (e.g., fire, timber harvesting) were identified. Because these environmental findings are descriptive, caution should be used when drawing conclusions on the basis of these data.

Conclusions

Previous studies have examined behavior and environmental risk factors for the acquisition of HPS (19-22). Certain behavior and occupations may increase the likelihood of exposure to the excreta of infected rodents; however, the overall level of risk seems low (21,22). Environmental risk factors may be divided into endemic and epidemic. Endemic environmental risk factors are tied to the static habitat structure of a geographic area. For example, the habitat of the Four Corners region allows population levels of *Peromyscus maniculatus* great enough to maintain SNV transmission (18,23). Some level of risk will likely always exist for exposure to SNV in the region; however, the level seems low (9). Epidemic environmental risk factors, also tied to the static habitat structure of a geographic area (i.e., they can only occur in disease-endemic areas), are primarily dynamic events associated with large-scale environmental changes of limited duration. For example, the precipitation pattern during the 1992 to 1993 El Niño, associated with increased rodent populations in the Southwest (7) and consequently with the 1993 outbreak of HPS in the Four Corners region, greatly increased the level of risk for human SNV infection in the outbreak area. By 1995, rodent populations (J. Mills, T. Yates, pers. comm.) and the number of HPS cases in this area dramatically decreased, and the level of risk for human SNV infection may have returned to endemic levels in 1995. Early recognition of these endemic and epidemic environmental risk factors might allow public health agencies to predict where and when the next HPS outbreak may occur and thus to effectively target prevention efforts.

While this report does not address data related to cases after 1995, the 1997 to 1998 El Niño may not have displayed the same precipitation pattern in the Four Corners region as the 1992 to 1993 El Niño. Limited data from Arizona's primary weather station for northern Arizona indicate 4.70 cm of precipitation in December 1997—the 98-year mean (1899–1997) for December is 4.88 cm (Office of the State Climatologist—Arizona State University, pers. comm.). In December 1992, the same station reported 17.22 cm of precipitation (Office of the State Climatologist—Arizona State University, pers. comm.). Future studies should examine and compare the two precipitation events, their lasting effects on rodent populations, and the subsequent effects on hantavirus epidemiology.

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The following articles on hantavirus contain preliminary results of 3 years of longitudinal mark-release-recapture studies in the southwestern United States, provide an analysis of the sensitivity of the field techniques and statistical analyses for detecting changes in rodent population densities, and discuss the role of longitudinal studies in understanding reservoir host ecology as it relates to human disease. Although the data presented in the articles are overall standardized, methods used to extract information vary by research institution.

Long-Term Studies of Hantavirus Reservoir Populations in the Southwestern United States: Rationale, Potential, and Methods

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Hantaviruses are rodent-borne zoonotic agents that cause hemorrhagic fever with renal syndrome in Asia and Europe and hantavirus pulmonary syndrome (HPS) in North and South America. The epidemiology of human diseases caused by these viruses is tied to the ecology of the rodent hosts, and effective control and prevention relies on a thorough understanding of host ecology. After the 1993 HPS outbreak in the southwestern United States, the Centers for Disease Control and Prevention initiated long-term studies of the temporal dynamics of hantavirus infection in host populations. These studies, which used mark-recapture techniques on 24 trapping webs at nine sites in the southwestern United States, were designed to monitor changes in reservoir population densities and in the prevalence and incidence of infection; quantify environmental factors associated with these changes; and when linked to surveillance databases for HPS, lead to predictive models of human risk to be used in the design and implementation of control and prevention measures for human hantavirus disease.

Hantaviruses (genus *Hantavirus,* family Bunyaviridae) are rodent-borne zoonotic agents that cause mild to severe hemorrhagic fevers throughout most of Europe, Asia, and the Americas. The epidemiology of these hemorrhagic fevers is largely defined by the distribution and ecology of the rodent hosts of the viruses. Hantaviruses have been identified at a dramatically increased rate in recent years; some 30 hantaviruses are now recognized throughout the world (1,2). With very few exceptions, each virus is associated with a single primary rodent host of the family Muridae. The rodent, in which the virus establishes a chronic infection, sheds infectious virus into the environment in urine, feces, and saliva (3,4); these characteristics are key to the transmission of the virus, both to humans (most frequently by inhalation of infectious aerosols [5]) and among rodents (frequently by aggressive encounters and biting [6,7]).

Human diseases due to hantaviruses, which have been recognized at least since World War I and probably occurred much earlier (8), were unknown in the Americas until recently. Although *Rattus norvegicus* infected with Seoul virus is common in many cities throughout the Americas (9), human disease associated with a

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rat-borne hantavirus was not documented in a U.S. city until 1994 (10). Prospect Hill virus, an indigenous North American hantavirus, was isolated from the meadow vole (*Microtus pennsylvanicus*) as early as 1982 (11) but has never been associated with human disease.

Since 1993, when hantavirus pulmonary syndrome (HPS) was recognized and its etiologic agent, Sin Nombre virus (SNV), was isolated and associated with the deer mouse (*Peromyscus maniculatus*) (12,13), at least 20 New-World hantaviruses, all associated with the same group of indigenous American rodents (family Muridae, subfamily Sigmodontinae) have been described, and HPS has been diagnosed from Canada to Patagonia. The severity (approximately 50% death rate) and wide geographic distribution of this rodent-borne zoonotic disease has prompted intensive collaboration between public health investigators and ecologists to elucidate the ecologic and epizootiologic features of infection in host populations and the factors that lead to human infection.

Because no specific treatment is yet available, prevention measures are essential in decreasing HPS-related illness and death. Developing effective preventive measures requires a detailed knowledge of the ecology and epizootiology of hantavirus infection in reservoir populations and the specific situations and mechanisms that result in the transfer of hantaviruses from hosts to humans.

Reservoir Studies

Reservoir studies, whose role in understanding, controlling, and preventing human disease has been reviewed, have resulted in a series of research goals that may facilitate the collection of data concerning reservoir ecology, a subject pertinent to human health (14). The first goal is to identify the reservoir host; others are a) to determine the area in which the disease may be endemic by identifying the geographic range of the host and the range of infection by the pathogen within the host range; b) to more precisely define relative risk to humans by determining the distribution of the host and pathogen among distinct habitats regionally; c) to investigate potential mechanisms of pathogen transmission within host populations by conducting cross-sectional surveys to define the prevalence of infection among various subpopulations of the host (e.g., male versus female, juvenile versus adult); d) to conduct long-term prospective studies to explain the temporal patterns of infection in host populations; and e) to integrate the data from these studies in a predictive model that will allow early identification of specific times and places where conditions can increase rodent populations or infection in rodent populations and elevate the risk for human disease. This model could be used to minimize the incidence of human disease through public education, habitat modification, or reservoir control.

Investigations of HPS cases in the United States have resulted not only in studies of the deer mouse and SNV but also in the identification of three additional host-virus relationships that maintain hantaviruses responsible for human disease (New York virus carried by the white-footed mouse, *Peromyscus leucopus* [Figure 1] [15]; Black Creek Canal virus carried by the cotton rat, *Sigmodon hispidus* [16]; and Bayou virus carried by the rice rat, *Oryzomys palustris* [17]). Most HPS cases in the United States have been caused by SNV.

Figure 1. White-footed mouse (*Peromyscus leucopus*). Photo by R.B. Forbes, Mammal Image Library of the American Society of Mammalogists.

Intensive studies of deer mouse populations have addressed most of the proposed goals. One of the most common and most extensively studied small mammals in North America, the deer mouse has a well-known geographic distribution (18). Antibody screening of deer mouse populations throughout North America has provided evidence of SNV infection throughout most of the species' range (Ksiazek et

al., unpub. data). Regional studies have shown differences in the prevalence of hantavirus infection among deer mouse populations in different habitats and helped define the varying disease risk to humans in these habitats (7). Finally, studies of the age- or size-specific prevalence of hantavirus infection among reservoir populations have shown that SNV, and other hantaviruses, are transmitted horizontally within reservoir populations, and that one important specific mechanism of transfer may be aggressive encounters and bites, most frequently among male animals (6,7,19). Although these cross-sectional studies have increased our understanding of host-virus ecology as it relates to human disease, they have not explained the temporal dynamics of host-virus ecology nor have they identified the environmental factors associated with these dynamics; only long-term prospective studies can provide this additional information.

Long-Term Studies

Long-term studies, widely regarded by ecologists as indispensable for understanding the temporal dynamics of vertebrate communities (20), are especially useful for assessing the effects of rare events (e.g., El Niño southern oscillation) and for detecting and observing processes that unfold slowly in communities or populations (e.g., establishment or disappearance of a reservoir species from part of its range; changes in reservoir population density; changes in community composition; introduction or extinction of a pathogen in a specific host population; and changes in the incidence or prevalence of infection within the host population).

Long-term studies of reservoir populations have helped elucidate the temporal dynamics of hantavirus infection in host populations for Seoul and Prospect Hill viruses (21) and identify characteristics of reservoir ecology associated with outbreaks of human disease. The numbers of cases of hemorrhagic fever with renal syndrome due to Puumala virus in Scandinavia and Argentine hemorrhagic fever due to Junín virus (an arenavirus with many epidemiologic similarities to hantaviruses) were correlated with cyclic changes in the density of reservoir host populations (22,23). Increases in population density are associated with improved reproductive success and survivorship that may be due to improved habitat. Changes in the environment may be associated with favorable weather patterns, accelerated vegetation growth, and availability of plant and small-animal foods (14,23). The 1993 HPS outbreak in the southwestern United States may have resulted from improvements in the quality of deer mouse habitat caused by the 1991-92 El Niño southern oscillation (24). When the environmental variables associated with increasing reservoir population densities are identified and quantified, a key component of a predictive model of human risk will be in place.

Despite their importance and utility, long-term studies of reservoir populations associated with zoonotic agents are rare. By definition, they require stable funding for many years, they are labor intensive, expensive, and may not produce significant results in the short term. The periodic shifts in environmental conditions that change host populations and increase risk for human disease may take many years.

The most common method for conducting long-term studies of small-mammal populations is the mark-release-recapture (MRR) technique. Animals trapped live on permanent trapping plots are measured, sampled (blood or oral swab), identified with a permanent mark or number, and released at the exact site of capture. The trapping plots are operated at predetermined intervals for several days. Animals recaptured in subsequent trappings are measured and sampled again so that changes in numbers of animals, body growth rates, movement, reproductive condition, and infection status can be monitored. Environmental variables such as weather conditions and vegetative cover also may be monitored on the trapping plots. Control plots, where invasive procedures are minimized, may be necessary for determining or correcting for the influence of sampling methods on animal survival or population size.

After the 1993 HPS outbreak, the Centers for Disease Control and Prevention (CDC) established a network of hantavirus and rodent monitoring sites in the southwestern United States to 1) monitor and quantify the seasonal and year-to-year changes in host population density and the prevalence and incidence of hantavirus infection, 2) identify and quantify the biotic and abiotic environmental factors associated with and likely influencing these dynamics, 3) identify mechanisms of virus transmission within reservoir populations, and 4) identify and

measure any effects of infection on individuals and populations of the host.

The studies should lead to predictive models of human risk for hantavirus infection and should facilitate prevention and control of human hantavirus disease.

Study Sites

Arizona, Colorado, and New Mexico were chosen as the general study area because of their high numbers of HPS cases at the time the study was being designed. In addition, four sigmodontine rodent species identified as hantavirus reservoirs inhabit at least parts of the three-state area (*P. maniculatus, P. boylii, Reithrodontomys megalotis,* and *S. hispidus*). Longitudinal MRR studies are being conducted on 24 trapping webs at nine sites in the three states (Figure 2): 10 webs at four sites in New Mexico, operated by the University of New Mexico; six webs at three sites in Colorado, operated by Colorado State University; four webs at one site in northern Arizona, operated by Yavapai College; and four webs at one site in southern Arizona, operated by the University of Arizona. Site selection criteria included presence of populations of *Peromyscus* spp. and evidence of infection by SNV or related viruses in these populations. The location of each trapping web was fixed precisely by global positioning system technology. A sketch of each trapping web site, including a description of the vegetation, was prepared.

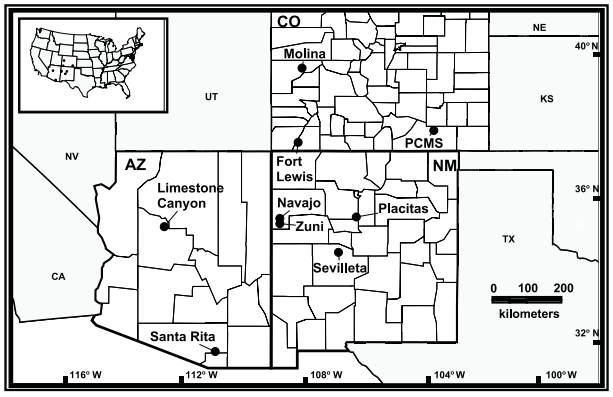

Figure 2. Geographic locations of nine sites where mark-release-recapture webs are being operated to study rodent reservoirs of hantaviruses in a three-state area of the southwestern United States. PCMS=Pinyon Canyon Maneuver Site (U.S. Army).

Placing Permanent Trapping Webs

Small-mammal populations were monitored through the use of permanent trapping webs (25) (Figure 3A). Each web covered 3.14 ha and contained 12 100-m transects radiating from a central point and resembling the spokes of a wheel (Figure 3B). Each web contained 148 Sherman (8 x 9 x 23 cm; H.B. Sherman Trap Company, Tallahassee, FL) and 24 Tomahawk (14 x 14 x 40 cm; Tomahawk Live Trap Company, Tomahawk, WI) live-capture traps, at 12 trap stations along each radiating spoke. The first four trap stations were at 5-m intervals and the remaining eight at 10-m intervals. Four Sherman traps were placed around the central point. In addition to a Sherman trap, one Tomahawk trap was placed at trap stations 7 and 12 in each radiating arm (Figure 3B). Two to four webs were located at each sampling site. At least one web at each site was designated a control web. At this web, rodent populations were monitored but not sampled by blood and oral swab collection so that the effects of sampling on small-mammal survivorship could be assessed. At the remaining webs, virus activity in small-mammal populations was monitored through monthly blood and oral swab samples from captured animals. After the second year of the study, the purpose of the control webs was achieved, so sampling of captured small mammals from these webs was initiated (25,26).

Trapping Schedules

All trapping web sites were visited monthly, except those in Colorado, which were visited every 6 weeks, as weather permitted. Webs were operated for 3 consecutive nights on each trapping occasion, generally coinciding with the new moon. Traps were set out in the evening of the first day and baited with peanut butter and rolled oats, cracked corn, or mixed grain. In cold weather, cotton or polyester fiberfill was placed in the traps to provide nesting material and reduce trap-associated deaths.

Captured rodents were collected, transported, and sampled according to standardized procedures (27,28). Briefly, traps were checked for captures early each morning. Investigators wearing rubber gloves collected the traps containing captured animals, labeled them with the web and trap station number, and placed them in double plastic bags for transport to a centralized outdoor processing station. Before

A

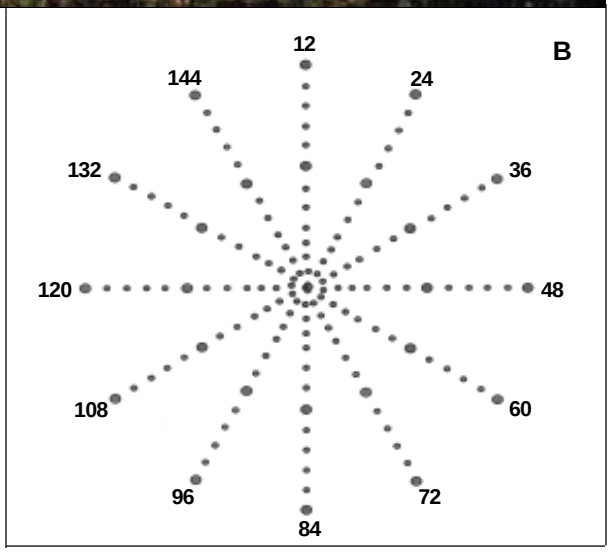

Figure 3. A. Characteristics of landscape and vegetation near Fort Lewis trapping web "A," southwestern Colorado.
Photo courtesy of C. Calisher.
B. Schematic representation of a trapping web showing the relative locations of the 148 trap stations. Small circles indicate the location of one Sherman trap, larger circles, one Sherman plus one Tomahawk trap. Diameter of the web was 200 m. After Parmenter et al. (25).

opening the bags containing captured small mammals, investigators donned protective clothing, including latex gloves, disposable surgeon's gowns, and respirators fitted with HEPA filters. Each captured animal was processed individually. The animal was first shaken from the trap into a plastic bag containing cotton or gauze soaked with inhalant anesthesia (methoxyflurane, Pitman-Moore, Mundelein, IL; or isoflurane, Abbott Laboratories, North Chicago, IL). To prevent potential cross-infection between animals, each was anesthetized in a clean plastic bag, and the anesthesia-soaked cotton was contained in a tea strainer that allows diffusion of the anesthesia, yet between animals can be wiped with a disinfectant. In one case, investigators used a specially adapted "nose cone" for anesthesia (Abbott et al., this issue, pp. 102-112). After being anesthetized, the animal was removed from the bag and placed on a clean surface. A standardized form was used at all trapping sites to collect the following data (28): unique capture number; date of capture; exact location of capture on the trapping web (trap station number); ear tag number; fate (first capture, recapture [different trapping session], or repeater [within same 3-day trapping session]); species; age (juvenile, subadult, or adult); mass; lengths of tail plus body, tail only, ear, and right hind foot; reproductive status including position of the testes (scrotal or abdominal) for males and condition of the vagina (closed or perforate) and description of the nipples (enlarged or small, lactating or not) for females; and the presence or absence of scars or wounds. For animals from the

sampling webs, oral swabs were taken with Dacron-tipped applicators cut with scissors at the level of the Dacron and inserted into 0.5 ml of virus medium (phosphate-buffered saline containing 20% fetal bovine serum, 2% penicillin and streptomycin, and 0.1% Fungizone) in a 2-ml cryovial. Approximately five drops of whole blood were collected into a second cryovial by a capillary tube inserted into the retro-orbital capillary plexus. Whole blood and oral swab samples were immediately placed in liquid nitrogen or on dry ice until transferred to -70°C freezers for storage. Animals recaptured on days 2 or 3 of the trapping session were not bled a second time to avoid trauma. Animals newly captured were marked with a uniquely numbered ear tag (some smaller animals were marked by toe clipping). The animal was then replaced in the original trap or in a clean, ventilated one-quart, screw-capped jar, and was allowed to recover fully from the effects of anesthesia and was released at the exact site of capture. A clean, baited trap was replaced at the site, and the original trap was returned to the processing site to be decontaminated before reuse. Animals from the control webs were treated similarly, except that blood and oral swab samples were not taken.

Investigators recorded environmental data, including a general description of the vegetation and depending on resources available, more detailed descriptions of vegetation at individual webs and weather conditions during the trapping session (detailed rainfall and temperature data were available from meteorologic stations near each trapping site).

Laboratory Analysis

Serologic testing was conducted at CDC, Atlanta, or at the Arthropod-Borne and Infectious Diseases Laboratory, Colorado State University, Fort Collins, CO, USA. Samples of whole blood were tested for antibody reactive with SNV recombinant nucleocapsid protein antigen by enzyme-linked immunosorbent assay according to a standardized protocol (29). Briefly, blood specimens were initially diluted 1:25 in 5% skim milk in 0.01 M phosphate-buffered saline with 0.5% Tween-20 and subsequently diluted to 1:100 through 1:6,400 in fourfold dilutions in microtiter plates. Samples were tested against the recombinant nucleocapsid antigen and a recombinant control antigen (29). A conjugate mix of anti-*Rattus norvegicus* and anti-*Peromyscus leucopus* (heavy and light chains) immunoglobulin G (IgG) (Kirkegaard and Perry, Gaithersburg, MD) was used to detect bound immunoglobulin. Adjusted optical densities (OD) for each dilution were calculated by subtracting the OD_{410} of the control antigen from the OD_{410} of the SNV antigen. Titers were assigned on the basis of an adjusted OD value exceeding 0.20 for each dilution. A second measure consisting of the sum of the adjusted OD values for all four dilutions was also calculated. Serum specimens were considered SNV-positive if their titer was 1:400 or their sum-adjusted OD was 0.95. The cut-off values were determined by assessment of rodents found to be SNV-positive by several serologic tests during the initial investigation of the 1993 outbreak (13) and have been reassessed periodically among large populations of rodents collected in North and South America. Antibodies to other North American hantaviruses are cross-reactive with SNV antigen. This assay would detect (but not distinguish among) infections by New York virus (from the white-footed mouse [15]), Prospect Hill-like viruses (from arvicoline rodents [30]), El Moro Canyon virus (from the Western harvest mouse [31]), Black Creek Canal virus (from the cotton rat [16]), and Bayou virus (from the rice rat [17]).

At this writing, analyses on oral swab specimens (antigen or antibody detection, polymerase chain reaction) have not been conducted. Blood and oral swab samples are archived in -70°C freezers at CDC, Atlanta, and at the Museum of Southwestern Biology, Albuquerque, New Mexico.

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Long-Term Hantavirus Persistence in Rodent Populations in Central Arizona

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For 35 months, we monitored hantavirus activity in rodent populations in central Arizona. The most frequently captured hantavirus antibody–positive rodents were *Peromyscus boylii* and *P. truei*. Antibody-positive *P. boylii* were more frequently male (84%), older, and heavier, and they survived longer on trapping web sites than antibody-negative mice. The number of antibody-positive *P. boylii* was greater during high population densities than during low densities, while antibody prevalence was greater during low population densities. Virus transmission and incidence rates, also related to population densities, varied by trapping site. The spatial distribution of antibody-positive *P. boylii* varied by population density and reflected the species preference for dense chaparral habitats. The focal ranges of antibody-positive *P. boylii* also demonstrated a patchy distribution of hantavirus.

We report results of the initial 35 months of one of several longitudinal hantavirus studies begun in the southwestern United States after the 1993 outbreak of hantavirus pulmonary syndrome (HPS) (Mills et al., this issue, pp. 95-101). This study monitors and quantifies the seasonal and year-to-year changes in rodent populations and the prevalence and incidence of hantavirus infection, identifies environmental factors associated with these dynamics, explores aspects of temporal and spatial viral transmission within reservoir populations, and examines the characteristics of infected animals.

Trapping and Processing

In January 1995, we established four 3.14-ha mark-recapture trapping webs in northcentral Arizona, elevation 1,648 m (Mills et al., this issue, pp. 95-101). The webs were located north of Prescott in Limestone Canyon ((35°31'N, 121°29'W). All sites were in juniper-pinyon and interior chaparral communities (1), although each site varied in physiognomy, aspect, slope, and plant species composition and distribution. Trapping web sites S-1 and C-1 were separated by a valley 150 m wide and were .6 km north of sites S-2 and C-2, which were set apart by a 100-m ravine and creek bed. All webs were operated from January 1995 to September 1996. Serologic samples were taken from rodents captured at S-1 and S-2, while C-1 and C-2 were initially operated as control sites to determine the effects of sampling on rodent survivorship. In October 1996, trapping was discontinued at C-2 (since our field data and others' [2] indicated that sampling had no effect on rodent survival), and blood collection and antibody testing were initiated at C-1 because of its microhabitat uniqueness and high rodent densities.

Web design and placement, trapping periods, mark-recapture techniques, animal processing, and serologic sampling procedures are described in Mills et al. (this issue, pp. 95-101). We anesthetized animals by securing the dorsal skin behind the head and slipping a nose cone with cotton wetted with isoflorane over the nose. Between animals, the nose cone was cleaned with disinfectant. When clearly anesthetized, the animal was placed on a clean table, measured, ear-tagged, and bled.

Serologic testing was conducted at the Centers for Disease Control and Prevention, Atlanta, Georgia. Samples of whole blood were tested for antibody reactive with Sin Nombre virus (SNV)-recombinant nucleocapsid protein antigen by enzyme-linked immunosorbent assay

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(ELISA) (3). The laboratory methods we used are described in Mills et al.; (this issue, pp. 95-101).

Data Analysis

Peromyscus boylii (brush mouse) and *P. truei* (pinyon mouse) were assigned to three categories on the basis of body mass at first capture. Body mass classes (derived from our field data and other sources [4]) were used as an indication of relative age: 6.0 g to 19.0 g (juvenile), 19.1 g to 22.0 g (young adult), and 22.1 g to >30.0 g (adult). We estimated the survival of trappable populations by using mark-recapture data to assess the number of times an animal was caught between the first and last capture. While not a measure of actual life span, average survival provides some indication of population turnover and longevity (5). The minimum number alive (the number of rodents captured in a month plus the number of rodents captured on at least one prior and one subsequent occasion) was used to estimate population sizes (5-7). The minimum number infected was calculated for antibody-positive rodents by using the same technique. Estimated standing prevalence was calculated by dividing the monthly minimum number infected by minimum number alive. These methods provide an estimate of the number of rodents alive and population sizes for a period, an estimate of the number of infected rodents, and comparisons of antibody prevalence between trapping web locations.

Field data were transferred to a computer database by using Excel (Microsoft Corp., Redmond, WA) and Lotus 1-2-3 for Macintosh (Lotus Development Corporation, Cambridge, MA). Statistical analyses were performed by using MINITAB (Minitab Inc, State College, PA) statistical software, the Mann-Whitney and two-sample *t* tests, one-way analysis of variance, and linear trend model (8).

Trapping Results

During 35 months of trapping at three grids, 844 rodents were captured 3,552 times. Blood samples were obtained from 553; from these rodents, 1,418 samples were collected (as a result of subsequent captures of the same rodents during progressive trapping sessions) and tested for hantavirus antibody (Table 1).

P. boylii was the most commonly captured species (70%), followed by *P. truei* (18%), *Tamias dorsalis* (9%), and *Dipodomys ordii* (2%). Irregular species (*Neotoma albigula*, *N. stephensi*, *Onychomys leucogaster*, and *Reithrodontomys megalotis*) accounted for 1% of the total captures. The highest rodent densities occurred at webs S-2 and C-1 (40% and 33% of all captures,

Table 1. Sin Nombre virus–antibody-positive mice and hantavirus prevalence at three mark-recapture webs, December 1995–November 1997[a]

Species	Trapping webs S-1	S-2	C-1[b]	Totals
Peromyscus boylii (Brush mouse)	76/286/109 (26.6%)	74/516/178 (14.3%)	3/56/22 (5.4%)	153/858/309 (17.8%)
Peromyscus truei (Pinyon mouse)	3/165/67 (2.0%)	5/133/55 (3.8%)	0/15/8 (0.0%)	8/313/130 (2.6%)
Tamias dorsalis (Cliff chipmunk)	0/73/40	0/83/29	0/19/9	0/175/78 (0.0%)
Dipodomys ordii (Ord's kangaroo rat)	0/3/2	0/33/13	0/7/3	0/43/18 (0.0%)
Onychomys leucogaster (Northern grasshopper mouse)	0	0/10/3	0	0/10/3 (0.0%)
Neotoma stephensi (Stephen's woodrat)	0/3/1	0/2/2	0/3/2	0/8/5 (0.0%)
Neotoma albigula (White-throated wood rat)	0/1/4	0/4/1	0/2/1	0/7/6 (0.0%)
Reithrodontomys megalotis (Western harvest mouse)	0	0/4/4	0	0/4/4 (0.0%)
All species	79/531/223	79/785/285	3/102/45	161/1,418/553

[a]Positive samples/number of samples tested/number individuals tested. Values in parentheses are hantavirus antibody prevalences for 35 months based on the number of samples tested.

[b]C-1 was initially a control web; serologic sampling began in October 1996.

respectively), while S-1 accounted for 27% of the total captures.

Population Dynamics

Population levels of the two most frequently captured rodent species, *P. boylii* and *P. truei*, were relatively high through the winter of 1995 to 1996 and then declined ($p < 0.05$) during the subsequent summer and autumn, remaining at low levels through 1996 to 1997 (Figure 1). The *P. boylii* population had the most persistent decline (76%) followed by *T. dorsalis* (64%) and *P. truei* (34% short-term reduction). Population levels of *P. boylii* were consistently higher than those of *P. truei*, except for the summer of 1997 (May through August); during this period *P. boylii* densities were at their lowest, 6.5 animals per 6.2 ha per month, while the *P. truei* populations increased to near high density levels (12.2 animals per 6.2 ha per month). For 4 months, far more *P. truei* were captured than *P. boylii* (Figure 1).

During the first 5 months, adverse weather conditions (rain, snow, high winds) hampered trapping efforts. Strong wind and wind gusts seemed the main factor contributing to reduced periodic capture rates (Figure 1).

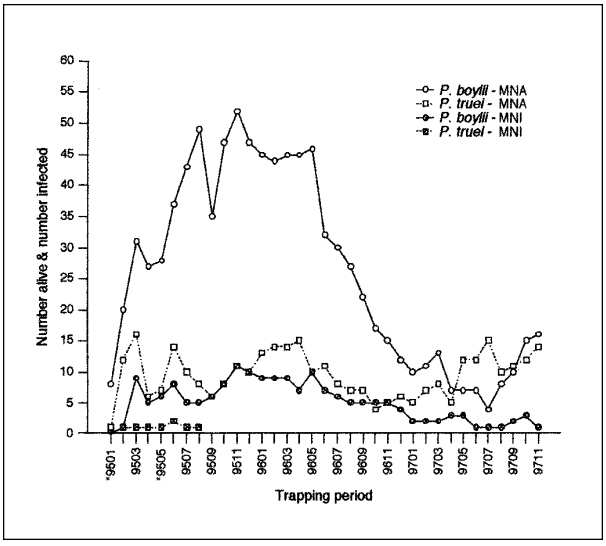

Figure 1. Minimum number of *Peromyscus boylii* and *P. truei* alive (MNA) and the minimum number infected (MNI) with Sin Nombre virus (antibody-positive) at two mark-recapture webs (6.2 ha).*
*Because of adverse weather conditions, we only trapped for 2 nights in January and May 1995.

Characteristics of Antibody-Positive Captured Rodents

Although data from C-1 were not included in comparative analysis because serologic sampling was not initiated at this site until October 1996 during low population densities (4.0 samples per month, range 0 to 8), of the 21 *P. boylii* captured and tested, 2 were hantavirus–antibody-positive (10%); 0 (0%) of 7 females and 2 (28%) of 14 males. After samples were collected from one antibody-positive *P. boylii* in October 1996, no antibody-positive samples were collected until the following October, when another *P. boylii*, which had survived for 12 months, became antibody-positive for the first time.

The 62 hantavirus antibody–positive rodents captured at the two sites represented two species: 58 *P. boylii* and 4 *P. truei* (Table 2). The prevalence of hantavirus antibody differed considerably by species: *P. boylii* had a prevalence of 20%, *P. truei* 3%. All four antibody-positive *P. truei* were trapped before September 1996 when population densities were high for all rodent species.

Antibody-positive *Peromyscus* were more often male and within the heaviest mass class (Table 2). Although approximately half of the *P. boylii* tested were male, 84% of the antibody-positive mice were male. The male-to-female ratio was similar to that of *P. truei,* despite the small sample size. We found more adults and fewer young among the antibody-positive *Peromyscus,* even though young-to-adult capture ratios were similar among seronegative mice.

Longevity of antibody-positive mice was considerably different between the two species, while longevity of antibody-negative mice was similar (Table 2). Antibody-positive male *P. boylii* tended to survive longer than antibody-positive female. Furthermore, antibody-positive male *P. boylii* lived longer than antibody-negative male *P. boylii* (4.4 months and 2.9 months, respectively; $t = 2.58$, $df = 48$, $p = 0.007$).

P. boylii Population Dynamics and Temporal Patterns of Infection

The number of captures per month and the number of samples per month were usually not the same—some animals were not sampled because of death, weakened physical condition, hypothermia, or escape. The number of animals tested for antibody to hantavirus, however, mirrored population trends. The *P. boylii* population declined dramatically during sum-

Table 2. Antibody-positive and antibody-negative *Peromyscus boylii* and *P. truei* at two mark-recapture webs,[a] December 1995–November 1997

Characteristic	No. (%) *P. boyii*			No. (%) *P. truei*		
	Positive	Negative	Totals	Positive	Negative	Totals
Sex						
Male	49 (32)	106 (68)	155 (54)	3 (4)	63 (96)	66 (56)
Female	9 (7)	123 (93)	132 (46)	1 (2)	50 (98)	51 (44)
Totals	58 (20)	229 (80)	287	4 (3)	113 (97)	117
Body mass class[b]						
I	2 (3)	75 (97)	77 (27)	0	31 (100)	31 (26)
II	9 (15)	51 (85)	60 (21)	1 (5)	18 (95)	19 (16)
III	47 (31)	103 (69)	150 (52)	3 (4)	64 (96)	67 (58)
Web-site longevity [months][c]						
Male	4.4 [1-16]	2.9 [1-26]		2.3 [1-5]	3.2 [1-18]	
Female	3.3 [1-13]	3.5 [1-18]		1 [1]	3 [1-15]	

[a]S-1 and S-2 webs.
[b]Classes assigned at first capture. I = 6.0g-19.0g; II = 19.1g-22.0g; III = 22.1g to >30.0g.
[c]Longevity is the mean number of months animals were captured, from first to last capture. Values in brackets are ranges.

mer and autumn 1996, stabilized at low levels during winter 1996 and 1997, and fell to minimal levels in spring 1997 (Figure 1) (Table 3).

For the 35-month sampling period, the mean number of antibody-positive *P. boylii* was 5.0 animals per 6.2 ha per month, range 0 to 11 (Figure 1). The number of antibody-positive *P. boylii* was higher during high population densities than during low densities (8.0 and 2.8 animals per 6.2 ha per month, respectively; $t = 4.83$, df = 21, $p < 0.001$). Numbers of antibody-positive animals were similar during 35 months at S-1 and S-2 (2.7 and 2.4 animals per 6.2 ha per month, respectively), even though population densities at S-2 were regularly higher than at S-1.

The mean antibody prevalence for the sampling period was 20.2% (range 0% to 43%) and was higher during low densities than high densities (Figure 2). At each site, antibody prevalence rates were also higher during low densities, but not significantly different from rates during high population densities. However, antibody prevalence varied between sites and was consistently higher at S-1 (Table 3). The highest mean monthly antibody prevalence occurred on S-1 during low population densities (37.0%) and was higher than prevalence on S-2 during the same period. The highest monthly antibody prevalence occurred at S-1 during minimal population densities, May 1997, when three of four captured *P. boylii* were antibody-positive (75%).

Table 3. Population densities and hantavirus-antibody prevalence in *Peromyscus boylii* at two mark-recapture trapping webs, by period

Web sites	Dec 1995-Nov 1997		High density[a]		Low density[b]	
	Density/ month[c]	Prevalence/ month[d]	Density/ month[c]	Prevalence/ month[d]	Mean/ month[c]	Prevalence/ month[d]
S-1 & S-2	26.1	20.2	43.6	18.4	11	25.4
	(4-52)	(0-43)	(32-52)	(10-22)	(4-22)	(12-43)
S-1	9.7	28.5	15.2	26.4	4.5	37.0
	(1-20)	(0-75)	(10-20)	(15-38)	(1-9)	(0-75)
S-2	16.4	14.2	28.4	14.3	6.5	15.0
	(3-42)	(0-33)	(13-42)	(6-19)	(3-13)	(0-33)

[a]June 1995 to June 1996
[b]September 1996 to September 1997
[c]Population density (number of individuals per 6.2 hectares), determined by minimun number alive. Values in parentheses are ranges.
[d]Antibody prevalence to hantavirus (%), determined by estimated standing prevalence. Values in parentheses are ranges.

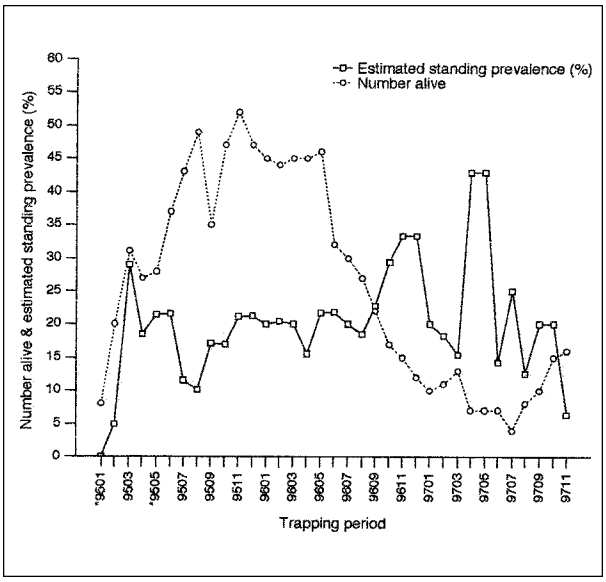

Figure 2. Minimum number of living *Peromyscus boylii* and the estimated standing prevalence of hantavirus antibody–positive mice at two mark-recapture webs (6.2 ha).*
**Because of adverse weather conditions, we only trapped for 2 nights in January and May 1995.

Maximum and minimum antibody prevalence occurred during similar periods at both sites. During low population densities, S-2 had 4 months without an antibody-positive sample, while antibody-positive animals were not captured from S-1 for 2 months. The months when no antibody-positive animals were captured were not the same for both sites; at least one positive sample was recorded each month, even during low population densities.

Longevity and Seroconversion of Infected Mice

Hantavirus antibody-positive *P. boylii* tended to survive longer (mean 4.2 months) than seronegative mice (mean 3.2 months) (t = 1.77, df = 138, p = 0.04) (Table 4). At site S-2, survival was similar between antibody-positive and antibody-negative mice, but at S-1, antibody-positive mice lived longer (4.8 months) than seronegative mice (3.0 months) (t = 2.58, df = 48, p = 0.007). At both sites, survival among male and female mice was not significantly different.

Initial acquisition of hantavirus antibody (seroconversion) was observed in 33% of the antibody-positive *P. boylii*. *P. boylii* acquired hantavirus antibody in all months except December, January, and March (Figure 3). Two transmission peaks, accounting for 79% of seroconversions, took place during the typical 7-month reproductive period, April through October (37% during April, May, and June; 42% during September and October). Seroconversions at S-2 were directly related to population levels, with 9 (90%) of 10 S-2 seroconversions taking place during high population densities in 1995. This relationship did not appear at S-1, where the number of

Table 4. Frequency of intervals between first and last capture of individual *Peromyscus boylii*, December 1995-November 1997

	No. *P.*	No. months in interval between first and last captures																			
Web sites	*boylii*[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	18	19	26	Mean[b]
Antibody-positive mice																					
S-1 & S-2	58	22	4	6	5	7	2		2	2	3	2		1	1		1				4.2
S-1	30	10	2	2	1	6	1		1	2	2	1		1			1				4.8
S-2	28	12	2	4	4	1	1		1		1	1			1						3.5
Antibody negative mice																					
S-1& S-2	250	117	48	24	11	11	6	7	7	3	4	3	3	1		2		1	1	1	3.2
S-1	90	47	15	8	3	4	2	2	2	2		3	1							1	3.0
S-2	160	70	33	16	8	7	4	5	5	1	4		2	1		2		1	1		3.3

[a]Total number of individual *P. boylii*.
[b]Mean number of months in interval.

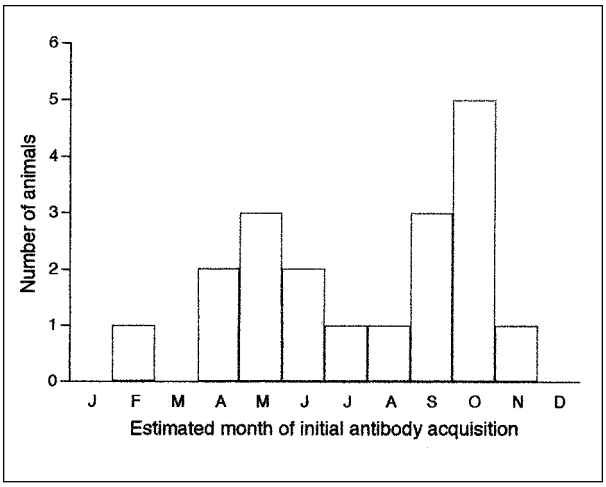

Figure 3. Initial antibody acquisition in *Peromyscus boylii* at two mark-recapture webs, by month, December 1995–November 1997.

seroconversions was similar during high and low population densities.

Incidence of Infection

The incidence rate for seroconversion per 100 mice per month was twice as high at S-2 (34.5) as at S-1 (17.0). The greater number of mice at risk and the number of months before seroconversion accounted for the higher incidence rate at S-2 (Table 5). S-1 had fewer *P. boylii* at risk, which did not seroconvert for an average of 5.4 months; S-2 had a larger number of mice at risk, which seroconverted after 2.2 months.

Table 5. Incidence rates of hantavirus infection in *Peromyscus boylii* that were recaptured and sampled at least twice, December 1995–November 1997, two web sites

Sites	No. at risk[a]	Sero-con-ver-sions	(Cumu-lative %)	Mouse-mo. of obser-vation[b]	Inci-dence[c]	Mean mo. before sero-con-version
S-1	43	9	(20.9)	53.0	17.0	5.4
S-2	90	10	(11.1)	29.0	34.5	2.2
S-1 & S-2	133	19	(14.3)	82.0	23.2	3.7

[a]Antibody-negative at time of first capture.
[b]Includes all time intervals between successive captures when mice were seronegative, and half the interval between captures when mice became seropositive.
[c]Seroconversions per 100 mice per month.

Spatial Patterns of Infected Mice

Distribution and movement of antibody-negative and antibody-positive *P. boylii* varied by population density and availability of shelter and food resources. At both trapping web sites, *P. boylii* distributions were associated with brushy chaparral plant species. The ranges of high density mice outline, in general, the distribution of thick chaparral stands (Figure 4).

Because plant species diversity and belts of chaparral stands were greater at S-2, *P. boylii* distribution was relatively continuous and widespread. Chaparral stands at S-1 were discontinuous, and *P. boylii* lived in rocky pockets of vegetation and were seldom trapped in different chaparral pockets if separated by open terrain (Figure 4). At both sites, *P. boylii* avoided open juniper-pinyon areas.

During periods of high population density, antibody-positive mice occupied scattered chaparral habitats of undergrowth areas of the sites and moved freely between web transects. Movement, however, appeared to be directly influenced by chaparral cover. During low population densities, antibody-positive mice withdrew to a few, well-defined refuges (Figure 4). The movement of antibody-positive *P. boylii* during low densities was also restricted; mice seldom moved between web transects.

Hantavirus Prevalence Rates and Patterns

The prevalence rates of *P. boylii* (20.2%) and *P. truei* (3%) in our study were similar to those found in other studies carried out in pinyon-juniper habitats (4). The short-term infection in *P. truei* may have been caused by spillover from syntopic *P. boylii* (the four antibody-positive *P. truei* were found only during spring and summer 1995, when *P. boylii* densities and the potential of interspecies contact were greatest). Six other rodent species coexisting with *P. boylii* should have had similar risks for hantaviral infection since they had been captured at trap stations used by *P. boylii* at one time or another (capturing two or three different species at one station during a single trapping session was not uncommon). The evident rarity of hantavirus infection in *P. truei* and the absence of infection in other sympatric rodents suggests that *P. boylii* is the primary hantavirus host in this area and that transmission to other rodent species may be unlikely during periods of average population densities. Similar relationships have been

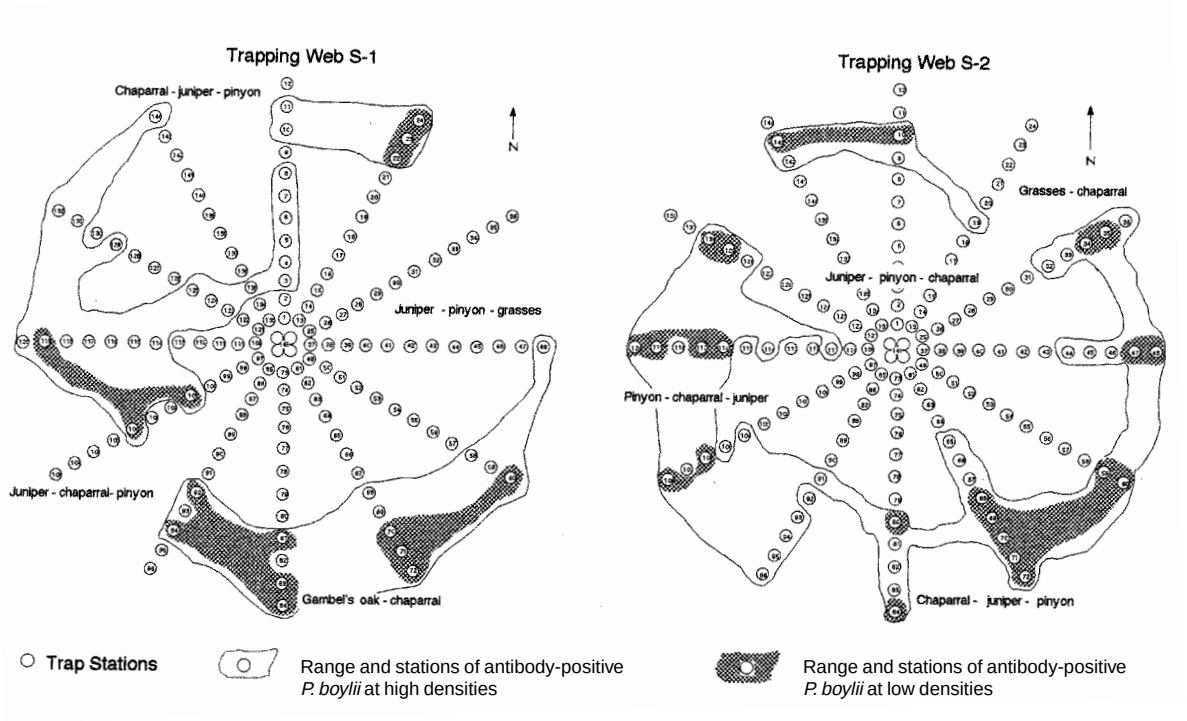

Figure 4. Ranges and trap stations of hantavirus antibody–positive *Peromyscus boylii* during high and low population densities. Each web covered 3.1 ha. Trap stations within ranges were occupied by antibody-negative and antibody-positive mice at various times. High densities represent 13 months (June 1995 to June 1996), and low densities represent 13 months (September 1996 to September 1997).

demonstrated in southern Arizona, where only species of *Peromyscus* had immunoglobulin G (IgG) antibody reactive with SNV (Kuenzi et al., this issue, pp. 113-117).

On the basis of long-term infection patterns and persistent virus shedding (9-11), we assume that hantavirus antibody–positive *P. boylii* are chronically infected and infectious (Mills et al., this issue, pp. 135-142). Studies using reverse transcription-polymerase chain reaction (RT-PCR) on blood samples from field-caught *P. maniculatus* from Nevada (12) mirror other studies of host-hantavirus associations in suggesting initial viremia, followed by a relatively rapid immune response that cleared virus from blood in approximately 1 month (animals remained antibody-positive for at least 7 months). However, as numerous studies have shown (9-11;13), the short duration of hantavirus RNA in blood does not reflect its residence in organs. Another study demonstrated that 97% of antibody-positive *P. maniculatus* were PCR-positive for viral RNA in organ tissues (13), which implies chronic infection, as has been demonstrated for other hantavirus-host associations. Nevertheless, the crucial experiments to demonstrate chronic infection and persistent shedding have not been done for *P. boylii*. We are attempting to develop methods to reliably and consistently collect urine from mark-release-capture animals in the field to address this problem.

Slightly more male than female mice (1.2:1) were tested for antibody to hantavirus; however, fewer male than female mice (1:1.2) were antibody-negative. The higher antibody prevalence in males may be due to territoriality, aggression toward other males during breeding periods, longer survival, and breadth of travel (4,14).

Factors Affecting Population Density

The population densities and distributions of rodents were related to seasonal and year-to-year availability of acorns, seeds, and juniper berries (mast). Acorns, pinyon seeds, juniper

berries, and grasses were abundant throughout the study sites during summer and autumn 1995, reflecting surplus winter precipitation the previous 3 years (15). Population levels of the rodent community—relatively high during autumn and winter 1995-96—may have been related to this abundance of seed crops. During the winters of 1995-96 and 1996-97, precipitation was well below normal, and the first winter drought resulted in complete mast failures by all chaparral species and pinyon pine. Juniper crops, evident in autumn 1996, were depleted soon after. The second winter drought resulted in the mast failure of oak species, pinyon, and juniper, but other chaparral species produced minimal crops during the spring and late summer (Abbott et al., unpub. data).

P. boylii population fluctuations (Figure 1), related to year-to-year mast resources and variations in seasonal female reproductive efforts, are consistent with fluctuations of mast-consuming *Peromyscus* and *Tamias* rodents, which show a positive correlation between mast production and breeding behavior (16-18). Comparable regional population fluctuations occurred during this same period in Colorado and southern Arizona (Calisher et al., this issue, pp. 126-134; Kuenzi et al., this issue, pp. 113-117). Female reproductive activity was consistently absent during the colder winter months of November through February. The reproductive period, April through October, typically unimodal and coinciding with seed development of syntopic vegetation, started at low levels in April and peaked in late summer and autumn. Mast of oak species, pinyon pine, and juniper usually ripen in late summer and early autumn while summer monsoons may cause other chaparral species to produce seeds in both spring and summer (Abbott et al., unpub. data).

This pattern of reproduction and food supply was evident during the 1995 breeding season; 40% of the female mice captured in spring showed signs of reproductive activity, compared with 76% of those captured in summer and 86% of those captured in autumn. The subsequent breeding season began normally, with 43% of females pregnant, but new pregnancies nearly halted during the summer months, decreasing 96% from the previous year. Only a few of the 64 female mice captured in June and July 1996 had a perforate vagina, and none showed signs of lactation or pregnancy. The autumn breeding effort declined by 56% from the previous year. During the 1997 breeding season, there were 84% fewer female mice than during the 2 previous years, but most were reproductively active, suggesting that a population recovery was under way.

Factors Affecting Hantavirus Prevalence

The number of hantavirus-infected mice was higher during the high population densities of 1995-96 (Figure 1). Month-to-month numbers of antibody-positive mice appeared more stable than those of antibody-negative mice. The number of high density–antibody-positive *P. boylii* was stable during the winter, with small peaks proportional to monthly capture success. The numbers of antibody-positive mice remained stable (though lower) during the subsequent precipitous 7-month population decline. Even during low population densities, antibody-positive mice were persistent at minimal, yet stable levels. This consistent presence of at least a few infected mice may reflect the resident nature of antibody-positive mice, characteristically older and able to survive for longer periods. Fifty-five percent of the antibody-positive mice survived on trapping web sites 3 months or longer and were considered resident, while 34% of the seronegative mice were resident.

The proportion of hantavirus antibody–positive *P. boylii* varied by population density and trapping web site (Figure 2) (Table 3). S-1 maintained the highest mean antibody prevalence; during low population densities, prevalence increased. Almost half of the *P. boylii* captured at S-1 tested positive during low density months when at least one mouse was antibody-positive. Population densities at S-2 were consistently greater than at S-1 and were associated with lower overall prevalence rates. Approximately 23% of the *P. boylii* captured at S-2 were antibody-positive during low density months when at least one mouse was antibody-positive. Positive linear correlations between population density and antibody prevalence have not been found in other species of *Peromyscus* (Calisher et al., this issue; pp. 126-134;11;19).

We observed that one third of the antibody-positive *P. boylii* acquired antibody. No mice reverted from antibody-positive to antibody-negative. Transmission of hantavirus was bimodal and associated with spring and autumn

reproductive activity (Figure 3). Thirty-seven percent of *P. boylii* seroconverted in the spring, and 42% in the autumn reproductive period. Mice that seroconverted were more frequently male, within the heaviest mass class, and survived longer than mice that remained antibody-negative. The trend for bimodal transmission may reflect intraspecific competition, greater movement, and aggressive behavior by resident antibody-positive males during peak reproductive periods (20). Similar transmission trends have been reported in rat populations (6). Consequently, risks of horizontal transmission may increase during the more active seasons.

Incidence of infection varied with population densities, recapture rates, and population dynamics. Rates of *P. boylii* seroconversion varied by site, but collectively, both sites had an average 14.3% incidence of infection among the population at risk during the study period (Table 5). The number of seroconversions at both sites was similar, but the number of mice at risk at S-2 was much larger, since population densities were regularly higher. Consequently, the cumulative proportion of mice seroconverting at S-2 was 47% lower than at S-1, whereas the incidence of seroconversions per 100 mice per month was 103% greater. Characteristics of the S-1 population (longer survival as antibody-negative animals, more restricted centers of activity, and continuous infection during periods of high and low population densities) may have been contributing factors to the difference in incidence rates between sites.

The focal ranges of antibody-positive *P. boylii* were patchy; they expanded and contracted over time (Figure 4). Hantavirus infection and distribution patterns were influenced by habitat structure, seasonal food availability, and the behavioral characteristics of infected mice. At both sites, *P. boylii* were associated with corridors and patches of chaparral understory within the juniper-pinyon woodland, and especially with dense stands of chaparral associated with rocky substrates and downed trees that provided optimal shelter. These favored sites were usually located on slopes and along creek channels. In southern Arizona, *P. boylii* were found in analogous habitat distributions; the species favored oak riparian vegetation, and most were captured in one portion of one trapping web (Kuenzi et al., this issue, pp. 113-117).

Diverse chaparral stands were more widespread and continuous at S-2. During high population densities, *P. boylii* occupied scattered chaparral areas throughout most of the web and were often trapped at sites several meters apart. The relatively high abundance of mice over a large area may explain the greater incidence of infection and lower antibody prevalence at S-2. The greater number of mice during high population densities and the greater turnover rate seemed to dilute the prevalence of infection and, at the same time, increase the risk for infection because of intensified encounters.

The patchiness of hantavirus infection was more evident and focalized at S-1. Chaparral stands were discontinuous; *P. boylii* occupied discrete chaparral pockets, seldom migrating from one pocket to another (Figure 4). During this study, S-1 had three prominent centers of hantavirus infection and three associated centers of *P. boylii* activity. The structure and disjunct nature of the activity centers (and associated centers of antibody-positive animals) may have contributed to higher antibody prevalences and greater cumulative seroconversion since the mice occupying these restricted habitats had a greater chance of encountering each other. During low population densities, the higher prevalence rates of 50% to 75% were related to antibody-positive male mice that were older, heavier, and able to reside for a longer period within the activity centers. Similar patterns of clustering or patchiness and hantavirus infection have been documented for cotton rats, *Sigmodon hispidus*, in Florida (21).

Along with high population densities, the longer stay of dominant male mice in optimal and reliable habitats may be a primary variable contributing to hantavirus infection. This assumption is based on three trends: animals that became antibody-positive survived longer than those that did not seroconvert; antibody-positive tended to survive longer than antibody-negative mice; and in patchy optimal habitats, resident mice tended to be dominant, male, and antibody-positive. Consequently, resident male mice may provide a reliable reservoir during low population densities and therefore ensure the survival of hantavirus within rodent communities.

Conclusions

Our preliminary results, and those of other recent studies (Kuenzi et al.,this issue, pp. 113-

117;18), have implicated precipitation, habitat structure, and food resources as ultimate environmental factors that influence reservoir population dynamics, viral transmission, and hantavirus persistence. The results of this and other recent studies have raised questions concerning proximate patterns of hantavirus maintenance, seroconversions, and transmission within specific reservoir species occupying different western regions (Mills et al., this issue, pp. 135-142). Additional data suggesting that sex ratios, size, and social organization affect temporal and spatial seroconversion relationships will be addressed in forthcoming articles. We hope that this ongoing study will collect sufficient data to explain the interplay of habitat resources, social hierarchies, intraspecific competition, and dispersal behavior and how these proximate factors influence hantavirus ecology and human risk.

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A Longitudinal Study of Sin Nombre Virus Prevalence in Rodents, Southeastern Arizona

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We determined the prevalence of Sin Nombre virus antibodies in small mammals in southeastern Arizona. Of 1,234 rodents (from 13 species) captured each month from May through December 1995, only mice in the genus *Peromyscus* were seropositive. Antibody prevalence was 14.3% in 21 white-footed mice (*P. leucopus*), 13.3% in 98 brush mice (*P. boylii*), 0.8% in 118 cactus mice (*P. eremicus*), and 0% in 2 deer mice (*P. maniculatus*). Most antibody-positive mice were adult male *Peromyscus* captured close to one another early in the study. Population dynamics of brush mice suggest a correlation between population size and hantavirus-antibody prevalence.

We examined the role of rodent species as natural reservoirs for hantaviruses in southeastern Arizona to identify the species infected with hantavirus, describe the characteristics of infected animals, and assess temporal and intraspecific variation in infection rates.

Trapping Procedures

Beginning in May 1995, we established four permanent trapping webs on the Santa Rita Experimental Range in the Santa Rita Mountains of southeastern Arizona (Pima County). The design of these webs, as well as details on mark-recapture trapping procedures, are described by Mills et al. (this issue, pp. 95-101). Elevations of the trapping webs are approximately 1,250 m to 1,379 m. All trapping webs contained approximately equal amounts of two main vegetation types, semidesert grassland (characterized by Lehmann lovegrass [*Eragrostis lehmanniana*], three-awn [*Aristida* spp.], prickly pear cactus [*Opuntia* spp.], and mesquite [*Prosopis velutina*]) and oak riparian (characterized by deciduous trees including Arizona white oak [*Quercus arizonica*] and netleaf hackberry [*Celtis reticulata*]; occurs in drainage areas where water flow is seasonally intermittent), occur at these elevations. Web 1 was operated from May 1995 through September 1996, when trapping was discontinued because of low trap success, and webs 2, 3, and 4 were operated from May 1995 through December 1997.

From May 1995 through September 1996, webs 1 and 4 were considered controls. Captured mice from these webs were identified, marked, weighed, and measured, but not bled. Beginning in November 1996, we began collecting blood samples from mice on web 4. The bleeding process had little effect on survival (1). The methods for obtaining blood samples and the serologic testing of samples for hantavirus antibodies are described in Mills et al. (this issue, pp. 95-101).

We examined population dynamics of common species infected with Sin Nombre virus (SNV) using data from three webs that were trapped continuously from May 1995 through December 1997. Using the minimum number of rodents known to be alive during a 3-day trapping session, we calculated an index of population size by taking the total number of rodents captured during each 3-day trapping session and adding to that sum the number of rodents captured on at least one previous and one subsequent session (2). The minimum number of hantavirus antibody–positive rodents was calculated in the same way. We estimated standing prevalence for each trapping session by dividing the minimum number of antibody-positive rodents by the minimum number of rodents known to be alive.

Capture histories were used to estimate survivorship of the trappable population. These estimates were calculated as the percentage of

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rodents known to be alive a given number of months after initial capture. Although we refer to these estimates as survival rates, they are more accurately described as trapping web residency rates, as deaths cannot be distinguished from emigration.

Trapping Results

Between May 1995 and December 1997, 1,234 rodents were captured a total of 3,226 times, and 1,231 blood samples were obtained (Table 1). Bailey's pocket mouse (*Chaetodipus baileyi*) was the most common species captured (57% of rodents captured). Common murid rodents captured included white-throated wood rat (*Neotoma albigula*) (10%) and four species in the genus *Peromyscus* (27%). The cactus mouse (*P. eremicus*) was the most common *Peromyscus* species captured (12%) followed closely by the brush mouse (*P. boylii*) (11.5%). Deer mice (*P. maniculatus*) and white-footed mice (*P. leucopus*) were also captured but in low numbers (<3% each). Other species captured infrequently (<1%) included the fulvous harvest mouse (*Reithrodontomys fulvescens*), yellow-nosed cotton rat (*Sigmodon ochrognathus*), desert pocket mouse (*C. penicillatus*), and Merriam's kangaroo rat (*Dipodomys merriami*).

Prevalence of Antibody-Positive Rodents

Only rodents in the genus *Peromyscus* had antibodies reactive with SNV; however, antibody prevalence varied considerably among species within this genus (Table 1). Most (13 of 17) antibody-positive rodents were brush mice. One cactus mouse and three white-footed mice were also antibody positive. With the exception of one white-footed mouse, all antibody-positive rodents were captured in oak riparian vegetation. Antibody-positive rodents were captured on all three webs from which animals were bled; however, most (65%) were first captured on web 2 early in the study (May to June 1995). The farthest distance between trap stations where these web 2–rodents were captured was approximately 190 m, and half were captured at three adjacent trap stations along one transect line.

All antibody-positive rodents were positive upon first capture, and most (58%) were never recaptured. Antibody-positive animals that were recaptured were caught an average of 3.8 times (standard deviation = 2.03, n = 7, range 2 to 8). All but one of the recaptured animals remained antibody positive on subsequent captures. The exception, a male brush mouse, was antibody negative on its three recaptures.

Characteristics of Infected Populations

Antibody-positive rodents were more likely to be male than female and were predominately adult (Table 2). The ratio of male to female among antibody-positive brush mice was significantly higher than that among the total sample (chi-square with Yates' correction = 7.97,

Table 1. Prevalence of antibodies to Sin Nombre virus among wild rodents in southeastern Arizona, May 1995-December 1997

Family/Species	Common name	No. rodents trapped and released (total captures)[a]		No. tested	No. positive	(%)
Heteromyidae						
Dipodomys merriami	Merriam's kangaroo rat	1	(2)	0	0	(0.0)
Chaetodipus spp.	Pocket mice					
C. baileyi	Bailey's pocket mouse	704	(715)	329	0	(0.0)
C. penicillatus	Desert pocket mouse	25	(27)	7	0	(0.0)
Subtotal		730	(744)	336	0	(0.0)
Muridae						
Neotoma albigula	White-throated wood rat	126	(126)	51	0	(0.0)
Onychomys torridus	Southern grasshopper mouse	7	(7)	7	0	(0.0)
Peromyscus spp.	White-footed mice					
P. boylii	Brush mouse	137	(142)	98	13	(13.3)
P. eremicus	Cactus mouse	151	(152)	118	1	(0.8)
P. leucopus	White-footed mouse	29	(30)	21	3	(14.3)
P. maniculatus	Deer mouse	6	(6)	2	0	(0.0)
Reithrodontomys fulvescens	Fulvous harvest mouse	16	(16)	12	0	(0.0)
Sigmodon ochrognathus	Yellow-nosed cotton rat	11	(11)	5	0	(0.0)
Subtotal		483	(490)	314	17	(5.4)
Total		1,213	(1,234)	650	17	(2.6)

[a]Total captures include rodents trapped and released and those that died during handling.

Table 2. Distribution of antibody-positive versus all brush mice, cactus mice, and white-footed mice, by sex and age

Characteristic	Brush mice		Cactus mice		White-footed mice	
	No. (%) positive	Total no. (%)	No. (%) positive	Total no. (%)	No. (%) positive	Total no. (%)
Sex						
Male	12 (92)	51 (52)	0	59 (50)	3 (100)	10 (48)
Female	1 (8)	47 (48)	1 (100)	59 (50)	0	11 (52)
Age						
Juvenile	0	12 (12)	0	22 (18)	0	2 (10)
Young adult	1 (8)	38 (40)	0	48 (41)	0	3 (14)
Adult	12 (92)	48 (48)	1 (100)	48 (41)	3 (100)	16 (76)

degrees of freedom = 1, p = 0.005), and significantly more adults were antibody positive than would be expected from the distribution of age classes among the total sample (chi-square = 9.69, df = 2, p = 0.002). Although the sample size is too small for significance testing, these patterns hold for white-footed mice as well (Table 2).

Brush Mice Population Dynamics and Temporal Pattern of Infection

The number of brush mice varied both by season and by year. The minimum number known to be alive was relatively high during the first 10 months of the study, May 1995 through March 1996 (Figure 1). The number of brush mice declined during the spring of 1996 and remained low until the fall, when the numbers increased but never reached the levels of the previous year. Captures for the next year followed a similar pattern with increased numbers during fall and winter (October through March), followed by a steady spring decline and summer low.

The minimum number of brush mice known to be infected was highest during the initial part of our study (Figure 1). Eleven of the 13 hantavirus antibody–positive mice were first captured between May and September 1995, gradually disappearing from the population. By October 1996, no animals were known to be infected on any of our trapping webs. One new antibody–positive brush mouse was captured in November 1996 and another in November 1997. Similarly, the estimated standing prevalence of hantavirus antibody ranged from 40% in May 1995 to 0% in both October 1996 and April through October 1997 (mean = 8.25%).

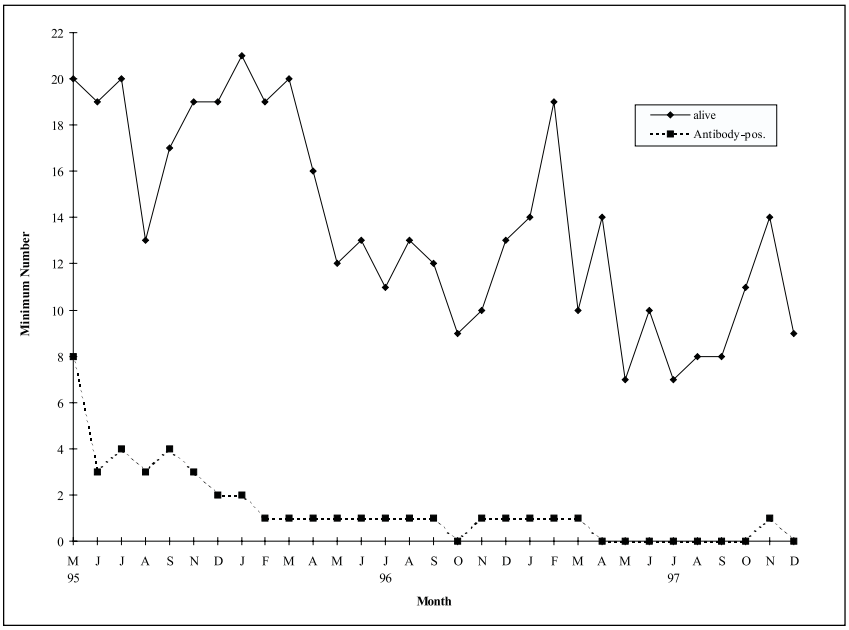

Figure 1. Population trends of brush mice, as determined by the minimum number known to be alive, Santa Rita Experimental Range, southeastern Arizona, May 1995–December 1997.

Male and female brush mice showed similar rates of survivorship with an approximately 50% turnover rate around 2 months after initial capture (Figure 2). Hantavirus antibody–positive mice did not survive quite as long; the 50% turnover rate occurred approximately 1 month after initial capture. By 6 months after first capture, approximately 80% of all rodents had disappeared. A small percentage of brush mice continued to be captured for more than 1 year after tagging.

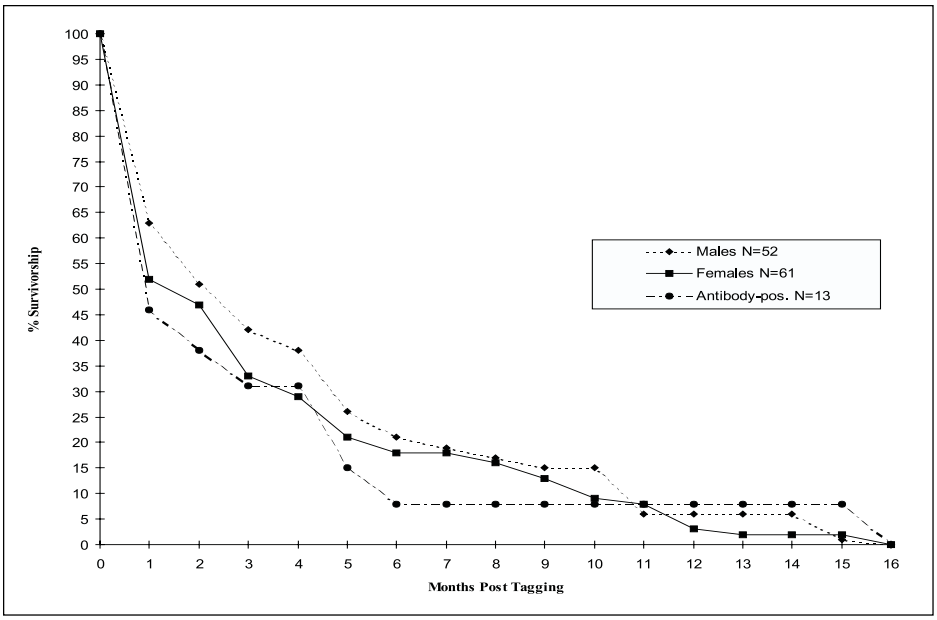

Figure 2. Survivorship functions (percentage of brush mice known to be alive after initial capture) based on recapture data, Santa Rita Experimental Range, southeastern Arizona, May 1995–December 1997.

Conclusions

The overall prevalence of antibodies reactive with SNV antigen varied considerably among wild rodents captured in southeastern Arizona between May 1995 and December 1997, from 0% for Heteromyidae to 5.4% for Muridae. Low prevalence within the heteromyids has been commonly documented (3-5). Of mice, only *Peromyscus* were seropositive at our study site. The mean antibody prevalence of 7% for all *Peromyscus* was similar to the mean prevalence reported from Kansas (6) and Montana (7), although lower than that at many other sites in Arizona and New Mexico (3,4). The low hantavirus-antibody prevalence at our site may be related to its location in Sonoran Desert semigrassland and its relatively low rainfall; Mills et al. (4) found that prevalence of SNV was lowest at altitudinal and climatic extremes.

The primary *Peromyscus* species with evidence of hantavirus infection at the Santa Rita Experimental Range was the brush mouse, recently shown to be an important carrier of SNV or an SNV–related virus throughout the southwestern United States (4). Even within a single species, overall prevalence of hantavirus antibodies has been reported to vary widely among different regions and habitats and in different seasons and years. In samples of deer mice from sites throughout the southwestern United States, Mills et al. (4) found antibody prevalence of 0% to 50%. Within states, overall prevalence in deer mice was 9.5% to 38.6% at 10 sampled sites in New Mexico (3) and 0% to 50% in 34 counties in California (5).

Several studies have indicated, as does ours, that the presence and number of antibody-positive mice are not evenly distributed. Although *Peromyscus* were trapped in both vegetation types within our study site, all but one of the antibody-positive mice were trapped in oak riparian vegetation, and most were trapped in one portion of one web. Similarly, Mills et al. (4) captured antibody-positive deer mice in only 21 of 41 sites where deer mice were captured, and hantavirus antibody–positive brush mice in only 9 of 17 sites. Our results suggest that the prevalence of antibody-positive animals may be correlated with different habitats and provide additional evidence for focality of hantavirus in "reservoir" populations (4).

While our sample sizes are too small to determine statistical significance, they suggest a correlation between population size and prevalence of hantavirus antibody. The number of antibody-positive animals was highest when the population was decreasing from an abundance of *Peromyscus* in the spring of 1995, the most recent peak. This finding is in contrast to local studies in the Channel Islands (8), Montana (7), and the regional study of Mills et al. (4), which found no relationship between antibody prevalence and density of deer mice. However, Childs et al. (3) found higher antibody prevalence in pinyon-juniper vegetation in 1993, when evidence suggests that rodent densities were unusually high (9).

Additional data from our long-term study and other studies should help determine

whether any relationship between density and antibody prevalence exists and if so, what the related temporal patterns are. Population sizes of rodents in the Sonoran Desert of southeast Arizona, as in other areas with climatic extremes, are highly variable. The number of *P. boylii* at Santa Rita Experimental Range was initially high but declined over the course of our study (perhaps because of changes in annual rainfall). To reproduce, many desert rodents require green vegetation (10), often not available in semidesert grasslands and xeroriparian areas. Total annual rainfall at Santa Rita Experimental Range was higher than normal in the 2 years before the start of our study. Since 1995, annual rainfall has been approximately 8 cm to 10 cm below the norm (unpub. data). Petryszyn (11) has linked high variability of *Peromyscus* populations in the Sonoran Desert with extreme fluctuation in winter rainfall. Others (12) have indicated local population expansion and retraction in response to wetter and drier conditions.

Finally, our results are consistent with those of other studies that show a higher prevalence of infection (as indicated by antibody) in male and sexually mature rodents. However, we did not observe direct signs of aggressive encounters or fighting among infected males, as observed by Childs et al. (13) for hantaviral infection in *Rattus norvegicus*.

Field studies of hantavirus infection and wild rodent populations provide a rare opportunity for public health officials, virologists, and ecologists to better understand the dynamics of rodent populations and the interactions between disease, humans, small mammals, habitat, and climatic factors. The few long-term datasets in ecology are invaluable for their contributions to the understanding of processes that vary in complex ways over time but are also relevant to management of both the natural environment and human health.

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Statistical Sensitivity for Detection of Spatial and Temporal Patterns in Rodent Population Densities

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A long-term monitoring program begun 1 year after the epidemic of hantavirus pulmonary syndrome in the U.S. Southwest tracked rodent density changes through time and among sites and related these changes to hantavirus infection rates in various small-mammal reservoir species and human disease outbreaks. We assessed the statistical sensitivity of the program's field design and tested for potential biases in population estimates due to unintended deaths of rodents. Analyzing data from two sites in New Mexico from 1994 to 1998, we found that for many species of *Peromyscus, Reithrodontomys, Neotoma, Dipodomys*, and *Perognathus*, the monitoring program detected species-specific spatial and temporal differences in rodent densities; trap-related deaths did not significantly affect long-term population estimates. The program also detected a short-term increase in rodent densities in the winter of 1997-98, demonstrating its usefulness in identifying conditions conducive to increased risk for human disease.

We analyzed the statistical capabilities of the long-term rodent monitoring program begun in 1994 to detect spatial and temporal changes in rodent densities and determine if the low death rates at all study sites resulted in biased (underestimated) rodent population density estimates. We also examined a short-term subset of the data (mid-1997 to early 1998) to test whether the program design could statistically detect a sudden rodent increase in density that may precede a hantavirus pulmonary syndrome outbreak.

Study Sites

We selected two monitoring sites in New Mexico for analysis because they provided the longest period of field sampling, the greatest range in rodent species richness, and the largest difference in habitat types. The first, a desert grassland site 90 km south of Albuquerque, on the Sevilleta National Wildlife Refuge, Socorro County, New Mexico, USA (34º 21.3'N, 106º 53.1'W, elevation 1,465 m), was dominated by one-seed juniper (*Juniperus monosperma*), honey mesquite (*Prosopis glandulosa*), and various desert grasses (*Sporobolus* spp. and *Bouteloua eriopoda*). The second site (Placitas), a pinyon-juniper woodland site located in Sandoval County in the foothills of the Sandia Mountains, Cibola National Forest, approximately 30 km north of Albuquerque, New Mexico, USA (35º 16.7'N, 106º 18.6'W, elevation 1,830 m), was dominated by pinyon pine (*Pinus edulis*), one-seed juniper, and blue grama grass (*Bouteloua gracilis*).

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Statistical Methods

The overall experimental design and field sampling procedures are described by Mills et al. (this issue, pp. 95-101). The rodent density estimates we analyzed were based on mark-recapture trapping data from three permanently marked trapping webs at each site (1,2). We used monthly trapping data collected from August 1994 to February 1998. Trapping and rodent handling methods were described by Parmenter et al. (3), and safety procedures during animal processing followed Mills et al. (4). Blood samples collected from *Peromyscus maniculatus* were analyzed by the Centers for Disease Control and Prevention for Sin Nombre virus (SNV). Rodent densities were calculated by the program DISTANCE (2). Each dataset was analyzed by three models (uniform, half-normal, and hazard)

with three possible model adjustments (cosine, polynomial, and hermite). Akaike's information criterion was then used to select the model that best fit the particular dataset (2). The three web density estimates for each trapping period were then partitioned into "proportional" densities representing each species, on the basis of the relative proportion of each in the total web sample. These species-specific densities (in numbers of mice per hectare) were analyzed by a repeated measures analysis of variance (RMANOVA) to test for differences between sites and through time and for site and time interactions. If significant F values were observed, we conducted within-trapping-period tests to detect differences among site means; we used Fisher's least-significant-difference method.

The effect of low death rates among rodents on estimates of population size was analyzed by minimum number alive (MNA) methods (5). For this analysis, we calculated the "observed" MNA values for each species during each sampling period on the basis of actual field data, which included occasional trap deaths of rodents. We then constructed a hypothetical MNA, assuming that no trap deaths occurred in the sampled populations. The hypothetical death-free MNAs were computed by extending the projected life span of each animal that died in the trap. The length of the extensions differed by species and site and was determined by the mean number of trapping periods during which each species would normally have been present on each site (this figure was based on the lifespans of all other mice of that species that did not die in the traps or during handling). For example, if *Neotoma albigula* at the Sevilleta National Wildlife Refuge site, Web 1, had a mean lifespan of three trapping periods (i.e., its initial capture period [time zero] plus three additional sampling periods), and a mouse died in a trap on its first capture, we would add one mouse to the observed MNA values for three additional trapping periods to arrive at the hypothetical MNA values. If the mouse died during its second trapping period, we would add two trapping periods to the MNA estimates, and so on. In addition, if the dead mouse was pregnant or lactating, we increased the hypothetical MNA by the average number of offspring that would have been produced; mean numbers of offspring for each species were determined from specimen databases at the University of New Mexico's Museum of Southwestern Biology. These offspring were included for the duration of the expected lifespan on each study site. Thus, if a pregnant female *N. albigula* died during the study, we would add two offspring (the mean litter size for this species in New Mexico) to the MNA estimates for the full three trapping periods of their life expectancy. This process created species-specific hypothetical MNA values that were either equal to or greater than the observed MNA values. The two MNA datasets were then compared by RMANOVA.

Spatial Differences in Rodent Densities

RMANOVA successfully distinguished rodent densities between sites for a number of species (Table 1). Ord's kangaroo rat and the

Table 1. Repeated-measures analysis of variance testing for differences among representative rodent population densities[a], Sevilleta National Wildlife Refuge and Placitas, 1994-1998

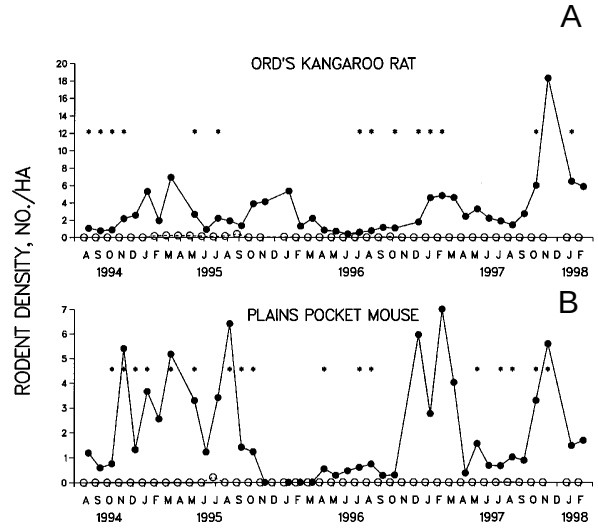
Species	Source	DF	F value	p
Dipodomys ordii	Site	1	7.52	0.0517
	Error (Site)	4		
(Ord's kangaroo rat)	Time	35	2.19	0.0007
	Time x Site	35	2.21	0.0006
	Error (Time)	140		
Perognathus flavescens	Site	1	25.69	0.0071
	Error (Site)	4		
(Plains pocket mouse)	Time	35	2.91	0.0001
	Time x Site	35	2.89	0.0001
	Error (Time)	140		
Peromyscus maniculatus	Site	1	0.28	0.6233
	Error (Site)	4		
(Deer mouse)	Time	35	2.80	0.0001
	Time x Site	35	1.18	0.2513
	Error (Time)	140		
Peromyscus leucopus	Site	1	0.11	0.7609
	Error (Site)	4		
(White-footed mouse)	Time	35	7.25	0.0001
	Time x Site	35	4.56	0.0001
	Error (Time)	140		
Peromyscus truei	Site	1	1.98	0.2328
(Pinyon mouse)	Error (Site)	4		
	Time	35	3.77	0.0001
	Time x Site	35	3.29	0.0001
	Error (Time)	140		
Neotoma albigula	Site	1	6.65	0.0614
	Error (Site)	4		
(White-throated wood rat)	Time	35	1.53	0.0431
	Time x Site	35	1.65	0.0221
	Error (Time)	140		
Reithrodontomys megalotis	Site	1	0.34	0.5906
	Error (Site)	4		
(Western harvest mouse)	Time	35	4.04	0.0001
	Time x Site	35	3.57	0.0001
	Error (Time)	140		

[a]Density=number of mice per hectare.

Plains pocket mouse (Heteromyidae) were much more abundant at the Sevilleta National Wildlife Refuge site than at Placitas (Figure 1A, B) and had greater statistical differences in the RMANOVA results (Table 1). In contrast, other rodent species (Muridae) had no overall differences by site (Table 1) and usually had similar densities, except for occasional episodes (Figures 1C-G). Although we observed no overall effect of site on density for these species, Fisher's least-significant-difference methods showed significant differences between sites during certain periods (Figures 1C,D,F,G), demonstrating that within a particular species, intersite differences could be discerned in both long-term sequences and during episodic, site-specific population irruptions.

Temporal Changes in Rodent Densities

The analyses also detected changes in rodent densities through time in all species examined (Table 1). Several species with generally low densities (e.g., the harvest mouse [Figure 1D], the white-footed mouse [Figure 1E], and the deer mouse [Figure 1G]) occasionally became locally extinct but periodically recolonized the sites. Other species (e.g., the pinyon mouse [Figure 1F] and the white-throated wood rat [Figure 1C]) were found consistently on both sites, although their densities fluctuated considerably. In all cases, RMANOVA found significant differences in these temporal patterns.



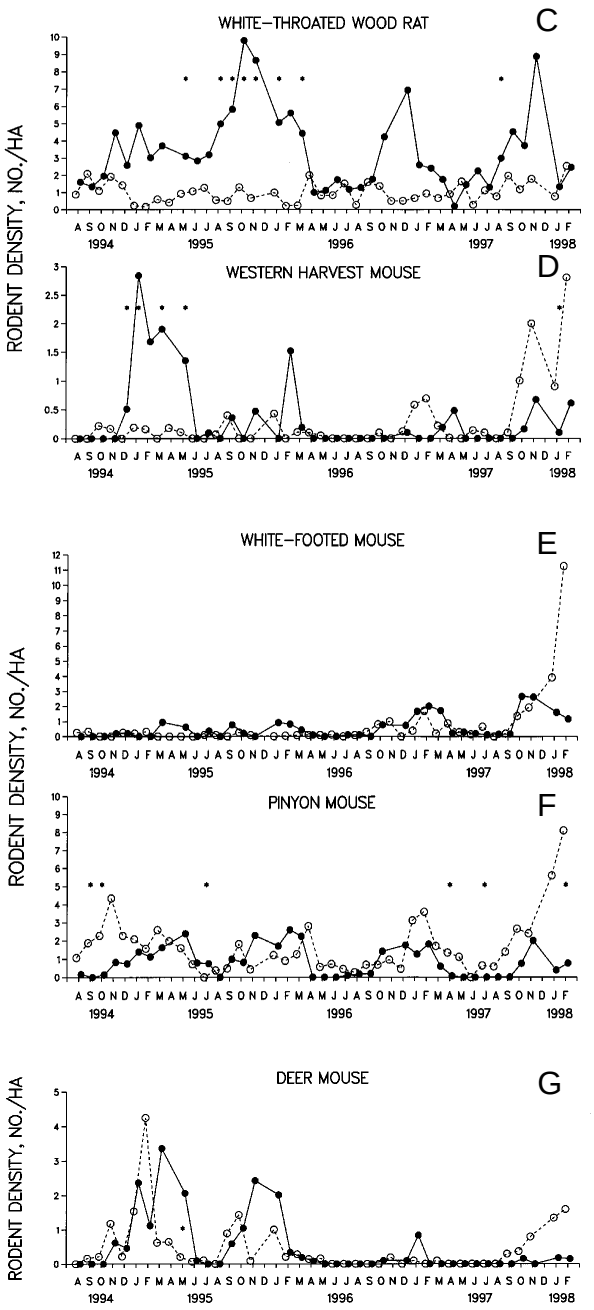


Figure 1. Mean densities of rodents at the Sevilleta National Wildlife Refuge (solid circles) and Placitas (open circles) study sites. Asterisks indicate significantly different means between sites (Fisher's least-significant-difference tests, $p < 0.05$). A. Ord's kangaroo rat (*Dipodomys ordii*). B. Plains pocket mouse (*Perognathus flavescens*). C. White-throated wood rat (*Neotoma albigula*). D. Western harvest mouse (*Reithrodontomys megalotis*). E. White-footed mouse (*Peromyscus leucopus*). F. Pinyon mouse (*P. truei*). G. Deer mouse (*Peromyscus maniculatus*).

Short-Term Rodent Population Increases

To determine the capability of the analyses to show statistically significant short-term increases in rodent densities (e.g., a rodent population "explosion"), we selected May 1997 to February 1998, a period characterized by a rodent density increase in some species (Figures 1A-G). We then tested the datasets for this period alone; RMANOVA results indicated significant increases in densities for the Plains pocket mouse, deer mouse, white-footed mouse, pinyon mouse, and Western harvest mouse (Table 2; Figure 1 B,D-G) during the winter of 1997 to 1998. Therefore, the monitoring program was capable of showing sudden, short-term increases in rodent densities that may precede a disease outbreak in humans.

Table 2. Short-term repeated-measures analysis of variance for differences among representative rodent population densities, Sevilleta National Wildlife Refuge and Placitas , May 1997 to February 1998

Species	Source	DF	F value	p
Dipodomys ordii	Site	1	5.75	0.0745
	Error (Site)	4		
(Ord's kangaroo rat)	Time	8	2.13	0.0616
	Time x Site	8	2.13	0.0616
	Error (Time)	32		
Perognathus flavescens	Site	1	27.09	0.0065
	Error (Site)	4		
(Plains pocket mouse)	Time	8	4.61	0.0008
	Time x Site	8	4.61	0.0008
	Error (Time)	32		
Peromyscus maniculatus	Site	1	2.70	0.1759
	Error (Site)	4		
(Deer mouse)	Time	35	2.45	0.0432
	Time x Site	35	1.57	0.1868
	Error (Time)	140		
Peromyscus leucopus	Site	1	2.25	0.2084
	Error (Site)	4		
(White-footed mouse)	Time	8	7.83	0.0001
	Time x Site	8	5.79	0.0001
	Error (Time)	32		
Peromyscus truei	Site	1	5.09	0.0870
	Error (Site)	4		
(Pinyon mouse)	Time	8	7.09	0.0001
	Time x Site	8	4.94	0.0005
	Error (Time)	32		
Neotoma albigula	Site	1	1.06	0.3618
	Error (Site)	4		
(White-throated wood rat)	Time	8	1.17	0.3443
	Time x Site	8	0.82	0.5944
	Error (Time)	32		
Reithrodontomys megalotis	Site	1	4.58	0.0990
	Error (Site)	4		
(Western harvest mouse)	Time	8	4.67	0.0007
	Time x Site	8	1.64	0.1530
	Error (Time)	32		

Density = numbers of mice per hectare.

Blood tests to determine the presence of SNV in deer mice showed generally low infection rates (Figure 2), with a maximum of only one mouse testing positive per trapping period at Placitas, and none at Sevilleta. The SNV-positive rodents were detected during periods of moderate abundance in 1994 and 1995 but not in the early stage of the population increase during the winter of 1997-98.

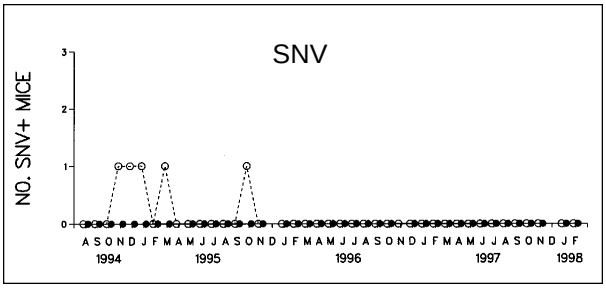


Figure 2. Seroprevalence of Sin Nombre virus (SNV) in the deer mouse populations at Sevilleta National Wildlife Refuge and Placitas study sites.

Low Death Rates among Rodents and Population Estimates

Four species had sufficient sample sizes for the MNA analyses, which were based on 28 trapping periods between August 1994 and January 1997 and included 7,024 rodent captures (Table 3). During field sampling, trap death rates were generally lower than 10% (3). A breakdown of the number of new animals, recaptured resident animals, and trap deaths indicated that immigration and reproduction rates were consistently higher than trap death rates (Figure 3A-D). Species showing territorial behavior (e.g., Merriam's kangaroo rat [Figure 3A] and the white-throated wood rat [Figure 3C]) had higher ratios of residents to immigrants than species without strongly defended territories.

In constructing the hypothetical MNA values, we used the following mean life expectancy values (number of trapping periods after initial capture): *D. merriami* = 2.16; *P. flavescens* = 1.11; *P. truei* = 0.68; *N. albigula* = 2.75. For female rodents of reproductive age, the following mean numbers of offspring were used (on the basis of University of New Mexico's

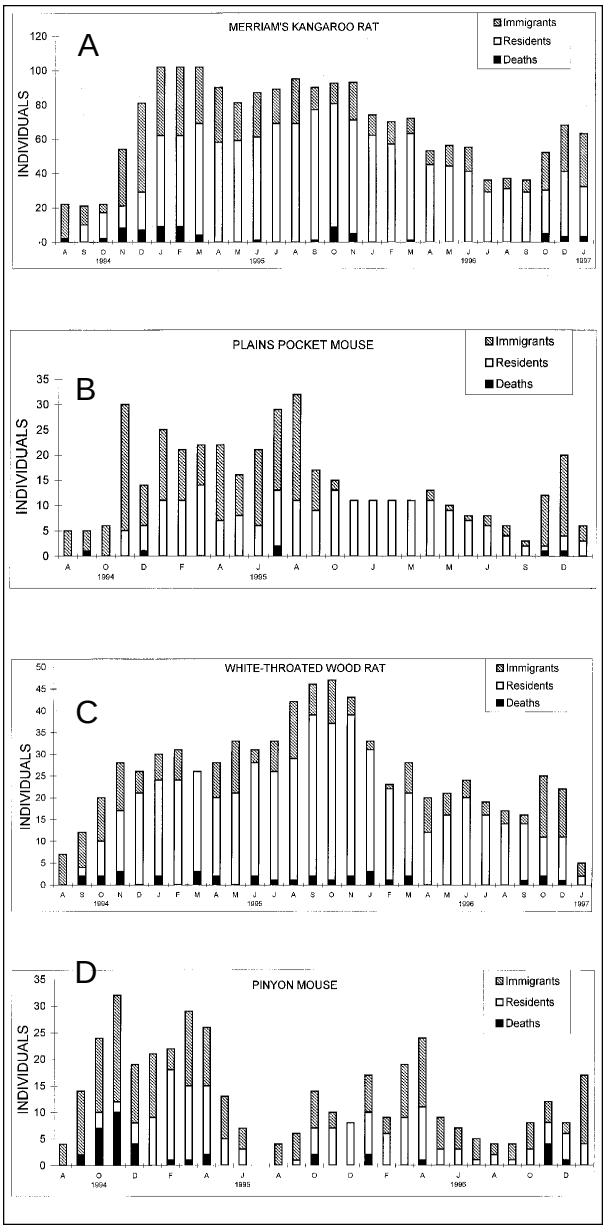


Figure 3. Composition of sampled populations at the Sevilleta National Wildlife Refuge study site. The number of immigrants greatly exceeds incidental trap deaths. A. Merriam's kangaroo rat (*Dipodomys merriami*). B. Plains pocket mouse (*Perognathus flavescens*). C. White-throated wood rat (*Neotoma albigula*). D. Pinyon mouse (*Peromyscus truei*).

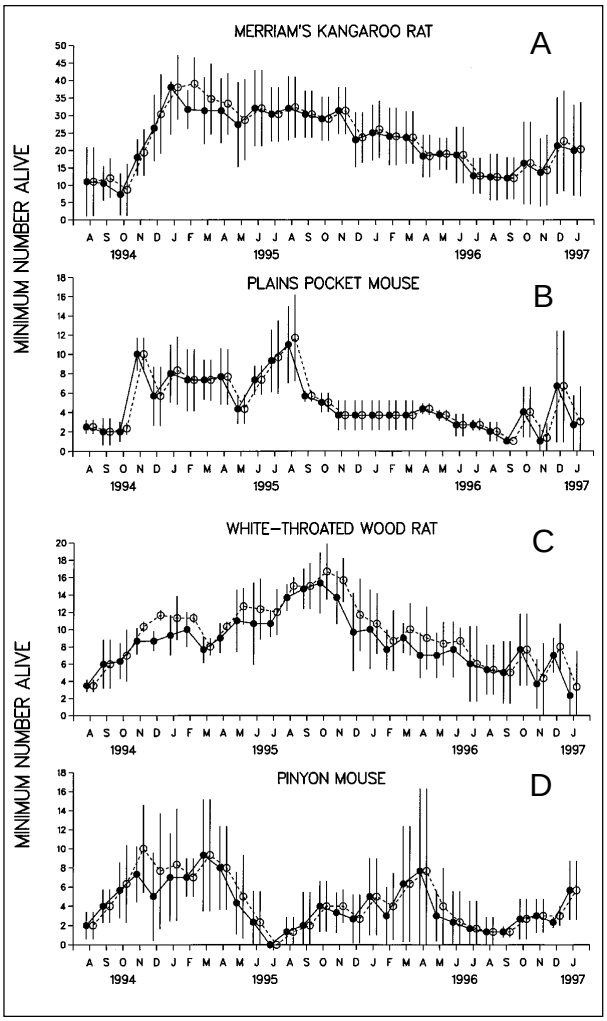


Figure 4. Mean minimum number alive (MNA) values of "observed" (solid circles) and "hypothetical" (open circles) for the following:
A. Merriam's kangaroo rat *(Dipodomys merriami*)
B. Plains pocket mouse (*Perognathus flavescens*)
C. White-throated wood rat (*Neotoma albigula*) at the Sevilleta National Wildlife Refuge study site
D. Pinyon mouse (*Peromyscus truei*) at the Placitas study site.
Values are means of three replicate trapping sites; error bars represent one standard deviation.

Table 3. Repeated-measures analysis of variance for differences between actual minimum number alive (MNA) estimates and hypothetical MNAs (zero deaths from trapping), New Mexico, 1994 to 1997

Species	Source	DF	F value	p
Dipodomys merriami	MNA Treatment	1	0.03	0.8672
(Merriam's kangaroo rat)	Time	27	21.19	0.0001
	Time x MNA	27	0.30	0.9997
	Error (Time)	108		
Perognathus flavescens	MNA treatment	1	0.08	0.7858
	Error (MNA)	4		
(Plains pocket mouse)	Time	27	7.99	0.0001
	Time x MNA	27	0.01	1.0000
	Error (Time)	108		
Peromyscus truei	MNA treatment	1	0.03	0.8661
	Error (MNA)	4		
(Pinyon mouse)	Time	27	6.24	0.0001
	Time x MNA	27	0.13	1.0000
	Error (Time)	108		
Neotoma albigula	MNA treatment	1	0.57	0.4914
	Error (MNA)	4		
(White-throated wood rat)	Time	27	11.82	0.0001
	Time x MNA	27	0.15	1.0000
	Error (Time)	108		

Museum of Southwestern Biology data): *D. merriami* = 2.03; *P. flavescens* = 2.75; *P. truei* = 3.46; *N. albigula* = 1.83. When analyzed with RMANOVA, observed MNA values were not different from hypothetical (Table 3). While highly significant temporal differences in MNA values were observed (Figures 4A-D), no treatment-by-time interactions were produced, which demonstrated that the low death rates during the monitoring program did not affect rodent population estimates.

Conclusions

Our analyses indicated that the field experimental statistical design was sufficiently sensitive to detect a range of differences in densities of rodent species across study sites and through time. Even relatively moderate levels of increases were detectable by our methods, although they were far less dramatic than those observed during the 1993 SNV outbreak (6). While rodent densities of certain species significantly increased during this study (1994 to 1998), the maximum densities were considerably lower than those observed at the Sevilleta National Wildlife Refuge site in 1993 during the SNV outbreak (6). In addition, seroprevalence in deer mice dropped to zero in 1996 (Figure 1G) and did not return, despite the higher densities observed in this species at these sites. Clearly, the population dynamics observed at this time were not equivalent to the rodent "outbreak" of 1993. Continued monitoring of these populations will be needed to determine the extent and importance of this apparent trend in rodent population ecology.

The statistical sensitivity demonstrated in this study is critical to the success of field monitoring programs, particularly those that function as early warning systems to alert health-care workers and researchers of impending outbreaks of disease (7,8). In the case of SNV, the monitoring program serves as both an early warning system and as a research database to which numerous environmental variables and the prevalence of SNV infections in rodents can be correlated.

In addition to the need for statistical power in distinguishing spatial and temporal patterns, the monitoring program must provide study site population estimates that can be directly compared. Standardization of techniques used by collaborating research groups ensures such comparability. In these hantavirus studies, the use of trapping webs and distance sampling theory (2) also allows for direct comparisons of rodent densities among species and across widely varying ecosystems. Trapping grids, and their associated population estimators, often produce results that may be suitable for local studies (internally consistent within a single experiment), but cannot be compared across ecosystem types and taxa because of site-specific characteristics or species-specific assumptions of capture probabilities. Trapping webs and distance sampling density estimators, however, have produced reasonably accurate density estimates in both a computer simulation study (9) and a field study (10). The accuracy of trapping webs and grids in estimating rodent densities is being evaluated more fully at the Sevilleta National Wildlife Refuge site.

All these methods require an appropriate sampling design and an understanding of the basic biology of the rodents. For example, the Plains pocket mouse has MNA values of four animals per month for the months of November, 1994 to March 1995 (Figure 4B). In contrast, its density for the same period is zero (Figure 1B), which indicates that no animals were captured during field sampling. The species' habit of

becoming inactive and remaining in underground burrows during cold winter weather accounts for this discrepancy. Thus, in ecologic and perhaps disease transmission terms, density data accurately reflect which species of rodents are active on a site, whereas MNA data show the combined survivorship of active and inactive species.

A potential source of estimation bias in rodent monitoring programs is the inadvertent influence of trapping and handling of rodents during field sampling. Capturing, anesthetizing, measuring, and collecting blood and saliva samples traumatizes small animals and may affect future trapping success, which, in turn, could bias the accuracy of the density or population estimators. Previous studies have shown various effects of trapping and handling on rodent body mass, ability to trap rodents in the future, and survival (3,11,12-17), but none have addressed the effect of low death rates on long-term population trends. While precautions are taken to ensure survival of sampled animals, occasionally a few die during sampling, especially those with a lower tolerance to the physical, physiologic, and psychologic stress of being captured and handled. Chronic loss of study animals from trapping or handling could underestimate their densities when compared to those of "natural" or undisturbed populations nearby. Results of our study indicate that death rates from trapping at these sites had no significant effect on long-term rodent population estimates.

The existing network of rodent population study sites seems successful in identifying local species-specific fluctuations in densities. Data from these sites can be used in addressing hypotheses on the relationships among environmental factors, rodent abundance, and SNV infections, as well as in providing an early warning for potential rodent population explosions that may increase the risk for other hantavirus pulmonary syndrome outbreaks in the southwestern United States.

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Natural History of Sin Nombre Virus in Western Colorado

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A mark-recapture longitudinal study of immunoglobulin G (IgG) antibody to Sin Nombre virus (SNV) in rodent populations in western Colorado (1994—results summarized to October 1997) indicates the presence of SNV or a closely related hantavirus at two sites. Most rodents (principally deer mice, *Peromyscus maniculatus*, and pinyon mice, *P. truei*) did not persist on the trapping webs much beyond 1 month after first capture. Some persisted more than 1 year, which suggests that even a few infected deer mice could serve as transseasonal reservoirs and mechanisms for overwinter virus maintenance. A positive association between wounds and SNV antibody in adult animals at both sites suggests that when infected rodents in certain populations fight with uninfected rodents, virus amplification occurs. At both sites, male rodents comprised a larger percentage of seropositive mice than recaptured mice, which suggests that male mice contribute more to the SNV epizootic cycle than female mice. In deer mice, IgG antibody prevalence fluctuations were positively associated with population fluctuations. The rates of seroconversion, which in deer mice at both sites occurred mostly during late summer and midwinter, were higher than the seroprevalence, which suggests that the longer deer mice live, the greater the probability they will become infected with SNV.

To monitor Sin Nombre virus (SNV) dynamics in natural rodent communities, we established longitudinal studies at two sites in western Colorado, each near a location where human hantavirus infections occurred in 1993. This article provides a summary of the data collected during the first 3 years of the studies. The results indicate that rodent populations in western Colorado have decreased since 1993; SNV or an SNV-like hantavirus persists at these sites; and prevalence of immunoglobulin G (IgG) antibody to SNV fluctuates with time and perhaps with weather patterns that modify the ecosystem.

The Study

Selected Sites

Each study area was selected on the basis of its proximity to residences of hantavirus pulmonary syndrome (HPS) case-patients, convenience for field work, and guaranteed cooperation by land managers. Sites at Fort Lewis (La Plata County, southwest Colorado) (N 37° 13' 30.9" latitude, W 108° 10' 51.1" longitude, altitude 2,438 m) and Molina (Mesa County, west central Colorado) (N 39° 09' 45.8" latitude, W 108° 03' 18.4" longitude, altitude 1,951 m) were within a few kilometers of case-patient residences.

Fort Lewis (approximately 22 km west of Durango, 8 km south of Hesperus) is 10 km north of Red Mesa, Southern Ute Indian Reservation, Colorado, where rodent trapping in 1993 showed that deer mice had an antibody prevalence rate of 50% to SNV and near where one of the persons who later died of HPS had been infected with SNV (1). Seroprevalence in *Peromyscus maniculatus*, the principal rodent reservoir of SNV, was approximately 50% to 19%, respectively, near study sites in La Plata and Mesa Counties (1).

We established trapping webs (Mills et al., this issue, pp. 95-101) in two protected areas

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(Fort Lewis A and Fort Lewis B) of the 2,550 ha-Colorado State University San Juan Basin Research Center, which serves as a model for cattle breeders and livestock geneticists. The natural characteristics of these sites have been preserved.

Fort Lewis is in the drainage of the La Plata River, south of Mount Hesperus in the La Plata Mountains. The general ecosystem of the area is montane shrubland (2) superimposed on intrusive igneous rocks forming laccoliths (3). The overstory vegetation at Fort Lewis A is predominately ponderosa pine (*Pinus ponderosa*) and Gambel's oak (*Quercus gambeli*); understory vegetation is primarily blue grama (*Bouteloua gracilis*), black grama (*B. eriopoda*), and floral components also seen at Fort Lewis B. At Fort Lewis B, 500 m from Fort Lewis A, overstory is essentially all Gambel's oak; understory is composed of blue and black grama or there is no overstory, with the microcommunity composed primarily of blue and black grama, small soapweed (*Yucca glauca*), tree cholla (*Opuntia imbricata*), and pasture sagebrush (*Artemesia frigida*).

The trapping sites near Molina (approximately 60 km east of Grand Junction) are within 2 km of the home of a 1993 case-patient. In 1993, deer mice had an antibody prevalence rate of 19% to SNV (4).

At Molina we established webs in two areas (Molina A and Molina B, 500 m apart) that are privately owned and have not been grazed by cattle for many years. The sites have no standing water sources, but an irrigation ditch, containing rapidly running water, flows during the summer at the west and north edges of Molina A.

The general ecosystem of the area is semidesert shrubland (2) superimposed on Mancos shale (3). At Molina A, we found principally Rocky Mountain juniper (*Juniperus scopulorum*), pinyon pine, small soapweed, and pasture sagebrush. Molina B is characterized by pasture sagebrush, Rocky Mountain juniper, Parry's rabbitbrush (*Chrysothamnus paryii*), and pinyon pine at the periphery.

All field data were recorded on hard copy and entered into EPI-5, a database and statistical program available from the Centers for Disease Control and Prevention (CDC) (5).

Sampling Methods

All materials were transported to the study sites or were available in towns near the sites. Under license of the State of Colorado's Department of Natural Resources, sampling was done every 6 weeks, weather permitting. Trapping webs were established according to methods agreed upon by collaborating groups (Mills et al., this issue, pp. 95-101). In brief, each web comprised 12 rows of 12 Sherman traps (7.6 cm x 8.9 cm x 22.9 cm; H.B. Sherman Traps, Inc., Tallahassee, FL) each, the first four traps in each row being placed 5 m apart, the next eight placed 10 m apart; rows were 30 degrees from each other. The location of each trap was marked with a construction flag. Rodents were anesthetized with Metaphane (methoxyflurane, Pitman-Moore, Mundelein, IL) during processing, marked with sequentially numbered stainless steel ear tags, and released at the capture site.

Webs A and B at each location were sampled for 2 or 3 consecutive nights, but rodents were neither bled nor swabbed at webs B until October 1996, when animals from both sites were sampled. The original intent had been to not take blood or oropharyngeal swab samples at either web B to determine, by comparison with data from the corresponding web A, the impact of these invasive procedures on the rodent populations. Because the death rates at webs A and B were essentially the same after 2 years (6; C.H. Calisher and B.J. Beaty, unpub. data), in October 1996, we began to take blood samples from rodents at both webs and to no longer collect oropharyngeal swabs. Rodents, principally deer mice, were processed and samples were placed on dry ice (-70°C), returned to the laboratory in Fort Collins, and placed in a mechanical freezer (-80°C) until they were tested for IgG antibody.

Sampling was conducted according to standardized protocols (Mills et al., this issue, pp. 95-101). To compare age categories, in the field we empirically classified captured animals as juvenile, subadult, or adult, according to Fitzgerald, Meaney, and Armstrong (2). For final determination, we separated animals into weight classes (10% to 40% of adult mean weight = juvenile, 41% to 80% = subadult, and 81% to 100% = adult).

After being tested at Colorado State University, blood samples and oropharyngeal swabs were shipped to Atlanta, Georgia, where confirmatory testing for IgG antibody to SNV was conducted with blood samples, and oropharyngeal swabs were stored for possible future testing.

Enzyme-Linked Immunosorbent Assays (ELISA) for IgG Antibody to SNV

ELISA was performed at Colorado State University as described (Mills et al., this issue, pp. 95-101). Results presented here were obtained at Colorado State University; testing at CDC provided confirmation. We initially screened whole blood samples at 1:100; antibody-positive samples were titrated to determine end points.

Population Densities

We estimated the population size at each sampling period by calculating the minimum number of rodents alive (7). The minimum number of rodents alive for a given trapping session was calculated by taking the total number of rodents captured during that session and adding to that sum all rodents that had been captured on at least one previous and one subsequent occasion. The minimum number of antibody-positive rodents was calculated similarly, and the estimated standing prevalence was calculated as minimum number of antibody-positive rodents/minimum number of rodents alive.

Findings

Over the 41-month trapping period at Fort Lewis and the 37-month trapping period at Molina, antibody reactive with SNV was detected in 29 (9.6%) of 302 deer mice at Fort Lewis and 36 (9.4%) of 385 at Molina; 4 (2.6%) of 155 of pinyon mice at Molina also had antibody (Table 1). For comparison, in 1993, prevalence of antibody to SNV in *P. maniculatus* was approximately 50% near Fort Lewis (La Plata County) and 19% near Grand Junction (Mesa County) (1). Of 112 least chipmunks (*Tamias minimus*), two Colorado chipmunks (*T. quadrivittatus*), and two western harvest mice (*Reithrodontomys megalotis*), none had antibody to SNV.

At Fort Lewis, trapping success (number of animals per total number of trap nights) was 0.3% to 7.6%, depending on the season (lowest rates, April–June; highest, August–October). Antibody-positive deer mice were found in 13 of 21 trapping intervals. Antibody prevalence (calculated when more than four deer mice were caught in a given trapping period) was 0% to 42.9% with a mean of 29 (9.5%) of 302. Antibody to SNV was detected in adult (10.5%), subadult (9.8%), and juvenile (12.5%) deer mice; the stages represented 63.9%, 13.1%, and 23%, respectively, of the deer mice captured. Males represented 48.8% of the deer mice (and 47.8% of recaptured deer mice) but 58.3% of the antibody-positive rodents.

Table 1. Antibody (enzyme-linked immunosorbent assay for immunoglobulin G) to Sin Nombre virus, Fort Lewis and Molina, Colorado, 1994-1997

Location	Species	No. positive/ No. tested	% Antibody-positive
Fort Lewis	*Peromyscus maniculatus*	29/302	9.6
	Tamias minimus	0/48	0
	P. truei	1/3	33
Molina	*P. maniculatus*	36/385	9.4
	P. truei	4/155	2.6
	P. leucopus	1/2	50
	Reithrodontomys megalotis	0/2	0
	T. minimus	0/64	0
	T. quadrivittatus	0/2	0

At Molina, trapping success was 2.6% to 17.9% and, as at Fort Lewis, depended on the season (lowest rates, May–June; highest, July–October). Antibody-positive deer mice were found in 12 of 17 trapping intervals. Antibody prevalence was 0% to 33% in deer mice (mean 9.4%) and 0% to 18.2% in pinyon mice (mean 2.6%). Antibody to SNV was detected in adult (11.3%), subadult (1.7%), and juvenile (4.4%) deer mice; the stages represented 73.1%, 15.3%, and 11.7%, respectively, of the mice captured. Males represented 45.5% of the deer mouse population, 46.3% of the recaptured deer mice, and 60% of the antibody-positive mice. Antibody was detected in four adult (three male, one female) pinyon mice (*P. truei*). Of 118 pinyon mice collected, 62 (50.8%) were female and 56 (49.4%) were male. We detected seropositive pinyon mice only during May and June 1995 and April 1996.

Wounds and Antibody

Because we were working with a large number of anesthetized rodents, we did not closely examine each animal for wounds, as had been done by Glass et al. (8). However, we noted the most obvious wounds (ear nicks, torn ears, scarred tail) and those likely not to have been caused by trapping, tagging, or processing, and

we evaluated the data for deer mice at webs A for Fort Lewis and Molina.

Of 233 adult deer mice at Fort Lewis, 20 had both antibody and wounds, 76 had no antibody but had wounds, 4 had antibody and no wounds, and 133 had neither antibody nor wounds; thus, wounds were associated with antibody to SNV among adult deer mice (Yates-corrected chi-square 17.71, p = <0.001). At Molina, of 339 adult deer mice, 8 had antibody and wounds, 23 had no antibody but had wounds, 21 had antibody and no wounds, and 287 had neither antibody nor wounds; again wounds were associated with antibody to SNV (Yates-corrected chi-square 10.67, p = <0.001).

Seroconversion

Fifteen deer mice and one pinyon mouse seroconverted (i.e., seronegative to seropositive or a fourfold or greater increase in titer) between captures (Figure 1). At Fort Lewis, 302 deer mice (150 female and 152 male) were captured. Of these, 37 female and 37 male mice were recaptured at least once. Five male and three female deer mice at Fort Lewis seroconverted. One deer mouse had antibody for the first time 14 months after it was initially captured. At Molina, 385 deer mice (212 female, 173 male) and 155 pinyon mice (85 female, 70 male) were captured. Of these, 33 female and 30 male deer mice and 12 female and 10 male pinyon mice were recaptured at least once. Five male and two female deer mice and one male pinyon mouse seroconverted. An additional three deer mice (two male, one female) at Molina were recaptured and had significant

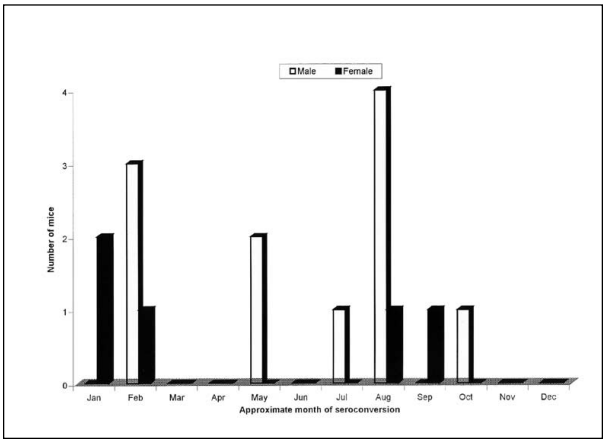

Figure 1. Approximate month of seroconversion in *Peromyscus* species at Fort Lewis and Molina, Colorado, June 1994–October 1997, by sex.

(3,200 to 25,600) but stable IgG antibody titers; we did not consider these as having seroconverted. The five male mice seroconverted at Fort Lewis during the summer (one between July and September 1994, two between July and September 1995, one at [estimated] midsummer 1995, and one between June and September 1997); two female mice seroconverted between October 1994 and May 1995, and one female mouse seroconverted during late summer (September to October) 1997. At Molina, one male deer mouse seroconverted in late spring (estimated May) 1995, one in late fall 1995, two male deer mice and a male pinyon mouse during the winter or early spring of 1995 to 1996, and one male deer mouse during late spring 1996; one female deer mouse seroconverted in late summer 1995 and one during the winter 1995 to 1996. Seropositive samples were titrated by IgG ELISA with fourfold dilutions. Titers were 100 to 102,400, with most of them at 6,400 to 25,600.

Incidence Rates

We calculated incidence rates of IgG antibody to SNV in deer mice recaptured and sampled at least twice at Fort Lewis and Molina (Table 2). At Fort Lewis A, the overall incidence was 4.6 new infections per 100 mice per month (4.8 for male, 4.4 for female); at Fort Lewis B, the overall incidence was 10.91 (9.1 for male, 18.2 for female); and at the two sites combined, the overall incidence was 6.1 (6.3 for male, 5.8 for female). At Molina A, the overall incidence rate in deer mice was 2.8 new infections per 100 mice per month (3.2 for male, 2.3 for female); at Molina B, no new infections were detected during the observation period; the incidence at the two sites combined was 2.6 (3.0 for male, 2.2 for female). Because sufficient numbers of pinyon mice were captured and a seroconversion was detected at Molina A, we were able to calculate the incidence of seroconversion: 0.6 overall (1.4 for male, 0 for female); the incidence at the two sites combined was 0.5 (1.3 for male, 0 for female).

Longevity

By recapturing animals, we were able to estimate the longevity of infected and uninfected mice at the sites. Most *Peromyscus* spp. (75.7% at Fort Lewis, 66.2% at Molina) were not recaptured after they were first caught. At Fort Lewis, of 118 female and 117 male deer mice, 79

Table 2. Incidence of immunoglobulin G antibody reactive with Sin Nombre virus in *Peromyscus maniculatus* (deer mice) recaptured and sampled at least twice at Fort Lewis (June 1994-October 1997) and *P. maniculatus* and *P. truei* (pinyon mice) recaptured and sampled at least twice at Molina, western Colorado (October 1994-October 1997)

Species/ location	Sex	No. at risk[a]	No. of new infections	Cum. % antibody-pos.	Mouse mos. of observation[b]	Incidence[c]
Deer mice/	Grid A	38	4	10.5	88	4.6
Ft. Lewis	male	17	2	5.9	42	4.8
	female	21	2	9.5	46	4.4
	Grid B	11	3	27.3	27.5	10.9
	male	6	2	33.3	22	9.1
	female	5	1	20.0	5.5	18.2
	Grids A + B	49	7	14.3	115.5	6.1
	male	23	4	17.4	64	6.3
	female	26	3	11.5	51.5	5.8
Deer mice/	Grid A	59	5	8.5	179.5	2.8
Molina	male	33	3	11.0	94	3.2
	female	26	2	7.7	85.5	2.3
	Grid B	7	0	0	12.0	0
	male	4	0	0	7.5	0
	female	3	0	0	4.5	0
	Grids A + B	66	5	7.6	191.5	2.6
	male	37	3	8.1	101.5	3.0
	female	29	2	6.9	90	2.2
Pinyon mice/	Grid A	22	1	4.5	157	0.6
Molina	male	10	1	10.0	71	1.4
	female	12	0	0	86	0
	Grid B	15	0	0	33.5	0
	male	7	0	0	9	0
	female	8	0	0	24.5	0
	Grids A + B	37	1	2.7	190.5	0.5
	male	17	1	5.9	80	1.3
	female	20	0	0	110.5	0

[a]No. of mice antibody-negative at first capture.
[b]Total time intervals between successive captures when mice were antibody-negative, plus half the interval when mice changed from antibody-negative to antibody-positive.
[c]New infections per 100 mice per month.

and 83, respectively, were caught only once; 61 were recaptured only within a 5-month period, eight within 6 to 9 months, and four 11 to 14 months after they were first captured. At Molina, of 164 female and 150 male deer mice, 115 and 93, respectively, were caught only once; 89 were recaptured only within a 4-month period, 15 only within 5 to 9 months, and 2 as long as 10 months after they were first captured. Of 63 female and 59 male pinyon mice, 49 and 43, respectively, were caught only once; 21 were recaptured only within a 4-month period, 5 only within 5 to 9 months, and 1 each for 10, 11, 19, and 20 months after they were first captured.

Longevity data of seropositive and seronegative deer mice at Fort Lewis and Molina are summarized in Table 3. Eighteen deer mice had antibody at two or three bleeding intervals from 1 to 7 months after first capture (mean = 2.4 months). Totals do not match the text above because blood samples were not collected from captured rodents at sites B until October 1996 and because we included separately periods of seronegativity and seropositivity for deer mice that seroconverted. Differences between mean longevities by sex, site, or antibody status were not statistically significant (Yates-corrected chi-square, $p = >0.2$).

Population Densities

Deer mouse populations and prevalence of antibody to SNV at Fort Lewis (Figure 2) were relatively low throughout this study, except in May and June 1995 when samples included only four deer mice and one deer mouse, respectively. Mean minimum number of rodents alive was 28 in 1994 but lower from 1995 to 1997 (10.8, 13.4, and 16.4, respectively). At Molina, populations were relatively stable between 1995 and 1997 (only one collection made in 1994), with mean minimum number of rodents alive values of 31.2, 20.4, and 25.4, respectively. As at Fort Lewis, estimated standing prevalence values were commensurately low (Figure 3).

Table 3. Longevity of hantavirus-infected and -uninfected male and female *Peromyscus maniculatus* at Fort Lewis and Molina, Colorado, June 1994–October 1997 expressed as number of months between first and last capture

Site	Sex	Sero-status	Total No.	No. months between first and last capture											Mean
				1	2	3	4	5	6	7	8	9	10	11	
Fort Lewis	F	+	3	2						1					3
	M	+	6	3		2				1					2.7
Molina	F	+	3	2		1									1.7
	M	+	6	4		1				1					2.3
Fort Lewis	F	-	27	15	5	2			2	1	1		1		2.5
	M	-	22	7	4	2	2	1	1	2		2	1		3.7
Molina	F	-	37	24	2	4	2		1	2		1		1	2.4
	M	-	27	13	1	4	2		3	1	1	2			3.2

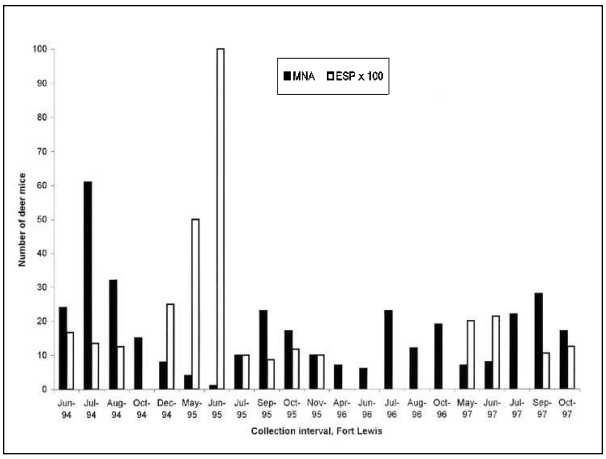

Figure 2. Minimum number of deer mice alive (MNA) (the number of individual mice captured in a month plus those mice captured on at least one previous and one subsequent occasion) and estimated standing prevalence (ESP) (minimum number infected divided by MNA), Fort Lewis, June 1994–October 1997.

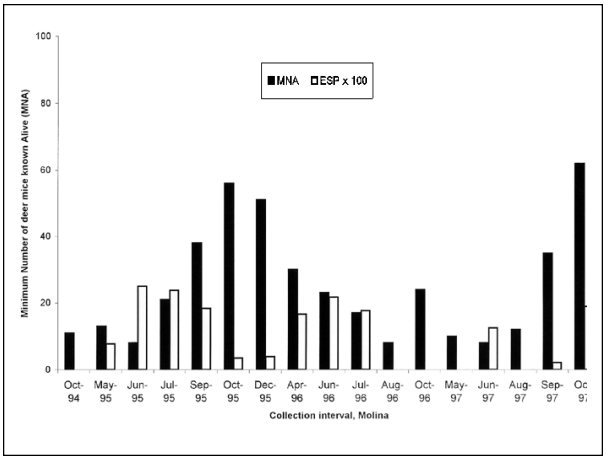

Figure 3. Minimum number of deer mice alive (MNA) (the number of individual mice captured in a month plus those mice captured on at least one previous and one subsequent occasion) and estimated standing prevalence (ESP) (minimum number infected divided by MNA), Molina, June 1994–October 1997.

Conclusions

On the basis of the high antibody titers of these seropositive samples, our findings elsewhere in Colorado (Calisher, Beaty, and Mills, unpub. data), and the findings of others studying hantaviruses in the Southwest (9), we presumed that IgG antibody to SNV in deer mice indicated infection with SNV and not with El Moro Canyon or another hantavirus. Although we did not attempt to isolate or detect hantaviral RNA in blood or other tissues from mice with antibody, the only hantavirus specifically identified in deer mice in western Colorado has been SNV (10).

The presence of IgG antibody to hantaviruses in rodents is presumed to indicate past infection and present infection, at least in the primary vertebrate hosts of hantaviruses (Mills et al., this issue, pp. 135-142). That is, rodents infected with hantaviruses with which they appear to be closely associated coevolutionarily (e.g., deer mice and SNV, Western harvest mice and El Moro Canyon virus, rice rats [*Oryzomys palustris*] and Bayou virus, Black Creek Canal virus and cotton rats [*Sigmodon hispidus*]) do not appear ill or otherwise affected by hantaviruses specific to them. In host-virus associations that have been studied, the specific hosts become infected early or later in life, are viremic for a short period, and excrete virus in their saliva, urine, and feces, perhaps for life (11-14).

Fighting (including exchange of blood and saliva) between infected and uninfected adult rodents has been suggested as the primary mechanism by which hantaviruses are amplified epizootically (8). Infected rodents become viremic and viruric and serve as subsequent sources of infection for others in the population. Earlier studies using Seoul virus and laboratory rats as a model system had indicated that while in newborn rats infection became persistent, in older rats it was transient (15). However, evidence using Black Creek Canal virus and

adult hispid cotton rats, Hantaan virus and *Apodemus agrarius*, and Puumala virus and *Clethrionomys glareolus* indicates that whereas viremia may diminish over time, virus can still be detected in various organs, including the salivary gland, for several months after infection (11-14). Given the relatively brief life span of rodents, infection and concomitant infectivity for a few weeks or months would provide a mechanism for seasonal, albeit not transseasonal, persistence of hantaviruses. Passive acquisition of maternal antibody may protect the offspring of infected dams early in their lives, but when antibody wanes, they enter the adult population as susceptibles. Infected later in life, they can become persistent shedders of virus and sources of infection for others in the population.

Deer mice infected with SNV when very young likely are able to serve as reservoirs of the virus for the remainder of their lives. Although our studies do not distinguish between death and dispersal, the life span of many deer mice at these sites may not be much more than a month. However, because some deer mice live for 1 or 2 years, longevity of even a small proportion of the deer mouse population may provide a transseasonal mechanism for virus persistence.

A second mechanism of virus transmission, an epizootic one, depends on short-term infections of deer mice infected as subadults or as adults. At periods of deer mouse population peaks (e.g., at the end of the breeding season, in late summer and fall, and during period of decreased availability of food), male mice fight one another for breeding partners, food, and territory. This premise is supported by results of serologic tests of recaptured deer mice at Fort Lewis and at Molina. At Fort Lewis, 48.8% of the deer mice and 47.8% of the recaptured deer mice were male, but 58.3% of the seropositive deer mice were male. At Molina, 45% of the deer mice and 46.3% of the recaptured deer mice were male, but 60% of the seropositive deer mice were male. These data support the hypothesis that male deer mice contribute more to the epizootic cycle of SNV than female deer mice. However, the lack of association between sex, wounds, and antibody at either Fort Lewis or Molina indicates that individual mice of either sex may fight and, through this mechanism or another, become infected with a hantavirus. That most mice with antibody to SNV are male supports the suggestion that fighting among mice, biting, and scratching can lead to hantavirus transmission from an infected to an uninfected, wounded mouse (4). The limited time these mice may be able to transmit virus might be sufficient to maintain virus infection in the population.

When deer mouse populations decrease precipitously because of decreased availability of food and water, the likelihood that SNV will disappear from the population increases. However, a few long-lived, persistently infected deer mice can serve as reservoirs until conditions are suitable for the populations to recover.

Our data appear to support such a unified hypothesis. Fluctuations in IgG antibody prevalence in deer mice at Fort Lewis and at Molina have lagged somewhat behind but have been similar to fluctuations in deer mouse population. In male deer mice at Fort Lewis and at Molina, most seroconversions (recent infections) occurred during the summer or fall, whereas in female deer mice, most occurred between fall and spring. During winter, Colorado deer mice reduce their home range, aggregate in nests, and enter short-term torpor—strategies that together temper reduced food availability and energy loss due to cold (2). Although we did not find deer mice that had been infected for more than 3 months, we recovered a few more than 1 year (some nearly 2 years) after they first were trapped; thus, under natural conditions and despite the usual declines caused by predation, cold, heat, and decreases in food, deer mice that reach adulthood can live as long as 2 years (2), a period sufficient to allow SNV to survive adverse conditions of low populations and the resulting decreased number of susceptibles. Furthermore, whereas the overall seroprevalence of IgG antibody to SNV in deer mice at Fort Lewis was 6.8% (12 of 165) and in deer mice and pinyon mice, respectively, at Molina 7.2% (15 of 193) and 5.5% (3 of 52), the rate of seroconversion among deer mice at Fort Lewis was 16.3% (8 of 41) recaptures, and among deer mice and pinyon mice, respectively, at Molina 9.9% (7 of 64) and 3.3% (1 of 29). These results suggest that the longer deer mice live, the greater the cumulative probability they will become infected with SNV.

The deer mouse, the most numerous mammal in North America, often described as a "quintessential generalist," can survive on any dry land habitat in its range and invade and exploit areas disturbed by flood, fire, avalanche,

landslides, mining, construction, extreme grazing, or land development. In ecologically stable areas, deer mice may be limited by the presence of more specialized rodent species (2), but they are found from forests to grasslands, canyons to deserts, farmlands to farm houses and suburban homes, moving into the latter more often in fall but able to take up residence whenever an opportunity presents itself. Thus, the movement of SNV-infected deer mice into human residences itself creates a risk factor for HPS. The Fort Lewis and Molina sites have not been affected by obvious ecosystem perturbations in recent years, and deer mouse populations at these sites are not high, yet are considerably decreased from the apparently inordinately high levels of 1993 (J. Mills, pers. comm. 1997). Each site seems ecologically stable, but subtle changes may have gone unnoticed.

Deer mice are omnivores, storing food for winter consumption but known to feed on acorns, nuts, insects, other small invertebrates, carrion, fungi, bone, and various plant parts, including seeds, leaves, and bark, roots, and tubers (2,16). In one study, seeds accounted for 69% to 76% of stomach contents of deer mice in Colorado, insects for 14% to 25% (2). This proportion depends on the season (i.e., availability of food supply); deer mice are more likely to feed on insects and insect larvae in spring, seeds and berries in fall.

Notwithstanding the nature of deer mice to consume a variety of foods, they rely heavily on acorns, when oaks (*Quercus* sp.) comprise a significant proportion of their habitat (16). Further, in the northeastern United States, the quantity of mast seems directly related to population size of white-footed mice (*P. leucopus*) and eastern chipmunks (*T. striatus*) (17). Although Gambel's oak is abundant at the Fort Lewis trapping sites and copious numbers of acorns were attached to the trees and on the ground in 1994, we did not observe acorns on trees there between spring 1995 and fall 1997. The chipmunk population at this site was, with the exception of a transient, moderate increase in June 1996, never high during our study period (June 1994 to October 1997). At the Molina sites, which do not have oaks, the chipmunk population declined considerably after June 1996 and did not return to its previous level. Chipmunks may serve as an indicator for the ready availability of acorns and other nuts or food in general.

The continued low population densities of deer mice at Fort Lewis and Molina are puzzling. If, for example, deer mouse population densities in surrounding areas are higher than at the study sites, one might expect deer mice from those areas to move into the area with the low population. However, at Fort Lewis 59% of adult and 5% of subadult deer mice were recaptured; at Molina 86% of adults and no subadult or juvenile deer mice were recaptured as adults. These data indicate that few, if any, young deer mice are immigrating to these sites or if they are, they did not survive long enough to be captured, and the survival rate of this species' young is not high.

Temperature fluctuations that affect habitat characteristics can influence rodent breeding seasons (e.g., rate of plant growth, total available nutritional biomass). However, analyses of available data (not presented) did not provide obvious evidence for such direct relationships. In contrast, a paucity of precipitation at Fort Lewis, between March 1995 and October 1996, and at Molina between May 1995 and April 1997, coincided with the usual breeding season of deer mice, least chipmunks, and other rodents at these sites, and with lower rodent population densities between the end of 1995 and the end of 1997; antibody prevalence fluctuated in parallel. Whether the two consecutive relatively wet years 1996 and 1997 will bring about conditions suitable to increase rodent populations near Fort Lewis and Molina and lead to an increase in HPS in the near term has not been determined. Availability of water may be the *sine qua non* of plant food availability, reproductive preparedness, gravidity rates, and attendant intraspecific fighting among individual mice within an increased population. From data (not shown) collected at these sites since October 1997, indications are that both rodent population densities and antibody prevalence are increasing at both sites. If precipitation excess correlates with rodent population, density increases, and the prevalence of hantaviruses, we will be able to predict increases in risk for hantavirus infection in the human population.

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Long-Term Studies of Hantavirus Reservoir Populations in the Southwestern United States: A Synthesis

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A series of intensive, longitudinal, mark-recapture studies of hantavirus infection dynamics in reservoir populations in the southwestern United States indicates consistent patterns as well as important differences among sites and host-virus associations. All studies found a higher prevalence of infection in older (particularly male) mice; one study associated wounds with seropositivity. These findings are consistent with horizontal transmission and transmission through fighting between adult male rodents. Despite very low rodent densities at some sites, low-level hantavirus infection continued, perhaps because of persistent infection in a few long-lived rodents or periodic reintroduction of virus from neighboring populations. Prevalence of hantavirus antibody showed seasonal and multiyear patterns that suggested a delayed density-dependent relationship between prevalence and population density. Clear differences in population dynamics and patterns of infection among sites, sampling periods, and host species underscore the importance of replication and continuity of long-term reservoir studies. Nevertheless, the measurable associations between environmental variables, reservoir population density, rates of virus transmission, and prevalence of infection in host populations may improve our capacity to model processes influencing infection and predict increased risk for hantavirus transmission to humans.

A series of ongoing studies of the natural history of hantavirus-host associations in the southwestern United States was conducted by four independent investigative teams in a variety of ecosystems. The studies, which have a common experimental design, describe several patterns common to all study sites; provide insight into hantavirus maintenance in natural reservoir populations; highlight differences among geographic regions, ecosystems, and closely related host-virus associations; and illustrate that different sigmodontine rodent species (even within the genus *Peromyscus*) may respond differently to the same environmental conditions at the same site.

Sin Nombre virus (SNV), whose host is the deer mouse (*Peromyscus maniculatus*), has been responsible for most, if not all, cases of hantavirus pulmonary syndrome (HPS) in the southwestern United States since 1993. Deer mouse population density and prevalence of SNV infection in deer mouse populations in the arid Southwest have declined sharply since the high levels documented in 1993 (1-3). Nevertheless, moderate population densities of deer mice persisting at the higher altitude web trapping sites in Colorado provided an opportunity to look at the natural history of this species over a wide range of conditions (Calisher et al., this issue, pp. 126-134).

The high prevalence of SNV-reactive antibody in brush mouse (*P. boylii*) populations observed during these and previous studies in the Southwest (2) led to the investigation and identification of a distinct hantavirus carried by brush mice (S. Nichol and A. Johnson, unpub. data). Before these studies were undertaken, it was not known whether antibody in brush mice represented spillover of SNV from the deer mouse reservoir (as may have been the case during the initial 1993 outbreak), unusual maintenance of the same hantavirus by two species of rodents, or (as molecular evidence now indicates) another example of cospeciation leading to a unique hantavirus maintained in a single rodent species. Although the status of the

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virus associated with *P. boylii* as a human pathogen is unknown, *P. boylii* is a common species in the Southwest, and its population density fluctuates dramatically with environmental conditions. Data on the brush mouse host-virus association can contribute to our understanding of hantavirus reservoir ecology.

We summarize major conclusions from the first 3 years of hantavirus reservoir studies in the southwestern United States, examine consistent patterns and salient differences, and discuss the implications of the studies for understanding reservoir host ecology.

Temporal Patterns

In Host Populations

Multiyear Patterns

In most rodent communities examined, sampling methods using trapping webs demonstrated periodic fluctuations in population densities; many populations were simultaneously adversely affected by periods of low rainfall. In the southwestern United States, unusually dry conditions directly followed the wet, exceptionally favorable conditions believed to have contributed to the increase in rodent numbers and the HPS outbreak of 1993-94.

Specific habitat characteristics critical to some hantavirus reservoir species are the availability of thick chaparral cover for brush mice (Abbott et al., this issue, pp. 102-112) and food supply, including acorns and other fruits and seeds (this issue, Calisher et al. pp. 126-134 and Abbott et al., pp. 102-112). As il lustrated by the different effects of drought on pinyon mice (*P. truei*) and brush mice in northern Arizona (Abbott et al., this issue, pp. 102-112), the response of a rodent population to ecologic conditions depends on its specific requirements for food, water, and habitat. A complete understanding of the ecologic requirements and adaptability of each reservoir species is required before the species' response to specific environmental conditions and its potential contribution to future outbreaks of HPS can be predicted.

Many species of murid rodents typically exhibit year-to-year fluctuations in population density. The Arvicolinae are a panarctic group containing several species that undergo fairly regular population cycles with a 3- to 4-year periodicity. Periodic fluctuations in populations of the bank vole (*Clethrionomys glareolus*), an arvicoline rodent and the reservoir for Puumala virus (a hantavirus that causes a mild form of hemorrhagic fever with renal syndrome [nephropathia epidemica] in Europe), have been related to nephropathia epidemica incidence in Sweden (4). The causes of these population cycles in arvicoline rodents are not well understood (5,6). All viruses known to cause HPS are carried by rodents of the New World murid subfamily Sigmodontinae. Although sigmodontine rodent populations do not fluctuate on a regular, cyclic basis, periodic, sometimes dramatic increases ("irruptions") occur in population density; these increases may be tied to unusual climatic events that result in highly favorable (if temporary) conditions for nutrition and reproduction. Such an increase involving deer mouse populations may have been associated with the first recognized outbreak of HPS in the southwestern United States in 1993 (7). Understanding the factors that control or influence the population dynamics of sigmodontine reservoir species is central to understanding the epidemiology of HPS.

Seasonal Patterns

In addition to the overall year-to-year trends in rodent population dynamics, some populations demonstrated seasonal patterns that varied by ecosystem: the size of brush mice and deer mice populations at desert grassland sites peaked in winter and waned in midsummer, while at high altitude sites in Colorado, it was generally highest in the fall. Models of disease risk to humans must consider altitude and biome, as well as regional weather patterns.

In Prevalence of Infection

Assumptions Concerning Antibody Analyses

In these studies, we assume that antibody-positive hosts (*P. maniculatus*, *P. boylii,* and *Reithrodontomys megalotis*) are chronically infected and infectious. Studies of other specific hantavirus-host associations, including Hantaan virus in *Apodemus agrarius* (8), Puumala virus in *C. glareolus* (9,10), and Black Creek Canal virus in *Sigmodon hispidus* (11), show a similar pattern: infection is followed by a brief period of viremia and then by the development of antibody and clearing of virus from blood. Nevertheless, in spite of the continuous presence of

circulating antibody, high-titer virus could be isolated from organs, and infectious virus was shed persistently or sporadically in urine, feces, and saliva for extended periods, probably the life of the host. The quantities of virus shed may be greatest during the early phases (2 to 4 weeks postinoculation) of infection (8). In a field study of SNV, 97% of antibody-positive *P. maniculatus* had viral RNA in organ tissue (1), which implies a similar pattern of chronic infection for the deer mouse–SNV association.

These studies confirm the cited laboratory investigations demonstrating the maintenance of antibody for the expected life span (approximately 1 year or less) of the host. Antibody was detected by enzyme-linked immunosorbent assay (ELISA) for up to 16 months in individual rodents, and no mice reverted from antibody-positive to antibody-negative. Nevertheless, loss of antibody may be observed in animals born with transient maternal antibody (2,12).

Finally, these studies used antibody as the only marker of infection. Mice sampled between infection and development of detectable antibody (probably 3 to 4 weeks [11]) are not recognized as infected; these may represent 2% to 7% of animals sampled (13,14). In one study (1), 55% of seronegative animals had viral RNA in blood samples; however, this study was unusual—it was conducted during epizootic conditions, which presumably involved very high rates of transmission in the reservoir population. In addition, the use of a heterologous antigen (Prospect Hill virus) to detect SNV antibody in the ELISA format may have slightly decreased the sensitivity of serologic tests. Thus, although the actual correction factor is imprecisely known and probably variable, the seroprevalence provided in these reports underestimate the true prevalence of infection in host populations.

Multiyear Patterns

These studies support previous investigations (2,12,15) demonstrating that rodents do not acquire hantavirus infection vertically but instead become infected (and presumably infectious) and develop antibody in an age- or size-related manner. Infection appears associated with life history and behavioral events surrounding the maturation of animals into sexually mature adults. Given the horizontal transmission of hantavirus within reservoir populations, increasing population densities should result in increased rodent-to-rodent contact, opportunities for virus transmission (to susceptible mice), and overall incidence and cumulative prevalence of infection within host populations. Such findings would be consistent with the mass action principle of disease transmission, which assumes that transmission is a function of density (16). Nevertheless, clear evidence of increased population densities leading to increased prevalence of infection in hantavirus host populations is lacking (2,14,17,18). Indeed, many datasets, such as that presented by Abbott et al. (this issue, pp. 102-112), show an inverse relationship between population density and antibody prevalence over time.

These same data, however, can provide insights into the interaction of temporal patterns of reproduction, changing population age structure, and virus transmission. For example, the successful breeding seasons for brush mice in northern Arizona (spring through fall 1995 and spring 1996) resulted in a population with a high proportion of juvenile and young mice not yet infected (as evidenced by antibody). Increasing population density resulted in increasing incidence of virus transmission (as evidenced by the high rate of seroconversion during this period), but the overall antibody prevalence in the population was continuously diluted and offset by the addition of uninfected juvenile mice. By summer 1996, however, local environmental conditions caused breeding to end and production of young to subsequently decline. Until noninfected susceptible young mice began to enter the population again during the summer of 1997, the population consisted of older residents that, by virtue of their age and cumulative life experiences, were commonly infected with hantavirus. Thus, the relatively high prevalence of infection during this period reflects the high rate of transmission during the previous fall, the subsequent decline in new births, and the resultant older age structure and accumulated life experience of the population.

Seasonal Patterns

The dynamics of changing population structure and virus transmission may also result in predictable seasonal patterns in the prevalence of infection. In strongly seasonal climates, the interplay of host demography and horizontal

virus transmission may result in a strongly seasonal alternation of peaks in population density and prevalence of infection. As an example of other hantavirus-host associations, in Sweden, bank vole population density was highest in the fall, but the prevalence of Puumala virus antibody was highest in the spring and correlated with vole density the previous fall and spring (4).

This delayed density-dependent prevalence of infection occurs in other reservoir populations in strongly seasonal environments, such as the high-altitude grids near Fort Lewis, Colorado (Calisher et al., this issue, pp. 126-134). Every year, except 1994, when populations may have been recovering from El Niño southern oscillation conditions and thus showed an atypical pattern, population density of *P. maniculatus* was lowest in the early spring (presumably because of the high number of winter deaths) and increased throughout the breeding season, into summer and fall. Furthermore, in 1995 and 1997 (no antibody-positive animals were captured in 1996), antibody prevalence was highest in the early spring and lower in the fall; this pattern could be the result of reproduction resulting in highest population density in the fall but with the juvenile dilution effect, which leads to low antibody prevalence. The spring population, consisting largely of overwintering adult mice, reflects the relatively high antibody prevalence expected in older animals. The high prevalence in spring presumably reflects virus transmission in the high density population of the previous autumn.

A study of hantavirus in rodent communities in Argentina provides additional evidence for the broad applicability of this pattern in temperate ecosystems. Several hantavirus reservoir species on the central Argentine pampa displayed the same spring-fall alternation of peaks in population density and antibody prevalence (19). Thus, the temporal asynchrony between reservoir population density and prevalence of infection on both the year-to-year and within-year scales can be explained by the interaction of seasonal changes in population structure and horizontal transmission of virus.

The explanations for this pattern suggest three corollary hypotheses: virus "overwinters" in temperate rodent communities as persistent infections in older adult animals, which serve as a reservoir for reintroducing virus into susceptible young animals in the spring; spring antibody prevalence is a function of the population density (infectious and susceptible) the year before (the high fall population densities and higher spring antibody prevalence at the Colorado trapping webs in spring 1995 provide tentative support for this hypothesis); and deviations from typical environmental conditions alter the pattern of infection in potentially predictable directions. For example, a mild winter might prolong the period of reproduction and transmission, simultaneously increasing population densities and improving overwinter survival. Such conditions might result in an atypically high prevalence of infection, as well as a higher-than-usual population base in the spring. Such a pattern might help account for the conditions of high population densities and high prevalence of infection during the initial phases of the HPS outbreak in the southwestern United States in the spring of 1993 (1). Expected changes in population density, antibody prevalence, and population age structure over a hypothetical multiyear cycle are shown in the Figure.

Prevalence of infection in reservoir populations, however, is only one of several factors that may be useful in predicting risk for human disease. The highest absolute numbers of infected rodents (but not prevalence) coincided with high population density (Abbott et al., this issue, pp. 102-112); thus, all other factors being equal, the highest risk for human contact with infected rodents would be during the period of highest rodent population density. Factors of the host-virus interaction (e.g., time course of infection and periods of maximum virus shedding), rodent behavior (e.g., entering human habitations), and human behavior (e.g., planting or harvesting in the spring and fall and opening and cleaning rodent-infested sheds or cabins in the spring) interact to modify specific temporal risk patterns.

In Virus Transmission

The two reports that documented a high incidence of infections as evidenced by first acquisition of antibody (this issue, Calisher et al., pp. 126-134 and Abbott et al., pp. 102-112) provide evidence for seasonal patterns in transmission—one (Abbott et al., this issue, pp. 102-112) clearly documented that the highest rates of seroconversion corresponded with highest population density. The apparently

different seasonal patterns of seroconversion of male and female animals in Colorado were unexpected (Calisher et al., this issue, pp. 126-134). The Colorado study suggests that winter transmission of virus occurs during communal "nesting." This may help explain why brush mice, living in desert and brushland habitats with milder winters, have a higher ratio of male to female antibody-positive mice. Virus transmission among brush mice may be more restricted to aggressive encounters, which would favor male infection; virus transmission among deer mice at high altitudes might also include opportunities for transmission during communal nesting (e.g., by aerosol or mutual grooming), which could diminish differences in transmission ratios between male and female mice.

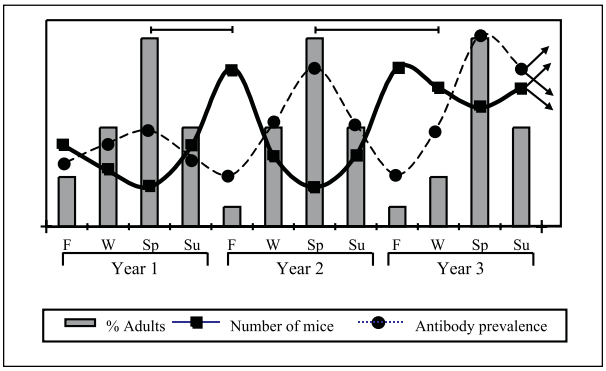

Figure. A hypothetical schematic of seasonal changes in hantavirus prevalence, rodent host population density, and population age structure.In the first autumn, after a normal breeding season, the high density population consists primarily of young not exposed to virus or recently exposed before development of antibody. Because of deaths in winter, populations decrease to a spring nadir. However, antibody prevalence is high in this population of overwintered adults exposed during the previous breeding season or during winter communal huddling. Unusually favorable conditions during the following spring and summer (first horizontal bar) result in higher population density the second fall, and the increased influx of young of the year (juvenile dilution effect) results in an even lower antibody prevalence than the previous fall. A typical winter results in a high number of winter deaths and a typical low density spring population, but seroprevalence the second spring is higher because of increased opportunities for transmission events in the high density population of the previous fall. The second extended favorable season (second horizontal bar) leads again to high population density and low seroprevalence in autumn. The reservoir population of the third spring demonstrates high antibody prevalence, because of high rates of exposure during the "crowded" conditions of the previous fall, and unusually high population density, because of extended breeding and high overwinter survival. Depending on environmental conditions, the population may abruptly "crash" (if it has exceeded the carrying capacity of the environment) or might continue to increase (e.g., if growing conditions the previous spring and summer resulted in abundant food supplies, such as a mast crop of acorns or pinyon nuts).

These studies indicate that hantavirus infection was resilient in the face of population fluctuations. Even when rodent populations were very low, some foci of infection were apparently sustained, presumably through persistent infection in a few long-lived residents (Abbott et al., this issue, pp. 102-112). However, other data indicate that infection may disappear completely from a population during periods of low density, only to reappear sporadically in a few infected mice (Kuenzi et al., this issue, pp. 113-117). The latter phenomenon could indicate that low levels of infection were continuously present in the population, but the sampling method was not sensitive enough to detect it; or it may indicate that virus periodically becomes extinct in semi-isolated populations that have declined in numbers. Virus might be reintroduced into a population through contact with, or dispersal of infected rodents from, adjacent populations, which suggests that reservoir species should be considered metapopulations in maintaining hantavirus infection.

Spatial Patterns

Several reports in this series provide evidence for spatial restrictions in the geographic distribution of rodent reservoir populations, as well as for focality of infection within populations. Although focality has been observed on a regional scale (2), the data by Kuenzi et al. (this issue, pp. 113-117) and Abbott et al. (this issue, pp. 102-112) demonstrated distinct "islands" of hantavirus infection apparently associated with preferred microhabitat for brush mice on web trapping sites, a pattern reminiscent of the concept of natural "nidality" of zoonotic disease as expounded by Pavlovsky (20). Characterizing preferred habitat types may help identify areas at increased risk for virus transmission to humans. However, these pockets of reservoir activity become blurred during periods of high reservoir population density (Abbott et al., this issue, pp. 102-112). Not only would it be more difficult to identify foci that pose a high risk for virus infection during reservoir population irruptions, but also the increased movement of individual rodents may lead to transfer of virus among previously distinct subpopulations, increasing the overall risk for human exposure.

Characteristics of Infected Populations

At all sites and for both brush and deer mice, infected animals were more frequently older males. These data are consistent with horizontal transmission of infection and suggest that the (or a) specific mode of transmission involves male more frequently than female animals. An alternative hypothesis, that males live longer than females (which would lead to greater cumulative probability of infection), is inadequate (Kuenzi et al., this issue, pp. 113-117); therefore, behavioral differences (e.g., greater home range, increased aggression, bites, wounding) may be the most likely explanation. Indeed male murid rodents may be more likely to have scars or wounds (indicators of aggressive encounters) than female rodents (19,21); the presence of scars has been associated with increased prevalence of infection for hantaviruses (12;19; Calisher et al., this issue, pp. 126-134).

Nevertheless, patterns of antibody prevalence differed distinctly by site and species. For instance, the male bias among infected animals was much greater for brush mice in Arizona (85% to 90% of infected animals were male [this issue, Abbott et al., pp. 102-112 and Kuenzi et al., pp. 113-117]) than for deer mice in Colorado (approximately 60% [Calisher et al., this issue, pp. 126-134]). These differences presumably relate to intersite or interspecies differences in social structure or behavior that influence hantavirus transmission. Are female deer mice more likely to fight or otherwise interact than female brush mice? Does winter communal nesting facilitate transmission among both male and female deer mice and not brush mice? Could venereal transmission be involved for deer mice? Some of these questions can be addressed only by continued data collection at the long-term trapping webs. For instance, collection and analysis of data concerning wounding and scars should document the relative frequency of aggressive encounters among males and females of all species. As demonstrated by Abbott et al. (this issue, pp. 102-112), comparing data on interactions of same sex and opposite sex mice involved in dual captures may also yield insights. In fact, preliminary analysis of scar frequency by Calisher et al. (this issue, pp. 126-134) indicates that male deer mice in Colorado may not experience more aggressive encounters than female mice; dual-capture results by Abbott et al. (this issue, pp. 102-112) show that male-male interactions among brush mice can be considerably more aggressive than female-female interactions. If communal nesting increases viral transmission between deer mice, the pattern of antibody prevalence among male and female mice may differ for deer mice captured at trapping webs in lower altitude sites in New Mexico and eastern Colorado. These data are being collected. Venereal transmission, which would be difficult to address in field studies, will require parallel studies in the laboratory.

Comparison of SNV Prevalence with Prevalence of Other Rodent-Borne Agents

The prevalence of infection with hantavirus shown by these studies is 0% to approximately 25%. Even under conditions of high rodent density in the areas of human disease outbreaks, the prevalence of SNV infection in deer mice reached only 30% (1). The high rate of population turnover and relatively short life span of most sigmodontine hosts results in populations frequently dominated by young mice not yet infected with hantavirus; the delay between infection and development of antibody further decreases the apparent prevalence of infection. However, when data are stratified by age and sex, antibody prevalence can be high. For example, 90% of male Norway rats >500 g in Baltimore, Maryland, had antibody to Seoul virus (15), and 88% of male cotton rats >200 g in southern Florida had antibody reactive with SNV (12). These prevalences are comparable to the highest prevalences for agents reported to be vertically transmitted such as some arenaviruses including Lassa (22) and lymphocytic choriomeningitis viruses (23).

The Future

Preliminary results from these studies indicate that some patterns, such as age- and male-associated infection, are clear. Nevertheless, upon closer inspection, the patterns differ between sites and species. Reservoir studies at one site, in one ecosystem, during 1 year, or of one host-virus system cannot provide the data necessary to piece together the natural history of hantavirus infection in North American reservoirs. Environmental conditions cannot be controlled in the field; therefore, adequate replication of field studies across time, space, and host-virus systems is critical. Although extensive, the studies reported in this series are

preliminary. Three years is a very brief period for detecting effects due to environmental changes (e.g., weather and landscape) and for detecting the impact of extremely rare events (e.g., a 20-year flood). The conditions that lead to rodent population irruptions may be infrequent, and there may be thresholds for either environmental conditions or population densities that lead to the increased numbers of infected rodents that are indicators of risk of virus transmission to humans. The ultimate usefulness of these studies depends upon their long-term maintenance.

The methods used in these studies appear sensitive enough to detect changes in reservoir populations associated with increased virus transmission. The sampling methods did not significantly increase deaths among study animals (this issue, Calisher et al., pp. 126-134 and Abbott et al., pp. 94-104; 24;25). The few unavoidable deaths associated with periodic bleeding of animals and mark-recapture studies do not affect most population estimates; the statistical analyses are sufficiently sensitive to detect intersite and temporal differences in population densities (Parmenter et al., this issue, pp. 118-125).

But can these studies provide early warning of conditions that predate and predict an increase in virus transmission and HPS? Data from the last few months of the study period show an abrupt increase in the population density of some reservoir species that coincides with habitat improvements, most likely resulting from increases in rainfall associated with an El Niño southern oscillation event beginning in 1997. The current environmental changes may provide a rare opportunity to document the weather and ecologic conditions associated with demographic changes in reservoir host populations that increase risk for virus transmission to human populations. Recent increases in reservoir populations have been associated with increased numbers of HPS cases in the southwestern United States. As of August 1998, approximately 14 cases have been reported in Arizona, Colorado, New Mexico, and Utah, in comparison to 2, 2, and 4, for the same period in 1995, 1996, and 1997 (A. Khan, unpub. data). The qualitative and quantitative data on reservoir populations and environmental variables collected during this period may also provide the necessary habitat-specific correlations so that satellite images can be related to specific environmental clues. When these links are established, the wide coverage offered by remote sensing platforms may provide the capability to predict increased risk in areas without direct reservoir monitoring.

Even though the variety of ecosystems and host-virus systems included in these studies may lead to models with broad applicability, they still represent a relatively small geographic area and a small percentage of the known hantavirus-host associations in the world. Similar studies in other areas of the United States provide comparisons (17), but similar studies of other sigmodontine reservoirs in South America and arvicoline and murine reservoirs in Europe and Asia are needed.

Finally, the value of these longitudinal studies will increase when these data are integrated with data from complementary field and laboratory studies. These mark-recapture studies are restricted to wild populations in natural environments, while most human cases of HPS are acquired in the peridomestic environment. Although the dynamics of natural populations ultimately influence the density and behavior of peridomestic deer mice, for example, the specific factors of human and rodent behavior that lead to peridomestic exposure can be elucidated only through studies in the specific environment of exposure.

The presence of IgG antibody reactive with SNV antigen is used as the marker of infection in these studies. Given the pattern of chronic infection and long-term shedding of virus in hantavirus-host associations (8,9,11), antibody is probably a good marker. Nevertheless, the specific dynamics and timing of infection, antibody development, and timing of maximum viral shedding are unknown for most hantavirus reservoir species. These data must be provided by controlled laboratory studies using artificially infected animals and (because laboratory infections may not always mimic natural infections [26]) field studies involving naturally infected animals. Natural or manipulative field studies might use captive populations in seminatural enclosures or excretory products collected (by use of metabolic chambers) from wild-caught animals in mark-recapture studies; the success of these studies will depend on the development of assays for infectious virus.

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Proficiency of Clinical Laboratories in and near Monterrey, Mexico, To Detect Vancomycin-Resistant Enterococci

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Early detection of vancomycin-resistant enterococci is important for preventing its spread among hospitalized patients. We surveyed the ability of eight hospital laboratories in and near Monterrey, Mexico, to detect vancomycin resistance in *Enterococcus* spp. and found that although laboratories can reliably detect high-level vancomycin resistance, many have difficulty detecting low-level resistance.

Since vancomycin-resistant enterococci (VRE) were first reported in the late 1980s, their geographic distribution and importance as nosocomial pathogens have continued to increase worldwide. In the United States, the percentage of states with a National Nosocomial Infections Surveillance System hospital reporting one or more patients with VRE infection increased from 27% (1989–1993) to 44% (1994–1995) (1). Among enterococci causing infection in these hospitals, the percentage resistant to vancomycin increased from 0.4% (1989) to 10.8% (1995) in intensive care unit patients and from 0.3% (1989) to 10.4% (1995) in nonintensive care unit patients (1).

Although early detection of VRE is important for preventing its spread among hospitalized patients, clinical laboratories in the United States have difficulty detecting VRE, especially those with intermediate or low-level resistance, characteristic of VRE with the VanB phenotype (2,3). Certain automated and manual antimicrobial susceptibility test systems are associated with the inability of laboratories to detect VRE. Laboratories outside the United States (e.g., in Argentina [4]) may have difficulty detecting VRE for largely the same reasons: limitations of susceptibility test systems. In addition, laboratories outside the United States, Canada, and Europe may face language and financial barriers to accessing information important for updating their methods and optimizing VRE detection.

No VRE have been reported from hospitals in Mexico. However, because VRE have been reported from hospitals in Texas, and VanB has been described as the predominant phenotype in at least one hospital in the Houston area (5), we assessed the ability of clinical laboratories in and near Monterrey (in northeastern Mexico) to detect VRE.

Study Protocol

The laboratory survey and data collection were performed in July-August 1997. Five strains of enterococci (two *Enterococcus faecium*, two *E. faecalis*, one *E. gallinarum*), with or without resistance to vancomycin, were coded as isolates 1 through 5 (Table). Four of these had been used in proficiency surveys in the United States (3) and Argentina (4). The fifth isolate came from the American Type Culture Collection (ATCC 29212). The isolates were distributed,

Table. Characteristics of enterococcal study isolates and results of enterococcal susceptibility testing, by category

Isolate no. and species	MIC (µg/ml)	Vancomycin phenotype	No. of labs[a] (n = 8) S	I	R
1. *E. faecium*	512	VanA	0	0	8[b]
2. *E. faecium*	64	VanB-like	2	1	5[b]
3. *E. faecalis*	16-32	VanB	2	1[b]	5[b]
4. *E. gallinarum*	8	VanC	1	5[b]	2
5. *E. faecalis*	≤4	Susceptible	7[b]	1	0

[a]Laboratories reporting susceptibility to vancomycin; S = susceptible, I = intermediate, R = resistant.
[b]Correctly identified.

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along with standardized susceptibility test results forms, to eight clinical laboratories (seven within and one near Monterrey).

Laboratories were blinded to the susceptibility patterns of the isolates and asked to test the five isolates for resistance to vancomycin with the antimicrobial susceptibility testing method they routinely used. The laboratorians recorded disk zone sizes or MICs and their interpretation of the results (susceptible, intermediate, or resistant), in addition to species identification methods used, zone size breakpoints used for disk diffusion, existence of an antimicrobial control program, and hospital demographic characteristics. No information was collected regarding the version of software program used with automated susceptibility test systems. The Centers for Disease Control and Prevention (CDC) analyzed the forms and classified errors in vancomycin susceptibility testing as very major (reporting a resistant strain as susceptible), major (reporting a susceptible strain as resistant), minor (reporting an intermediate or resistant strain as susceptible or intermediate, respectively), or very minor (reporting a susceptible or intermediate strain as intermediate or resistant, respectively).

MICs were determined by broth microdilution and disk diffusion testing (3). In addition, a polymerase chain reaction assay was used to confirm the presence of the *vanA* resistance determinant in organism 1 (3).

Study Findings

The eight participating laboratories each serviced one hospital with a median bed count of 148 (70 to 185). All but one hospital had neonatal, pediatric, and adult intensive care units; half were teaching hospitals. Only two laboratories reported an antimicrobial use control program in place in their hospital.

The antimicrobial susceptibility testing methods used were the Sceptor system (Becton-Dickinson Microbiology Systems, Cockeysville, MD) (three laboratories); Vitek (bioMerieux, St. Louis, MO) (two); standard disk diffusion (two); and Microscan Autoscan (Dade International, West Sacramento, CA) (one). Of the two laboratories that used disk diffusion, one used outdated breakpoint zone sizes; the other used breakpoints currently recommended by the National Committee for Clinical Laboratory Standards (NCCLS).

Vancomycin Resistance Detection

All laboratories correctly detected the high-level vancomycin resistance in isolate 1 (high-level vancomycin resistance [MIC 512 μg/ml] of the VanA phenotype) (Table). Only five laboratories reliably detected the low-level resistance (MIC 64 μg/ml) typical of the VanB-like phenotype possessed by isolate 2; laboratories made two very major errors reporting this resistant isolate as susceptible. Six laboratories correctly categorized isolate 3 with the VanB phenotype (MIC 16-32 μg/ml) as intermediate or resistant; two laboratories committed minor errors by reporting this isolate as susceptible. Five laboratories correctly categorized isolate 4; one laboratory committed a minor error, and two laboratories committed very minor errors. Of the eight laboratories, seven correctly identified vancomycin susceptibility in isolate 5.

Of the 15 test results from the three hospitals using the Sceptor system, 3 (20%) were errors (all minor). Of the 10 results from the two hospitals using disk diffusion, four (40%) were errors. The laboratory using outdated NCCLS zone-size breakpoints committed one very major and one very minor error; the laboratory using the current NCCLS breakpoints committed two very minor errors. The two hospitals using the Vitek automated system generated 10 vancomycin susceptibility test results; two were errors (one very major and one minor).

Species Identification

Of the participating laboratories, three used the Sceptor system to identify species, two used the Vitek system, one used Microscan Autoscan, one used the Pasco system (Difco, Wheatridge, CO), and one did not report its identification method. Seven laboratories correctly identified isolates 1 and 2, both *E. faecium*; five laboratories correctly identified isolate 3, an *E. faecalis;* none correctly identified isolate 4, an *E. gallinarum* (six laboratories identified it as *E. faecalis* and two as *E. faecium*); and all laboratories correctly identified isolate number 5, an *E. faecalis*.

Conclusions

Because VRE may be transmitted easily by health-care workers from infected to uninfected patients, who may become colonized and serve as reservoirs for transmission, the delay caused by failure of the laboratory to detect VRE from an

infected patient may facilitate the emergence of VRE in a hospital or a group of hospitals in a geographic region. Early studies suggested laboratories were more likely to miss intermediate- or low-level vancomycin resistance in enterococci if they relied on commercial automated test systems (3,6). More recent studies suggest persistent problems with the Vitek instrument (2). In addition, disk diffusion is not sufficiently sensitive in detecting low- and intermediate-level resistance to vancomycin when used as the sole method (2,4). Despite revision of NCCLS-recommended breakpoint zone sizes (7), disk diffusion remains relatively insensitive, possibly because susceptible and resistant zone sizes cluster around breakpoints. In addition, laboratory personnel may not consistently follow recommendations to read plates with transmitted light after a full 24 hours' incubation (7). Microscan has shown improved performance (2), likely due to software and hardware revisions (6).

Of the 40 test results in our study, 9 (23%) were erroneous compared with results at CDC; 2 (5%) were very major errors. In comparison, of 335 vancomycin susceptibility test results in 67 New Jersey laboratories, 111 (33%) were erroneous, with 20 (6%) very major errors (3). Of 25 vancomycin susceptibility test results in four Argentine laboratories, 11 (44%) were erroneous, with 2 (8%) very major errors (4). Although our results compared favorably with those of the New Jersey and Argentina studies, which used four test strains we used, the rate of very major errors is unacceptably high, especially for low-level resistance.

Some errors may have been caused by inadequately skilled personnel. One laboratory was using outdated zone-size breakpoints, despite 1992 revisions, a reminder that instituting contemporary methods in some laboratories may be difficult and lead to incorrect epidemiologic data. However, the overall lower error rate compared with previous studies and the association of errors with certain test methods suggest that limitations of test methods were primarily responsible for inaccuracies. Too few vancomycin susceptibility tests were performed to ascertain relative performance of test methods. However, the 20% error rate and lack of major errors obtained by the Sceptor system, compared with higher error rates and very major errors from laboratories that used the Vitek and disk diffusion methods, are consistent with previous results (8).

NCCLS recommends the use of an agar screen plate consisting of brain heart infusion agar containing 6 μg/ml of vancomycin to detect low- and intermediate-level vancomycin resistance (9). We recommend that laboratories in the Monterrey area use a supplemental brain heart infusion screening agar to test for vancomycin resistance in enterococci isolated from selected clinical specimens (e.g., blood, urine, sterile body sites). Because screening agar has a sensitivity of 100% and specificity of 96% to 99% (2), we recommend that isolates that grow on the screening agar be reported as resistant unless repeat testing with a reference MIC method suggests otherwise.

Some enterococci (e.g., *E. gallinarum* and *E. caseliflavus*), which have intermediate-level vancomycin resistance known as the VanC phenotype, rarely cause human infection. Isolation of this form of VRE, therefore, does not have the same public health importance as that of vancomycin-resistant *E. faecium* or *E. faecalis*. Therefore, to focus efforts on controlling antimicrobial resistance in species likely to cause serious human infection, laboratories must correctly identify VRE to the species level. Both *E. gallinarum* and *E. casseliflavus* can be differentiated from other enterococci on the basis of their motility. *E. casseliflavus* may be easily differentiated from *E. faecium* or *E. faecalis* by its yellow pigment; in contrast, *E. gallinarum* may not be reliably identified unless a motility test is performed with the appropriate media (10). A new conventional biochemical test for methyl-α-D-glycopyranoside (MDG) may be even more reliable than motility in differentiating *E. gallinarum* from *E. faecium* and *E. faecalis* (11).

The laboratories' difficulty in detecting low- and intermediate-level vancomycin resistance and in correctly identifying enterococci to the species level, especially *E. gallinarum,* suggests a need for additional tests to aid in the early detection and correct identification of VRE. Although no VRE have been reported from Mexico, the study area is close to areas in the United States where VRE have been reported and the VanB phenotype may be predominant (5).

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Staphylococcus aureus with Reduced Susceptibility to Vancomycin Isolated from a Patient with Fatal Bacteremia

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A *Staphylococcus aureus* isolate with reduced susceptibility to vancomycin was obtained from a dialysis patient with a fatal case of bacteremia. Comparison of the isolate with two methicillin-resistant *S. aureus* (MRSA) isolates obtained from the same patient 4 months earlier suggests that the *S. aureus* with reduced susceptibility to vancomycin emerged from the MRSA strain with which the patient was infected. Atypical phenotypic characteristics, including weak or negative latex-agglutination test results, weak or negative-slide coagulase test results, heterogeneous morphologic features, slow rate of growth, and vancomycin susceptibility (by disk diffusion test) were observed.

Three cases of infection by *Staphylococcus aureus* with reduced susceptibility to the glycopeptide antibiotics vancomycin and teicoplanin (glycopeptide intermediate *S. aureus*, GISA) have been reported from Japan, Michigan, and New Jersey (1,2). We report the third case in the United States of *S. aureus* with reduced susceptibility to vancomycin. We found that this GISA apparently emerged from a methicillin-resistant *S. aureus* (MRSA) isolate that had infected/colonized the patient during the 3 months before his death. This report highlights key phenotypic characteristics of interest to both clinicians and microbiologists.

The first (in New York State) *S. aureus* isolate with reduced susceptibility to vancomycin was obtained from the blood of a 79-year-old man with a history of coronary artery disease, obstructive pulmonary disease, hypothyroidism, and renal failure requiring chronic hemodialysis. Bacteremia developed in December 1997, and an MRSA was isolated from the patient's blood on December 31. He received vancomycin while in the hospital, improved clinically, and was discharged. He was readmitted to the hospital in January 1998 with an infected jugular catheter, which was removed. An MRSA isolate was obtained from the catheter on January 10, and MRSA was also isolated from the patient's blood. He improved clinically and was subsequently discharged on vancomycin therapy. From December 1997 to February 1998 he received 3.5 g of intravenous vancomycin, followed by a 7-day course of 3.5 g of oral ciprofloxacin. In March 1998, the patient was admitted to the dialysis center with signs of congestive heart failure and sepsis and was transferred to the hospital. He received vancomycin (1 g), ceftriaxone (2 g), and tobramycin (200 mg) but died the following day. Over the last 4 months of his life, the patient received 8.0 g of vancomycin for various infections or for surgical prophylaxis of thrombectomy repairs. An autopsy was not performed.

The vancomycin MIC of the isolate, obtained from blood collected before the administration of antibiotics on March 20 at the patient's last hospital admission, was determined by the hospital laboratory to be 8 μg/ml by Etest (3) and MicroScan conventional panels (Dade Behring, Inc., MicroScan). This MIC was confirmed by broth microdilution at the New York State Department of Health and the Centers for Disease Control and Prevention (4). The MRSA isolates obtained from the patient in December and January were susceptible to vancomycin by

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broth microdilution (MIC <2 μg/ml) and Etest (3 μg/ml and 4 μg/ml, respectively). All three isolates appeared to be susceptible to vancomycin when tested by the disk diffusion method, with zones of 17 mm to 18 mm (5), and were susceptible to teicoplanin by broth microdilution (MIC 2-4 μg/ml). Broth microdilution and Etest results showed that in addition to vancomycin and methicillin, the GISA isolate is also resistant to penicillin, erythromycin, ciprofloxacin, and rifampin; the two MRSA isolates from December and January were also resistant to the latter four antibacterial agents, although at higher levels than the GISA isolate for penicillin (>32 μg/ml and 6 μg/ml, respectively) and erythromycin (256 μg/ml and 16 μg/ml, respectively). All three isolates were susceptible to clindamycin, trimethoprim/sulfamethoxazole, gentamicin, chloramphenicol, and tetracycline. Although the susceptibilities of the recently described GISA isolates to antimicrobial agents vary, all are resistant to clindamycin (6), whereas the GISA isolate was susceptible.

The GISA isolate has two colony types, one of which is smaller than the typical *S. aureus* colony. The MRSA obtained from the patient in January also demonstrated colony size heterogeneity, unlike the MRSA obtained in December, which had only the larger typical colony type. Coagulase activity of the GISA isolate was detected at 24 hours by tube test but was only very weakly detected by slide test (NYSDOH) or was negative (UHMC, CDC). Atypical (weakly positive [Staphyloslide, BBL] or negative [Staphaurex, Murex]) results were obtained by latex-agglutination tests.

As part of a contact investigation, cultures were obtained from the nares and hands of dialysis center and hospital employees, healthcare aides, dialysis center patients, and family members. Nares of 45 persons and the hands of 19 of the 45 were cultured; 14 methicillin-susceptible *S. aureus* (MSSA) and two MRSA isolates (one from a nephrologist and one from a dialysis patient) were obtained. However, no other GISA isolates were found. There was no evidence that the GISA was spread to other patients or employees.

The *S. aureus* isolates obtained from the patient in December, January, and March, as well as the MRSA from the nephrologist and the dialysis patient, were compared by pulsed-field gel electrophoresis (PFGE). The three isolates from the patient had the same PFGE type (Figure). The isolates from the nephrologist and the dialysis patient (not shown) had PFGE types unrelated to the GISA type and unrelated to each other. This suggests that GISA emerged from the MRSA strain that had infected the patient in December, presumably as a result of vancomycin therapy. Although the patient was admitted to the hospital in March with a high fever and evidence of pneumonia and the GISA isolate was obtained from blood specimens during hospitalization, it was not possible to determine whether vancomycin resistance in this organism was responsible for the patient's death.

The third reported in the United States, this case of GISA is similar to previously reported cases. The patient described above had been infected with MRSA for several months and had received prolonged treatment with vancomycin. Laboratories should retain MRSA isolates because they may be valuable in outbreak investigations. As in this case, MRSA isolates, together with subsequent isolates, can be used in studying the emergence of glycopeptide resis-

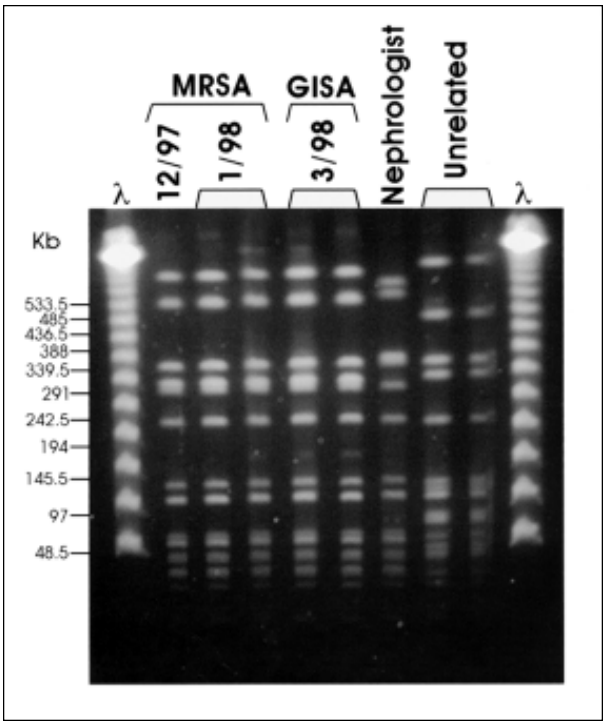

Figure. Pulsed-field gel electrophoresis (PFGE) profiles of *Sma*I digested DNA. Case patient MRSA isolates from December 1997 and January 1998, GISA isolate from March 1998. Nephrologist MRSA isolate from March 1998 and unrelated MRSA isolates.

tance in MRSA. The GISA isolate grew slowly, had heterogeneous morphologic features, weak or negative slide-coagulase test results, and weak or negative latex-agglutination test results. GISA appeared to be susceptible to vancomycin by disk diffusion, as was reported for the previous isolates (6). The disk diffusion test does not appear to be accurate for determining vancomycin susceptibility in these organisms. Microbroth dilution or the Etest method should be used instead. Atypical phenotypic characteristics have been reported for every other GISA, although not all isolates exhibited the same atypical characteristics (1,2,6,7). Laboratorians should be aware of these atypical characteristics so that GISA are not misidentified. Evidence for cell-wall reorganization has been reported for GISA isolates (1,7). These changes in cell-wall structure may be responsible for the atypical phenotypic characteristics and decreased susceptibility to vancomycin.

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Candida dubliniensis Candidemia in Patients with Chemotherapy-Induced Neutropenia and Bone Marrow Transplantation

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The recently described species *Candida dubliniensis* has been recovered primarily from superficial oral candidiasis in HIV-infected patients. No clinically documented invasive infections were reported until now in this patient group or in other immunocompromised patients. We report three cases of candidemia due to this newly emerging *Candida* species in HIV-negative patients with chemotherapy-induced immunosuppression and bone marrow transplantation.

Although *Candida albicans* remains the most common opportunistic yeast pathogen in patients with AIDS and other immunocompromised persons, species less susceptible to fluconazole are becoming more common (1). Recently, a newly described species, *Candida dubliniensis,* was isolated from oropharyngeal lesions in patients with AIDS living in Dublin, Ireland (2). *C. dubliniensis*, phenotypically very similar to *C. albicans* in producing both germ tubes and chlamydospores, has since been recovered from the oral washings of approximately 25% of 94 HIV-positive Irish patients with or without AIDS and 3% of 150 HIV-negative Irish persons (3,4), which suggests that this species belongs to the indigenous microflora of the oral cavity, albeit in a minority of healthy persons. Subsequent reports indicate that the species has a worldwide distribution (4). The role of *C. dubliniensis* as a pathogen has been limited to oral candidiasis. We now report three cases of candidemia due to *C. dubliniensis* in patients not infected with HIV. The yeasts were initially identified as *C. albicans* because each produced germ tubes and chlamydospores; this identification became suspect when equivocal carbohydrate assimilation patterns were obtained.

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Case 1

Graft-versus-host disease of the skin, liver, and digestive tract developed in a 39-year-old woman with chronic myelogenous leukemia after an allogeneic hematopoietic stem cell transplant in September 1995, during which she was treated with cyclosporine and high-dose prednisolone. Germ tube–producing *Candida* spp., later identified as *C. dubliniensis,* were isolated from stool samples obtained for routine testing. The white-cell count was 2.7 x 10^9/L (72% granulocytes); 4 days later fever and ascites developed, and *C. dubliniensis* was cultured from three separate blood cultures (two sets obtained by venipuncture and one by the central venous line) taken on the same day (MIC fluconazole, 0.25 μg/ml). Ascitic fluid obtained by a sterile puncture also grew *C. dubliniensis*. Ascites was probably related to hypoalbuminemia. An echogram showed no radiologic evidence of liver candidiasis, although alkaline phosphatase was elevated (222 U/L; normal < 120 U/L). Treatment was started intravenously with fluconazole, 800 mg/day; 3 days later, *C. dubliniensis* were still recovered in one of five blood cultures taken over 2 days, but from then on, blood

cultures were yeast-negative. Because the patient was in stable condition, the central line was not removed. Cytomegalovirus (CMV) disease also developed, which could explain the elevated alkaline phosphatase, with severe thrombopenia (<20 x 10^9/L). The patient was given ganciclovir and hyperimmune gamma-globulin (Cytotect); *Staphylococcus epidermidis* bacteremia also developed, and the patient died 3 weeks after the onset of candidemia, with severe graft-versus-host disease stage IV, complicated by candidemia and CMV disease. Permission for autopsy was not granted.

Case 2

In July 1995, a 5-year-old boy was treated with cytotoxic chemotherapy for relapsed nasopharyngeal rhabdomyosarcoma. Two episodes of bacteremia caused by *Streptococcus mitis* and *S. epidermidis* followed, and the boy was treated with ceftazidime and later ciprofloxacin (combined with vancomycin) for 3 weeks. Cultures of stools and oral specimens yielded germ tube–producing *Candida* spp. later identified as *C. dubliniensis*. Four days before the onset of candidemia the patient became febrile; *Staphylococcus aureus* and *C. dubliniensis* were cultured from sputum. At this time, the child was not aplastic (leukocyte count 2 x 10^9/L). Flucloxacillin and ceftazidime were started. When fungal blood cultures were taken, the patient was very ill, had profuse diarrhea and high fever, and was leukopenic with a total leukocyte count of 0.3 x 10^9/L (granulocytes <0.1 x 10^9/L; thrombocytes 12 x 10^9/L); 1 day later, three blood cultures, taken over 24 hours through a central line, yielded *C. dubliniensis* (MIC fluconazole, 0.5 µg/ml). Treatment with 12 mg/kg fluconazole was started immediately. *C. dubliniensis* were still being recovered from two blood cultures 2 days after treatment began, but after that, cultures remained sterile, and the patient gradually improved. The central line was removed 20 days after the last positive blood culture but was not submitted for culture. The patient was treated with fluconazole for 1 month (3 weeks intravenously, and 1 week orally) and was discharged 2 months after the onset of candidemia. No yeasts were recovered from fecal cultures and oral washes, but 1 month after discharge, oral washes again sporadically grew *C. dubliniensis*. The patient received further radiotherapy without any evidence of candidemia but died 1 year later of relapsed rhabdomyosarcoma.

Case 3

An 8-year-old girl with sickle cell disease combined with ß-thalassemia and recurrent hemolytic crisis received an allogeneic hematopoietic stem cell transplant in January 1995. One year before, she had a splenectomy because of hypersplenism. The conditioning regimen for the transplant consisted of busulfan, cyclophosphamid, and antithymocyte globulin administered with a Hickman catheter. Cyclosporine and methotrexate were given as prophylaxis against graft-versus-host disease. A suspension of cotrimoxazole and amphotericin B was given as antiinfective prophylaxis. Seventeen days after transplant, sepsis syndrome and renal failure developed while the patient was still profoundly granulocytopenic (< 0.1 x 10^9/L). Germ tube–producing *Candida* spp. later identified as *C. dubliniensis* were isolated from two sets of blood cultures drawn 6 hours apart from a peripheral vein and from two sets through the central venous catheter (MIC fluconazole, 0.25 µg/ml). The patient had no signs of oral candidiasis, and yeasts were not recovered from cultures (oral washes and stools). At this time the patient had already been treated for 72 hours with imipenem and vancomycin. Because of persistent fever unresponsive to broad-spectrum antibacterial agents, intravenous amphotericin B (30 mg) was empirically added. Once the results of the positive blood cultures became known, 5-flucytosin (100 mg/kg) was added to the regimen. After initiation of amphotericin B, later blood cultures remained negative for yeasts. The Hickman catheter was removed 14 days later when the patient had recovered from neutropenia. Catheter tip cultures remained negative. However, low grade fever persisted. Nonetheless, because the patient's condition was stable, treatment was changed to oral fluconazole (50 mg t.i.d.) for another 2 weeks and the patient was discharged. The cause of persistent fever was not identified, but approximately 6 months later, the patient recovered.

Microbiologic Results

All yeast isolates were initially identified by germ tube and chlamydospore formation as *C. albicans*, but carbohydrate assimilation patterns by commercial test kits (Auxacolor,

Sanofi Pasteur, Paris and API 20C, Analytab Products Plainview, New York) gave equivocal results. Furthermore, the isolates did not elaborate ß-glucosidase, grew very weakly at 42°C, and failed to grow at 45°C (6); they produced dark green colonies on CHROMagar Candida plates (Becton Dickinson, Etten-Leur, The Netherlands) typical of *C. dubliniensis* (4,5) and abundant chlamydospores on rice-cream agar after 24 hours (2). In contrast with *C. albicans*, the yeasts isolated from our patients' specimens hybridized poorly with the *C. albicans*–specific Ca3 fingerprinting probe (5) and gave characteristic arbitrary primer phosphatase-polymerase chain reaction patterns for *C. dubliniensis* with primer RP02 (5'-GCGATCCCCA-3'). Each *C. dubliniensis* isolate yielded two major bands at 0.4 kb and 1.0 kb, with up to five weak bands ranging from 0.9 kb to 1.3 kb. In contrast, with *C. albicans*, the two major bands were never observed. Instead, each *C. albicans* isolate yielded approximately 15 bands of various intensity, ranging from 0.65 kb to 2.4 kb. Furthermore, banding patterns obtained with RP02 were clearly different from *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis*. In vitro susceptibility testing for fluconazole (powder provided by Pfizer BV, Capelle a/d IJssel, The Netherlands) was performed by the broth microdilution method with RPMI-1640 with L-glutamine, buffered with MOPS incubated at 35°C, and read after 48 hours according to NCCLS M-27A (7). *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included as quality control strains. The isolates from patients 1 and 2 were deposited as CBS 8500 and CBS 8501 at the yeast division, Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands.

C. dubliniensis was first described 3 years ago (2) and is genetically and phylogenetically distinct from *C. albicans* (8). Hitherto, its pathogenic role has been mainly restricted to oropharyngeal infections in HIV-infected persons and AIDS patients (3,5). In a recent study of *C. dubliniensis,* one isolate recovered from a blood culture and one from postmortem lung tissue was examined (6); however, no clinical data were described to allow determination of the pathogenic role. The cases we have described show that *C. dubliniensis* can cause candidemia in immunocompromised patients. However, these may not be the first cases of invasive disease due to this yeast. Identification and differentiation from other germ tube–producing yeasts on the basis of phenotypic characteristics has been problematic (8); therefore, the incidence and prevalence of this organism and its role in invasive disease have been difficult to determine. For instance, a strain of *C. stellatoidea* originally isolated in 1957 from the sputum of a patient with bronchopneumonia and deposited in the British Culture Collection of Pathogenic Fungi has been shown to be *C. dubliniensis* (2,3), and an isolate of *C. albicans* (from sputum of a Dutch patient) deposited in the culture collection of CBS in 1952 has been shown to be *C. dubliniensis* (Meis, unpub. obs.). In both cases, it has not been established whether the *C. dubliniensis* isolates were responsible for invasive infections.

Fluconazole appears to be less active against *C. dubliniensis* than against *C. albicans* (4) since *C. dubliniensis* is usually associated with recurrent episodes of candidiasis and protracted exposure to azole antifungal drugs in patients with AIDS. Fluconazole showed excellent in vitro activity against each of the *C. dubliniensis* isolated from the blood cultures of our patients; each patient responded well clinically. Nevertheless, it is too early to estimate the true susceptibility of this species to fluconazole. This requires the correct identification of the species, which now seems necessary, given its ability to cause invasive disease in patients treated for malignant diseases.

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Household Transmission of *Streptococcus pneumoniae*, Alberta, Canada

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Proven or presumptive multidrug-resistant *Streptococcus pneumoniae* pneumonia was diagnosed simultaneously in three married couples in Alberta, Canada. The pair of isolates from each couple had identical antibiotic resistance profiles, serotypes, and pulsed-field gel electrophoresis patterns. One or more of these cases could have been prevented by *S. pneumoniae* vaccine.

Outbreaks of *Streptococcus pneumoniae* (antibiotic resistant and nonresistant) have been reported from child-care centers, nursing homes, hospitals, military camps, homeless shelters, and penal institutions (1-6). Simultaneous cases within households have rarely been reported (7-11); such cases require common exposure and transmission, as well as similar likelihood of disease in the hosts or increased virulence in the pathogen.

In December 1996 and January 1997, three married couples with multidrug-resistant *S. pneumoniae* (MDRSP) were admitted to Foothills Medical Centre in Calgary. The couples were not admitted on the same day. None of the couples lived with children, although couple C had daily contact with children. All patients received appropriate antibiotic therapy after their culture and antibiotic sensitivity results were known. We reviewed each patient's health record (Table) and were able to contact two of the three couples for further information.

S. pneumoniae were identified by standard methods. MICs were determined by E-Test (AB Biodisk, Solna, Sweden) and classified as susceptible (S), intermediate resistant (I), or fully resistant (R) to each antibiotic, according to National Committee for Clinical Laboratory Standards guidelines (12). Serotyping of *S. pneumoniae* was performed by the Quellung reaction technique at the National Centre for Streptococcus, Edmonton. Electrophoretic fingerprinting of *S. pneumoniae* was performed by pulsed-field gel electrophoresis (PFGE) of DNA digested with *Sma*1 (BRL, Gaithersburg, MD). The PFGE patterns were classified as indistinguishable, related, or different according to criteria suggested by Tenover (13).

The diagnosis of *S. pneumoniae* pneumonia in couple A was confirmed by positive blood cultures, chest X-ray lobar pneumonia, and disease-compatible clinical findings. Patient 1 in couple A was a health records clerk at Foothills Medical Centre. Her illness was complicated soon after admission by empyema, which was drained; the fluid was *S. pneumoniae*-negative. Vertebral osteomyelitis was suspected from clinical evidence 18 days after admission and was confirmed by bone scan; no diagnostic culture was obtained. Osteomyelitis in this patient was presumably caused by *S. pneumoniae*. The initial 7-day course of cefuroxime (to which *S. pneumoniae* was resistant) may not have cleared the infection and thus allowed secondary seeding to bone.

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Table. Clinical and laboratory features of three couples with *Streptococcus pneumoniae* pneumonia

	Couple A		Couple B		Couple C	
Feature	Patient 1	Patient 2	Patient 1	Patient 2	Patient 1	Patient 2
Age (yrs)	62	61	72	71	39	37
Chronic conditions	Hypertension, diabetes	Gout, 3 previous MIs[a]	Hypertension, CAD[b]	COPD[c]	Recurrent sinusitis	Recurrent sinusitis
Smoker	No	No	Yes	Yes	Yes	Yes
S. pneumoniae vaccine	No	No	Unknown	Unknown	No	No
Recent antibiotics	None	None	Unknown	Unknown	>3 courses in previous year	>3 courses in previous year
Others in home	None	None	None	None	None	None
Initial complaints	URTI[d] symptoms, cough, fever	URTI[d] symptoms, cough, fever	URTI[d] symptoms, cough, fever, chest pain	URTI[d] symptoms, cough, fever, chest pain, eye discharge	Burn, recent URTI[d] symptoms, cough, fever	Burn, recent URTI[d] symptoms, cough, fever
Physical exam	Febrile, ↑HR[e], ↑RR[f], severe distress, ↓breath sounds	Febrile, ↑HR[e], ↑RR[f], ↓breath sounds	Febrile, ↑HR[e], ↑RR[f], ↓breath sounds, ↓O_2 saturation	Febrile, ↑HR[e], ↑RR[f], ↓breath sounds	Febrile, ↑distress on ventilator, ↓breath sounds, crepitations	Febrile, ↑distress on ventilator, ↓breath sounds, crepitations
Chest X-ray (admission or as noted)	Right upper lobe consolidation	Right lower lobe consolidation	Bibasilar consolidation	Extensive right-sided consolidation	Day 3 – extensive bilateral consolidation	Day 2 – extensive bilateral consolidation
Admitting diagnosis	Right lobe pneumonia	Bilateral pneumonia	Pneumonia	Lobar pneumonia	Burn	Burn
Discharge diagnosis	Right upper lobe pneumonia	Right lower lobe pneumonia	Pneumonia	Lobar pneumonia	Burn, complicated by pneumonia	Burn, complicated by pneumonia fatal sepsis
Complications	Empyema, osteomyelitis	None	None	None	None	Died
Source of isolate	Day 1 - blood	Day 1 - blood	Day 1 - sputum (4+[i])	Day 1 - sputum (3+[i])	Day 3 - ETT[g] (4+[i])	Day 2 - BAL[h] (10^5 CFU/mL[i])
Gram stain	Not applicable	Not applicable	GPC resembling *S. pneumoniae*[j]	GPC resembling *S. pneumoniae*[j], GNB[k]	GPC resembling *S. pneumoniae*[j]	GPC resembling *S. pneumoniae*[j]
Other potential pathogens when pneumonia diagnosed	None	None	None	*H. influenzae* (3+[i])	GNB[k]	*H. influenzae* (10^3 CFU/mL[i])
Antibiotic susceptibility[l]						
Penicillin	2 R	1.5 I	1.5 R	2 I	1.5 I	1 I
Cefuroxime	4 R	6 R	3 R	4 R	6 R	4 R
Ceftriaxone	1 I	0.5 S	0.75 S	0.38 S	0.75 S	0.75 S
TMP/SMX[m]	≥32 R	≥32 R	≥32 R	≥32 R	≥32 R	≥32 R
Erythromycin	0.25 S	0.25 S	16 R	16 R	0.25 S	0.25 S
Serotype	14	14	9V	9V	9V	9V
PFGE pattern[n]	AA	AA	BB	BB	BC	BC

[a]Myocardial infarction.
[b]Coronary artery disease.
[c]Chronic obstructive pulmonary disease.
[d]Upper respiratory tract infection.
[e]Heart rate.
[f]Respiratory rate.
[g]Endotracheal tube.
[h]Bronchoalveolar lavage.
[i]For sputum or ETT aspirates, 3+ & 4+ reflect growth on the third and fourth set of streaks, respectively, on the culture plate; for BAL, sample fluid is an approximately 100-fold dilution of lung fluid.
[j]Gram-positive lancet-shaped cocci found singly, in pairs or in short chains.
[k]Gram-negative coccobacilli.
[l]Antibiotic susceptibilities reported as MIC (micrograms/mL) and as S (susceptible), I (intermediate) or R (resistant) (NCCLS criteria).
[m]TMP/SMX (trimethoprim/sulfamethoxazole).
[n]Pulsed-field gel electrophoresis.

Couple B (who could not be reached for further information) had had recent visitors from Texas (one a hospital worker) with upper respiratory tract infections. *S. pneumoniae* pneumonia was presumptively diagnosed in this couple on the basis of symptoms, signs, and chest X-rays compatible with the diagnosis of pneumonia, as well as sputum samples, which had gram-positive lancet-shaped cocci identified on Gram stain and grew *S. pneumoniae*. From the sputum of patient 2 in couple B, gram-negative bacilli were identified on Gram stain; *Haemophilus influenzae* was also isolated. Thus, this patient may have been coinfected, or primarily infected, with *H. influenzae*. The patient's blood cultures were negative; a blood culture was not performed on patient 1 in couple B.

Couple C was admitted with severe burns and inhalation injuries after the stove in their two-room trailer exploded. They had had recurrent sinusitis and other respiratory infections in the previous year since moving to their trailer, which had poor air circulation. Patient 1 of this couple was taking antibiotics at the time of admission, and patient 2 had recently completed a course of antibiotics. The diagnosis of pneumonia (patient 1 on day 3 of admission and patient 2 on day 2) was made on the basis of recent upper respiratory symptoms and fever, diminished breath sounds, crepitations, and disease-compatible chest X-ray findings (previous films had been normal), which made pneumonia more likely than noninfectious conditions such as acute lung syndrome. The presumptive diagnosis of *S. pneumoniae* as the etiologic agent in the case of patient 1, couple C, was made on the basis of the initial endotracheal tube aspirate, which had gram-positive lancet-shaped cocci identified on Gram stain and grew *S. pneumoniae*. Only gram-positive lancet-shaped cocci were identified from the initial bronchoalveolar lavage of patient 2 on Gram stain, and *S. pneumoniae* grew in much greater numbers than *H. influenzae*. Blood cultures, performed for couple C only after antibiotic therapy was started, were negative. Patient 2 died of septic shock 20 days after admission, with *Candida albicans* in his blood. The bronchopneumonia never resolved clinically, although *S. pneumoniae* was not isolated from any further cultures. Thus, *S. pneumoniae* may have been a contributing factor to, but not likely the direct cause, of this patient's death.

The identical susceptibility patterns, serotypes, and PFGE patterns indicate that both partners in each couple were infected with the same multidrug-resistant *S. pneumoniae* strain. Couples A and B apparently had community-acquired pneumonia. Although couple C contracted pneumonia 48 to 72 hours after admission, each partner entered the hospital already infected with MDRSP; the infecting organisms were identical, and no other recognized cases of nosocomial MDRSP occurred at Foothills Medical Centre at the time of their admission (they were admitted 1 month before couple B, who were also infected with serotype 9V MDRSP). Couple A may have been exposed to MDRSP as a result of one partner's work in a tertiary-care hospital; couple B as a result of one partner's exposure to a health-care worker with respiratory symptoms. At the time of these cases, the prevalence of penicillin-nonsusceptible *S. pneumoniae* infections in Calgary was approximately 10% (A.P. Gibb, unpub. data).

None of these patients had received *S. pneumoniae* vaccine, yet each had one or more risk factors for infection (advanced age, exposure to young children, smoking, and chronic lung or heart disease). Couple C had a history of recent antibiotic use, the predominant risk factor for antibiotic-resistant infections.

In Canada, the *S. pneumoniae* vaccine is recommended for all persons ≥65 years old and persons ≥2 years with identified risk factors (14). Despite the vaccine's reasonable effectiveness, its use has been very low in Canada until recently (fewer than 12 doses per 10,000 population distributed annually [15,16]). The vaccine has been provided free of charge to persons with medical indications, but not to healthy persons 65 years of age and older and not as part of a routine vaccination schedule (17). Some provinces (including Alberta, beginning in 1998) have begun to routinely provide the vaccine to all persons at risk. The current incidence of invasive *S. pneumoniae* infections in Calgary is 20 per 100,000 per year overall and 87 per 100,000 per year in those older than 64 years of age (J.D. Klein, unpub. data).

Outbreaks of *S. pneumoniae* disease occur in institutions with crowding, poor air quality, or increased host susceptibility (2,4,6). These factors may also exist within households (9,11). Couple C, for example, lived in a very crowded space with poor air circulation.

The rate at which secondary *S. pneumoniae* infections occur in household contacts of index patients with invasive disease is not known, but rare cases have been reported (7-11). Factors contributing to secondary infections include the likelihood of nasopharyngeal infection due to exposure to the index patient or a common source, susceptibility to the strain of the index infection, and likelihood that colonization will lead to disease rather than to development of asymptomatic immunity. Data on contemporaneous nasopharyngeal carriage of the outbreak strain by household contacts are limited. A recent study from Gambia found carriage in 8.5% of household contacts, compared with 21% in an older U.S. study (18,19). In healthy adults, the prevalence of circulating *S. pneumoniae* antibodies is low (4% to 34%, depending on the serotype); however, two thirds of adults have protective antibody within 1 month of colonization (20). Approximately 15% of children who acquire a new *S. pneumoniae* strain nasopharyngeally in a nonoutbreak setting acquire clinical disease (usually otitis media); this rate is unknown for adults (21). In contrast, during a recent nursing-home pneumonia outbreak, 23% of residents were infected with the *S. pneumoniae* outbreak strain, and 4% became ill (22). The median age of residents was 85 years; only 4% had received *S. pneumoniae* vaccine.

Increased use of *S. pneumoniae* vaccine may prevent MDRSP pneumonia within households and among persons living in crowded conditions.

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Preventing Zoonotic Diseases in Immunocompromised Persons: The Role of Physicians and Veterinarians

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We surveyed physicians and veterinarians in Wisconsin about the risk for and prevention of zoonotic diseases in immunocompromised persons. We found that physicians and veterinarians hold significantly different views about the risks posed by certain infectious agents and species of animals and communicate very little about zoonotic issues; moreover, physicians believe that veterinarians should be involved in many aspects of zoonotic disease prevention, including patient education.

The bond between humans and animals has been recognized for many years, and pet ownership has been associated with both emotional and health benefits (1-4). However, pet ownership may also pose health risks through the zoonotic transmission of infectious diseases, especially in the immunocompromised (5). Animal-associated pathogens of concern to immunocompromised persons include *Toxoplasma gondii*, *Cryptosporidium* spp., *Salmonella* spp., *Campylobacter* spp., *Giardia lamblia*, *Rhodococcus equi*, *Bartonella* spp., *Mycobacterium marinum*, *Bordetella bronchiseptica, Chlamydia psittaci,* and zoophilic dermatophytes (2,6). However, with the exception of *Bartonella henselae* and zoophilic dermatophytes, infections in humans are more commonly acquired from sources other than pets, and the infectious disease risk from owning pets is considered low (2,7). Nonetheless, HIV-infected persons may still be advised not to own pets (8).

Since human medicine often does not delve deeply into the role of animals in the transmission of zoonotic agents (7,9) and veterinary medicine does not cover the clinical aspects of human disease, zoonotic disease control requires involvement of both physicians and veterinarians. We examined how frequently physicians and veterinarians encounter zoonotic diseases, what role physicians think veterinarians should play in zoonotic disease prevention, how often physicians and veterinarians communicate about zoonoses issues, and what physicians and veterinarians perceive as the disease risk of immunocompromised persons from pets.

Our sample populations were drawn from membership lists of the Wisconsin Veterinary Medical Association (WVMA) and the State Medical Society of Wisconsin (excluding retired practitioners). Veterinarians (n = 526) were chosen by a systematic sampling of every third name on the WVMA membership list. Since veterinarians in all types of practice may encounter zoonotic problems, sampling was not stratified by specialty. Physicians (n=698) were chosen by specialty most likely to involve both zoonotic diseases and immunocompromised patients (all physicians who listed infectious disease [n = 38] or hematology/oncology [n = 103] as specialties), as well as randomly selected cohorts of pediatricians (n = 100), and general internal medicine physicians (n = 500). Duplicate names were removed.

Each participant was mailed a cover letter; a number-coded, postage-paid return envelope; and a physician- or veterinarian-specific survey. Nonresponders received a second survey 3 weeks after the first. For questions with a response scale of 1 to 5, the sample size was large enough for a 2-sample Z-test to statistically compare mean responses between physicians and veterinarians. Additionally, responses were analyzed by veterinary practice type and physician

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specialty. Statistically significant differences in responses by specialty are noted in the text. A 2-sample Z-test employing the standard large sample approximation to binomial data was used to compare the proportions of responses by physicians and veterinarians to the question about zoonotic pathogens of concern to immunocompromised patients.

Surveys were completed by 327 veterinarians and 322 physicians (overall response rate of 53%). Responses from veterinarians were as follows: 142 (43%) small-animal practice, 65 (20%) large-animal practice, 98 (30%) mixed-animal practice, and 22 (7%) exotic-animal practice. The distribution of respondents by practice type is very similar to the distribution by practice type across the State of Wisconsin (46% small animal, 19% large animal, 35% mixed, and 0.3% exotic [data courtesy of M. Mardock, Wisconsin Veterinary Medical Association]), except for an overrepresentation of exotic-animal practitioners. Nationally, 8% of veterinarians are in large animal practice exclusively, 29% in mixed practice, 58% in small animal practice and 5% "other" (data from the Veterinary Economics Statistics Brochure of the AVMA, September 1998). The distribution of responses from physicians included 24 (7%) in infectious diseases, 48 (15%) in hematology/oncology, 53 (16%) in pediatrics and 197 (61%) in general internal medicine (including 16 who specifically categorized themselves as pulmonologists and 11 as rheumatologists). Among our random selection of pediatricians and general internists, the respondent ratio of 3.7 internists for every 1 pediatrician is slightly higher than the statewide ratio of 2.5 (Wisconsin physician data courtesy of M. O'Brien, State Medical Society of Wisconsin) and the national ratio of 2.0 (10).

The survey results indicate that veterinarians (Table 1) encounter zoonotic diseases in their practices or discuss them with their clients more frequently ($p < 0.00001$) than physicians (Table 2). Among veterinarians, small-animal practitioners encounter zoonoses more frequently than veterinarians as a whole (mean = 2.80, $p = 0.05$), and large-animal veterinarians less frequently (mean = 3.41, $p = 0.001$). Among physicians, infectious disease specialists encoun-

Table 1. Survey of veterinarians

Questions	Responses
How often do you encounter or discuss zoonotic diseases in your patient population?	
1=Several times/day; 2=Daily; 3=Weekly; 4=Occasionally; 5=Never	$\overline{X}$ = 3.02[a] (±0.05)[b]
How often do physicians contact you for advice on the animal aspects of transmission and risks of zoonotic diseases?	
1=Several times/week; 2=Several times/month; 3=Several times/year; 4=Rarely; 5=Never	$\overline{X}$ = 4.30 (±0.04)
How often do you contact physicians regarding a zoonotic disease?	
1=Several times/week; 2=Several times/month; 3=Several times/year; 4=Rarely; 5=Never	$\overline{X}$ = 4.21 (±0.04)
If you know that a client is immunocompromised, do you offer consultation on zoonotic disease prevention?	
- Yes	n=96[c]
- No	n=9
- The situation has never arisen	n=205
How much risk to immunocompromised patients is associated with owning or having contact with the following animals?	
1=Highest risk to 5=Lowest risk	
- Reptile	$\overline{X}$ = 2.28 (±0.09)
- Bird	$\overline{X}$ = 2.49 (±0.07)
- Kitten (<6 months of age)	$\overline{X}$ = 2.81 (±0.07)
- Puppy (<6 months of age)	$\overline{X}$ = 3.02 (±0.07)
- Farm animals	$\overline{X}$ = 3.05 (±0.07)
- Cat	$\overline{X}$ = 3.28 (±0.06)
- Dog	$\overline{X}$ = 3.86 (±0.06)

[a]Mean of all respondents.
[b]Standard error of the mean.
[c]Absolute number of veterinarians answering "yes", "no" or "the situation has never arisen".

Table 2. Survey of physicians

Questions	Responses
How often do you encounter or discuss zoonotic diseases in your patient population?	
1=Several times/day; 2=Daily; 3=Weekly; 4=Occasionally; 5=Never	$\overline{X}$ = 4.16[a] (±0.03)[b]
How comfortable do you feel in advising patients specifically on the animal aspects of transmission and the risks for zoonotic diseases?	
1=Very comfortable to 5=Not comfortable	$\overline{X}$ = 3.69 (±0.05)
Should veterinarians be involved in advising clients about the potential for zoonotic disease?	
1=Veterinarian should have primary responsibility;	$\overline{X}$ = 2.77 (±0.05)
3=Responsibility should be equal; 5= Physician should have primary responsibility	
How involved should veterinarians be in the following areas in reducing transmission of zoonotic disease agents to immunocompromised patients, providing that client confidentiality is maintained?	
1=Very involved to 5=Not involved	
- General maintenance of animal health	$\overline{X}$ = 1.62 (±0.06)
- Additional zoonotic disease screening of animals	$\overline{X}$ = 1.78 (±0.06)
- Zoonoses education for patients	$\overline{X}$ = 2.08 (±0.06)
- Consultation for physicians	$\overline{X}$ = 2.12 (±0.06)
How often do veterinarians contact you regarding zoonotic diseases?	
1=Several times/week; 2=Several times/month; 3=Several times/year;	$\overline{X}$ = 4.74 (±0.03)
4=Rarely; 5=Never	
How often do you contact veterinarians for advice on the animal aspects of transmission and risks of zoonotic diseases?	
1=Several times/week; 2=Several times/month; 3=Several times/year;	$\overline{X}$ = 4.55 (±0.03)
4=Rarely; 5=Never	
How much risk to immunocompromised patients is associated with owning or having contact with the following animals?	
1=Highest risk to 5=Lowest risk	
- Bird	$\overline{X}$ = 2.37 (±0.07)
- Kitten (<6 months of age)	$\overline{X}$ = 2.47 (±0.08)
- Cat	$\overline{X}$ = 2.58 (±0.07)
- Reptile	$\overline{X}$ = 2.64 (±0.09)
- Farm animals	$\overline{X}$ = 2.94 (±0.08)
- Puppy (<6 months of age)	$\overline{X}$ = 3.28 (±0.08)
- Dog	$\overline{X}$ = 3.69 (±0.06)

[a]Mean of all respondents.
[b]± Standard error of the mean.

ter zoonoses more frequently than the overall population of physician respondents (mean = 3.44, p = 0.001), but these specialists still encounter zoonoses problems less frequently than veterinarians (p = 0.05).

When physicians were asked (on a scale of 1 to 5 with 1 = very comfortable and 5 = not comfortable) how comfortable they felt about advising patients on the role of animals in the transmission of zoonotic agents and associated risks, with the exception of infectious disease specialists (whose mean comfort level = 1.92 was significantly [$p \leq 0.0001$] better than that of the overall population of physicians), they responded that they were not very comfortable in this role (mean = 3.69, Table 2); moreover, physicians indicated that veterinarians should play an equal or greater role in advising patients about zoonotic diseases (Table 2). In particular, they suggested that veterinarians should be involved not only in controlling zoonotic disease pathogens in animals, but also in providing information for patients and physicians (Table 2). However, the survey demonstrated a nearly complete lack of communication between physicians and veterinarians about zoonotic disease issues (Tables 1,2). In addition, patients themselves do not appear to view veterinarians as a source of zoonotic disease information. Of 310 veterinarians, 96 indicated that they offer special consultation about additional steps for zoonotic disease prevention if they are aware of

the fact that a client is immunocompromised; however, for 205 of 310 respondents, the client's health was never discussed (Table 1).

In the second portion of the survey, we examined the views of physicians and veterinarians on the possible disease risks (from specific animals or pathogens) to immunocompromised persons. Various animals were ranked on a risk scale of 1 to 5 (1 = highest risk to 5 = lowest risk, with an option to respond "unsure"). Veterinarians assigned a higher risk than physicians to reptiles ($p = 0.004$) and puppies ($p = 0.01$); physicians assigned a higher risk than veterinarians to cats ($p \leq 0.00001$) and kittens ($p = 0.001$) (Tables 1,2). Physicians and veterinarians were also asked to list the two zoonotic pathogens of greatest concern for immunocompromised persons (Table 3). The two most frequently named pathogens were *Salmonella* spp. and *Toxoplasma gondii.* Within this ranking, *Salmonella* spp. were listed more frequently ($p = 0.001$) by veterinarians than physicians, and this concern may explain why veterinarians thought that reptiles pose the greatest risk to the immunocompromised (Table 1). (Because of the high prevalence in reptiles of *Salmonella* infection, immunocompromised persons are advised not to own or handle reptiles [2,11]). In contrast, *T. gondii* was listed as a potential disease risk more often by physicians ($p = 0.001$), which is consistent with physician's concern about immunocompromised persons owning kittens and cats as pets (Table 2).

In summary, our survey results indicate that physicians and veterinarians hold very different views about the disease risks from certain animals and infectious agents and communicate very little about zoonotic disease prevention. The perceived risks posed by specific pathogens raise some questions. First, for both *Salmonella* spp. and *T. gondii*, contact with pets is not the only, or even the most important, source of infection for humans. Contaminated foods are the most common vehicle of *Salmonella* spp. infection (12); undercooked meat is also a common vehicle of *T. gondii* infection. Up to 25% of lamb and pork samples contain *Toxoplasma* tissue cysts (13). Therefore, although cats are the definitive hosts for *T. gondii*, cat ownership is not associated with an increase in *Toxoplasma* seroconversion among HIV-infected persons (14). Secondly, a number of the infectious disease agents (e.g., *Borrelia burgdorferi*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Pneumocystis carinii*) listed as zoonotic disease risks by both physicians and veterinarians are not truly zoonotic, but rather shared infections. Both animals and humans are infected, but animals are not the direct vehicles of infection for humans. In addition, cytomegaloviruses of humans and animals are not infectious across species. Finally, it is surprising that infection with *B. henselae,* the causative agent of cat scratch disease, which also causes bacillary angiomatosis, peliosis hepatis, and other conditions in immunocompromised persons, was listed relatively infrequently by both physicians and veterinarians (Table 3). Exposure to kittens has been clearly implicated as a significant risk factor in the epidemiology of *B. henselae* (15). Both physicians and veterinarians need to recognize the role of this pathogen in the zoonotic infection of immunocompromised persons and the role of cats in its transmission.

Table 3. Responses of physicians and veterinarians when asked to "List the two zoonotic pathogens you believe should be of greatest concern for immunocompromised individuals"

Pathogen	Physicians	Veterinarians
Toxoplasma gondii	n[a] = 144	n[b] = 74[c]
Salmonella spp.	n = 61	n = 111[c]
Cryptosporidium parvum	n = 54	n = 86
Mycobacterium spp.	n = 29	n = 18
Chlamydia psittaci	n = 24	n = 31
Bartonella spp.	n = 15	n = 10
Histoplasma capsulatum	n = 13	n = 1[c]
Giardia lamblia	n = 12	n = 14
Pasteurella spp.	n = 9	n = 6
Borrelia burgdorferi	n = 8	n = 6
Pneumocystis carinii	n = 8	n = 2
Cytomegalovirus	n = 8	n = 0[c]
Blastomyces dermatitidis	n = 7	n = 9
Rabies virus	n = 6	n = 2
Campylobacter spp.	n = 5	n = 10
Escherichia coli	n = 2	n = 11[d]
Streptococcus spp.	n = 1	n = 9[d]
Dermatophytes	n = 1	n = 24[c]
"Unsure"	n = 29	n = 9
Total number of participants responding to this question	n = 259	n = 271

[a]Total number of times each agent was listed by physicians.
[b]Total number of times each agent was listed by veterinarians.
[c,d]The number of times these organisms were listed by physicians and veterinarians were significantly different. ([c]$p \leq 0.001$; [d]$p=0.02$).

Our finding that 205 of 310 veterinarians never knew a client's immunocompromised condition is consistent with a previous study in which only 21% of HIV patients felt most comfortable in asking their veterinarian about the health risks of pet ownership (16). Through approaches such as small signs in exam rooms, zoonotic disease brochures in reception areas, comments in practice newsletters, and affiliation with support groups in the community, veterinarians can encourage immunocompromised persons to avail themselves of the diagnostic and preventive measures that can be provided for zoonotic agents.

Our results suggest that communication between physicians and veterinarians about zoonotic diseases is largely absent. Enhancing such communication could help prevent transmission of zoonotic agents. In addition to directly contacting veterinary practitioners in their community, physicians can also contact their state health departments for information, since some health departments have public health veterinarians on staff. Links between the professions on a broader scale (e.g., through combined veterinary/medical student training and continuing education) to foster a broader consensus about zoonotic disease risks and prevention should also be encouraged.

Acknowledgments

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Mycoplasma penetrans Bacteremia and Primary Antiphospholipid Syndrome[1]

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Mycoplasma penetrans, a rare bacterium so far only found in HIV-infected persons, was isolated in the blood and throat of a non-HIV-infected patient with primary antiphospholipid syndrome (whose etiology and pathogenesis are unknown).

Antiphospholipid syndrome (APS), first described in 1983 to 1986, is characterized by a wide variety of hemocytopenic and vaso-occlusive manifestations and is associated with antibodies directed against negatively charged phospholipids. Features of APS include hemolytic anemia, thrombocytopenia, venous and arterial occlusions, livedo reticularis, pulmonary manifestations, recurrent fetal loss, neurologic manifestations (stroke, transverse myelitis, Guillain-Barré syndrome); and a positive Coombs test, anticardiolipin antibodies, or lupus anticoagulant activity (1). The factor(s) causing production of the antiphospholipid antibodies in primary antiphospholipid syndrome (PAPS) remain unidentified (2).

A substantial number of patients with *Mycoplasma pneumoniae*–induced respiratory disease have anticardiolipin antibodies (3). Furthermore, many clinical criteria for APS have also been well documented in patients with *M. pneumoniae* infection, including Guillain-Barré–like illness and other central nervous system manifestations, hemolytic anemia, positive Coombs test, thrombocytopenia, and arthritis (4).

In this report, we describe the case of a patient with clinical features of PAPS and a documented bacteremic infection due to *M. penetrans* (5).

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Case

One month before hospital admission, a previously healthy non-HIV-infected 17-year-old woman (blood group O Rh+) who had not previously received a blood transfusion and had not had sexual experience had acute onset of arthritis of both ankles, generalized arthralgias, fever, progressive asthenia, and hemolytic anemia (hemoglobin 87 g/L, unconjugated bilirubin 25.1 μmol/L). In the 30 days before hospital admission she had not received medications other than nonsteroidal antiinflammatory drugs for 5 days. Three days before hospital admission, she became ill with respiratory distress, generalized weakness, anorexia, and inability to walk.

On admission to the hospital (day 1), physical examination showed severe pallor, a swollen cervical lymph node, slight edema of both legs, tachycardia, and no hypertension. Laboratory data showed severe hemolytic anemia (hemoglobin 34 g/L, lactate dehydrogenase 5.1 μmol/s/L), leukocytosis (24.5 x 10^9/L), thrombocytopenia (28.0 x 10^9/L), and normal renal function. A Coombs test was positive at 4ºC, 22ºC, and 37ºC. Blood and bone marrow smears did not show a neoplasic process. The nonsteroidal antiinflammatory drugs were suspended, and treatment was started with a combination of methylprednisolone (1 gm bolus q24h intravenously [i.v.] for 3 days) and trimethoprim/sulfamethoxazole (80/400 mg q12h orally) on day 2, but her condition deteriorated. On day 3, the antibiotic treatment was changed to ceftazidime (1 gm q8h i.v.).

[1]Presented in part at the 11th International Congress of the International Organization for Mycoplasmology. July 14–19, 1996. Orlando, FL, USA.

Transfusion was not attempted because serologic tests indicated the lack of compatibility; (there was a strong positive mismatch incompatibility in 55 different blood samples and a mild mismatch in one sample). Another transfusion was partially rejected because of unidentified nonspecific antibodies. On day 4 severe respiratory distress and hypoxemia developed, requiring a ventilator, and the patient was admitted to the intensive care unit. Livedo reticularis was noted, and methylprednisolone (125 mg q8h i.v.) was administered. Venereal Disease Research Laboratory tests were negative as were tests for lupus erythematosus. The patient had anti-dsDNA antibodies (Kallestad Quantafluor *Crithidia lucilae* Sanofi Diagnostic Pasteur, Inc.) but positive anticardiolipin antibodies by enzyme-linked immunosorbent assay (ELISA) (100 GPL units) (negative test = <10 GPL units) (ImmunoWell, Cardiolipin Antibody Immunoglobulin [Ig]G ELISA; and Reaads Medical Products, Inc.), which remained positive 4 and 12 months later, and antiplatelet antibodies by immunofluorescence (Anti-Human IgG H-chain Fluorescein conjugated, OTIY-05 Behring Diagnostics). Laboratory data showed hemoglobin 33 g/L, leukocyte 23.6 x 10^9/L, a prolonged activated partial thromboplastin time >150 seconds (control <42 seconds) and prothrombin time of 26.1 seconds (control 14.0 seconds), International Normalized Ratios value = 3D 2.09, and the presence of lupus anticoagulant (LA) antibodies (prolonged Russell viper venom time and confirmed by the STACLOT LA ELISA test, Reaads Medical Products, Inc.). Respiratory secretions were culture-negative and negative by immunofluorescence for respiratory syncytial virus, adenovirus, influenza A, influenza B, parainfluenza 1,2,3, and *Chlamydia*. Serologic analysis indicated that the patient had no antibodies against HIV, hepatitis B surface and core antigens (HbsAg, HBc), or hepatitis C virus. No acid-fast bacilli or other bacteria were observed on blood and tracheal aspirate smears. In addition, thoracic radiography showed only bilateral diffuse pulmonary infiltrates, which was not suggestive of an anaerobic infection.

On day 2 of hospital admission, blood and throat samples were cultured for aerobic flora and mycoplasma. *M. penetrans* in pure culture was isolated from the patient's blood (isolate HF-1) and throat (isolate HF-3). Later *M. penetrans* was isolated from tracheal aspirate in pure culture (isolate HF-2). Treatment was initiated on day 6 with clindamycin 600 mg q8h i.v. and vancomycin 500 mg q6h i.v. The patient also received transfusion of two units of washed red blood cells.

By the microbroth dilution method (6), the HF-1 isolate was sensitive to clindamycin, clarithromycin, azithromycin, erythromycin, tetracycline, doxycycline, ofloxacin, and chloramphenicol but resistant to vancomycin and gentamicin. After 3 days of treatment, the patient improved clinically and was released from the intensive care unit on day 9; thoracic radiographs were clear.

The unique evidence of thrombosis was a low-degree paresthesia of both legs while the patient was receiving anticoagulant therapy; when the condition developed, anticoagulant therapy was increased. The patient received physiotherapy to correct paresis and reduced sensation in the left foot and ankle region. She left the hospital after 26 days, with minimal evidence of peripheral neuropathy as a sequela.

M. penetrans infection was detected in the patient's specimens prior to culture and was confirmed by specific polymerase chain reaction (PCR) (7) (Figure 1A, 1B). Similar results were obtained by another pair of PCR primers also within the 16S rRNA gene and designed for the specific detection of *M. penetrans* (data not

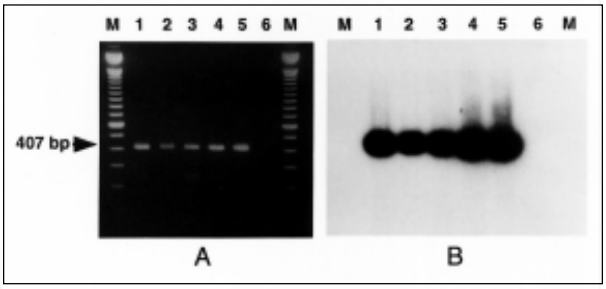

Figure 1. Polymerase chain reaction (PCR) detection of *Mycoplasma penetrans* in clinical samples. A. *M. penetrans* PCR genomic amplification with the primers MYCPENET-P and MYCPENET-N (7) and analyzed by electrophoresis in 2% agarose gel. Lysates from the following original samples: throat swab (lane 1); tracheal aspirate (lane 2); blood (lane 3); first blood subculture (HF-1 isolate) (lane 4); *M. penetrans* GTU-54-6A1 (lane 5), showing the amplification product of 407-bp; and negative control (lane 6). B. Southern blotting of the same material. Hybridization with the internal oligonucleotide (MYCPENET-S) probe confirmed the specific amplification of *M. penetrans* genetic sequences.

shown). Samples from both original specimens and broth cultures were tested by PCR for other human mycoplasmas (8,9), but none were detected (data not shown).

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein patterns of different extracts (whole cell lysate, Triton X-114 extracts) for the isolate HF-1 and the type strain GTU-54-6A1 were almost identical (Figure 2A). Upon close examination, minor differences were found, in particular for antigens approximately 38 kDa in both SDS and Triton X-114 extracts. Ultrastructural examination of the HF isolates by transmission electron microscopy showed mycoplasma cells with morphologic features typical of *M. penetrans* (Figure 2B).

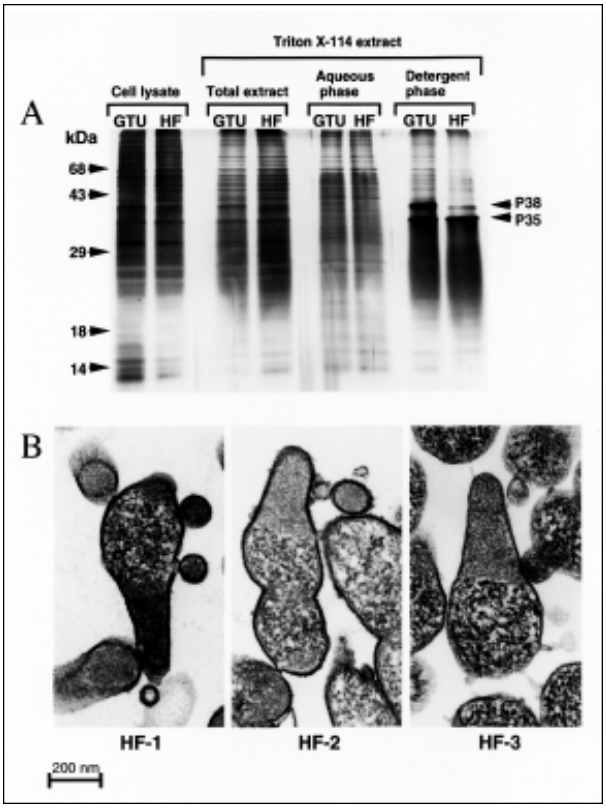

Figure 2. HF isolates belong to the species *Mycoplasma penetrans*. A. Comparison of protein patterns from the type strain of *M. penetrans* and the isolate HF-1. Mycoplasma cells were directly lysed with SDS (cell lysate), or antigens were extracted with the neutral detergent Triton X-114 (total extract). Antigens were further separated after partitioning between the aqueous and detergent phases. The two mycoplasmas compared are the *M. penetrans* type strain GTU-54-6A1 (GTU) and the isolate HF. B. Ultrastructural features of the *M. penetrans* isolates HF-1, HF-2 and HF-3. The HF isolates were passaged four times in SP-4 broth without antibiotics and processed for transmission electron microscopy (TEM). Ultrathin sections were stained with osmiun tetroxide and ruthenium red and observed by TEM. The three isolates show the typical elongated, flask-shaped morphology of *M. penetrans*, with the two divided internal compartments. The cytoplasm is limited by a single triple-layered unit membrane that is covered with capsular material.

Serologic assays (ELISA and Western blot) with Triton X-114-extracted antigens and other *M. penetrans* polypeptides from whole-cell lysates both from the type strain GTU-54-6A1 and from our isolate HF-1 were done as previously described (10). A lack of reactivity against the Triton X-114–extracted antigens of *M. penetrans* was observed by both methods. However, with whole-cell extracts from both type strain and the HF-1 isolate, a 20-kDa polypeptide was immunodetected by Western blotting with three serum samples collected on days 2, 4, and 9 of hospitalization. The 20-kDa polypeptide is an *M. penetrans* product, but whether the observed reaction corresponds to a cross-reacting epitope is not known. The patient's samples were also negative for antibodies against *M. pneumoniae, M. genitalium,* and *M. fermentans* by ELISA (11) (data not shown).

Conclusions

Since *M. penetrans* was first reported in 1993 as an emerging infectious agent, *M. penetrans*–specific antibodies have been detected more frequently (18.2% to 35.4%) in HIV-infected than in non-HIV-infected persons (0.4% to 1.3%) (10). Until this case, *M. penetrans* had only been isolated eight times (5,10), always from the urine of HIV-infected persons (10).

The results indicating that the isolates HF-1, HF-2, and HF-3 belong to the *M. penetrans* species are as follows: 1) clinical samples and the mycoplasmal isolates obtained from them were positive in the *M. penetrans*-specific PCR assay; 2) protein patterns of the HF isolates and the type strain of *M. penetrans* GTU-54-6A1 were almost identical; 3) serum samples from different patients (10), which contained *M. penetrans*-specific antibodies on the basis of a reaction with the p35 antigen from the type strain of *M. penetrans* also reacted with a similar Triton X-114–extracted polypeptide from the HF-1 isolate; and 4) HF isolates exhibited typical morphologic features of *M. penetrans,* which are unique among mycoplasmas isolated from

humans. The fact that serum from other patients reacted with a similar polypeptide from HF isolates indicates that this protein is produced by this strain of *M. penetrans*. The lack of *M. penetrans* strong humoral response in the HF patient was a factor in favor of dissemination of the mycoplasma, hence its isolation from the blood. A possible association between *M. penetrans* and PAPS should be considered.

Snowden et al. (1990) found antiphospholipid antibodies in more than 50% of patients with *M. pneumoniae* pneumonia, especially those with severe infections requiring hospitalization (3). Catteau et al. (1995) described two cases of Stevens-Johnson syndrome associated with *M. pneumoniae* infection and the presence of antiphospholipid antibodies (12). Our patient had manifestations typical of PAPS (2). Thus, this report is the first of *M. penetrans* isolation in a non-HIV-infected patient and the first of a blood and respiratory tract infection with *M. penetrans*.

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Infectious Diarrhea in Tourists Staying in a Resort Hotel

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An outbreak of infectious diarrhea with 70 laboratory-confirmed cases (58 with *Giardia lamblia*) and 107 probable cases occurred in U.K. tourists who stayed in a hotel in Greece. After a cluster of six cases in persons who had stayed at the hotel was reported, the Communicable Disease Surveillance Centre began active case ascertainment. This outbreak illustrates the value of an approach to surveillance that integrates routine surveillance data with active case ascertainment.

A large outbreak of infectious diarrhea in residents of the United Kingdom who became infected while staying at a hotel in Greece was investigated in the month after their return. The outbreak was first identified by the Communicable Disease Surveillance Centre (CDSC) in June 1997 when a cluster of six microbiologically confirmed cases of giardiasis (all in patients who had stayed at the same hotel in Greece) were reported by a local public health epidemiologist. The cluster was identified after a school sought advice about excluding a child with diarrhea. These six patients reported that many other hotel guests had also been ill.

British guests who arrived at the hotel between 22 May and 9 June 1997 (the period of reported illness) were included in the investigation. The names of these guests were obtained from the tour operator (who sent, through travel agents, a letter to each booking group advising them of the outbreak and asking them to contact CDSC); other local public health epidemiologists and environmental health officers; and a list of ill people compiled by one particularly concerned guest.

In July, a standard questionnaire was administered by telephone to a member of each group (with contact information available) that had booked their holiday together. If the group consisted of more than one family, attempts were made to interview one member from each family. Fact sheets about giardiasis were distributed.

Of 86 booking groups on the tour operator's list, 51 groups (59%) (239 persons) were contacted and interviewed; 35 groups (41%) (138 persons) could not be contacted. Those contacted resided throughout the United Kingdom; 128 (54%) were male, and 132 (55%) were 16 years or older. Of the 239, 224 (94%) were ill while on holiday or within a few days of return; diarrhea was more commonly reported (95%) than stomach cramps (82%) or vomiting (64%). The median duration of diarrhea (13 days, interquartile range 4 to 27) exceeded that of vomiting (1 day, interquartile range 1 to 2).

Of the 224 persons who were ill, 70 (31%) were categorized as definite cases (those with a pathogen identified in stool specimen reported by mid-July); 107 (48%) as probable cases (those with no pathogen identified by mid-July but diarrhea lasting 4 or more days); and 47 (21%) as possible cases (those with no pathogen identified by mid-July and diarrhea lasting less than 4 days). Of the definite cases, *Giardia lamblia* was identified in 58. Other pathogens were identified, and some cases had dual infections (Table 1).

Epidemic curves for both diarrhea and vomiting suggested a point-source outbreak with peak onset from 5 to 7 June (Figure 1). Without

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Table 1. Pathogens identified in definite cases

Pathogen	No. of definite cases[a]	(%)
Giardia lamblia	58	(83)
Cryptosporidium parvum	11	(16)
Campylobacter spp.	4	(6)
Salmonella spp.	3	(4)
Entamoeba histolytica	2	(3)
Rotavirus	1	(1)

[a]Sum exceeds 70 because of co-infections.

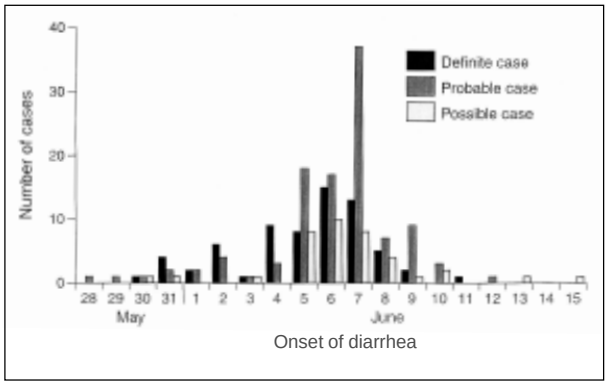

Figure 1. Epidemic curve for diarrhea, by category of case.

knowing the date of exposure, we could not calculate the exact incubation period; instead, we estimated the upper limit of the incubation period by calculating the interval between arrival at the hotel and onset of illness (Figure 2). The short incubation period (most persons became ill within 3 days) and high proportion of cases reporting vomiting at an early stage suggest that some initial illness was caused by a viral gastroenteropathic pathogen. Supporting this hypothesis was the isolation of small round-structured virus (Norwalk virus) from one stool specimen obtained in Greece and of rotavirus in the United Kingdom.

No stool specimens were examined for *G. lamblia* in Greece. During telephone interviews in the United Kingdom, members of 20 groups were advised to provide further stool specimens for testing for *G. lamblia*. By the end of the investigation, at least one stool specimen had been examined for ova, cysts, and parasites for 142 (63%) of the cases. Of the 58 confirmed cases of giardiasis, 51 were treated with metronidazole; 40 cases were treated empirically.

During the telephone interviews, travelers were asked about problems with the food or

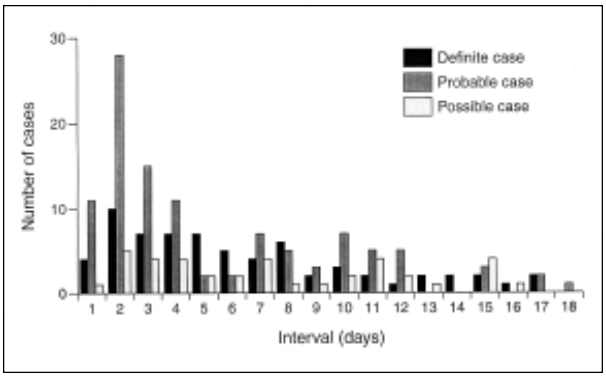

Figure 2. Interval between arrival at hotel and onset of illness (vomiting or diarrhea), by category of case.

water in Greece: 54 reports were documented from 51 groups. Of the reports, 32 (59%) identified problems with food (tasted strange, inadequately cooked or heated, left out uncovered); 27 (50%) identified problems with room water (sewage smell and discoloration, principally around 4 June); and none identified problems with drinking water (travelers drank bottled water). However, 7 (13%) identified problems with drinks reconstituted with tap water. None identified problems with swimming pool water. Statistical analysis showed that attendance at children's activities and problems with food were not associated with certainty of diagnosis. However, date of departure, consumption of reconstituted orange juice, consumption of raw vegetables and salads, and reports of problems with water in the hotel room were significantly associated with illness (Table 2).

This outbreak of giardiasis was the largest reported in the United Kingdom since 1985 (1) and the first identified in tourists returning from abroad. The investigation, which relied on active case finding, was complicated by occurrence of illness in more than one country and multiplicity of pathogens identified. Evidence suggests that two principal illnesses, both due to sewage contamination of the water supply, were responsible for the outbreak: an epidemic viral gastroenteritis and giardiasis.

The epidemic curve suggests a point-source outbreak (although some of the viral gastroenteritis may have been transmitted person to person before the peak incidence). If exposure to *G. lamblia* occurred at the same time as that to the pathogen responsible for the viral gastroenteritis, then with its longer incubation period (5

Table 2. Possible risk factors for illness

Risk factor, chi-square, and p values	Number (%) of guests							
	Definite cases (n=70, 36 children)		Probable cases (n=107, 39 children)		Possible cases (n=47, 21 children)		Not ill (n=15, 11 children)	
	yes	no	yes	no	yes	no	yes	no
Departure after 5 June (chi-square=7.3, p=0.007)	65 (93)	5 (7)	99 (93)	8 (7)	38 (81)	9 (19)	11 (73)	4 (27)
Attendance at children's activities (chi-square=0.01, p=0.92)	23 (64)	13 (36)	25 (64)	14 (36)	15 (71)	6 (29)	6 (55)	5 (45)
Consumption of raw vegetables and salads (chi-square=3.9, p=0.04)	52 (74)	18 (26)	78 (73)	29 (27)	33 (70)	14 (30)	6 (40)	9 (60)
Consumption of reconstituted orange juice (chi-square=8.4, p=0.004)	67 (96)	3 (4)	100 (93)	7 (7)	43 (91)	4 (9)	10 (67)	5 (33)
Problem with water in room (chi-square=7.2, p=0.007)	49 (70)	21 (30)	54 (50)	53 (50)	17 (36)	30 (64)	9 (60)	6 (40)
Problem with food (chi-square=0.3, p=0.58)	43 (61)	27 (39)	76 (71)	31 (29)	26 (55)	21 (45)	9 (60)	6 (40)

to 25 days vs. 24 to 48 hours for viral gastroenteritis (2), illness due to giardiasis would be expected to merge with recovery from the viral illness. A biphasic epidemic curve would arise with the second peak due to cases in persons who developed symptoms due only to giardiasis.

The following evidence supports the water-borne nature of the outbreak: 1) the epidemic curve suggests a point-source outbreak; 2) several water-borne pathogens were identified in cases (although environmental investigations in Greece at the time of the outbreak found no pathogens, *G. lamblia* was not specifically sought); 3) 129 guests reported that their room water smelled of sewage or was discolored around 4 June, and such reports were more likely from guests in groups with definite cases; 4) consumption of reconstituted orange juice was associated with certainty of diagnosis; 5) although all guests reported avoiding tap water, exposure was almost universal through reconstituted drinks and wet glasses; and 6) the water supply was chlorinated but not filtered, except at certain points within the hotel.

Most nationwide outbreaks of gastrointestinal disease in the United Kingdom, as well as additional cases, are identified through nationally collated laboratory reports. In 1996, of the 5,517 laboratory reports of *G. lamblia* to CDSC from England and Wales, 995 (18%) were associated with foreign travel. This outbreak, however, was initially identified by a local public health epidemiologist; most cases were ascertained largely through the tour operator. Laboratory reports resulted in identification of only 10 cases; a nationwide request for information on related cases resulted in identification of cases from only six groups. Tour operators are likely to collaborate with outbreak investigations, particularly since a European Directive has made them liable for acts and omissions of those with whom they contract to care for their clients (3). In this instance, the tour operator organized an inspection and environmental sampling of the hotel, initiated a survey of food exposure of hotel guests, offered alternative accommodation, informed their clients of the outbreak after their return to the United Kingdom, advised them to obtain an appropriate stool examination, withdrew from assigning clients to the hotel for 1 month while the situation was monitored, and offered guests financial compensation.

Ill travelers may have been more likely than healthy travelers to respond to the tour operator's request to contact CDSC. The number of definite cases, however, was likely to be underrecognized. Guests were classified as having a protozoal illness only if stool specimens were examined specifically for ova, cysts, and parasites; 36% of cases did not have stool

specimens examined in this way, and many others had only one stool specimen tested (intermittent excretion of cysts means that the estimated sensitivity of one stool specimen is 50% to 70% [4]). Only half of the laboratories in the Public Health Laboratory Service routinely examine stool specimens for ova, cysts, and parasites (5); most doctors must specifically request the investigation to ensure it is performed.

Given limited health-care resources, the benefits of such an outbreak investigation must be weighed against the costs. In this outbreak, the benefits included identification and appropriate management of cases, as well as provision of information to the tour operator to direct preventative action (which is important, because the investigating body in the country of residence of outbreak patients cannot act directly). Increasing international collaboration in surveillance and investigation of communicable disease outbreaks, particularly within the European Union (EU) (e.g., Enter-Net [6]), may facilitate action by public health professionals in the host country after illness in visitors. The United States/EU Task Force on Communicable Disease is committed to extending Enter-Net beyond Europe, and several countries including the United States, Canada, Australia, South Africa, and Japan are interested in joining.

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A Midcourse Assessment of Hantavirus Pulmonary Syndrome

Our understanding of infectious diseases follows a natural course—the initial discovery of the cause, the exploration of the natural history and biology of the etiologic agent, and finally the cure or solution. The discovery of Sin Nombre virus (SNV), one of the viruses causing hantavirus pulmonary syndrome (HPS), is unparalleled in terms of the rapid progress of the scientific investigation leading to its description. A cluster of cases of fatal adult respiratory syndrome was recognized in the Four Corners region of the United States in May 1993, and within a few days serologic evidence confirmed hantavirus infection (1). The outbreak occurred in the wake of the Institute of Medicine's report Emerging Infections: Microbial Threats to Health in the United States, in which acute respiratory disease was first on the list of clinical syndromes requiring high-priority surveillance (2).

The scientific community has now entered the midcourse phase of HPS research, including the exploration of the natural history of the American hantaviruses. Seven articles in this issue describe studies of rodent reservoirs of Sin Nombre and related viruses. They illustrate the multidisciplinary nature of such studies, which require ecologic methods, in addition to the newer molecular biology techniques that have helped hantavirologists detect and characterize the viruses.

These and other studies have elucidated much about the natural host relationships of hantaviruses. Multiple hantavirus genotypes exist in virtually all parts of North and South America; each hantavirus genotype has a single rodent species as its principal reservoir. Evidence exists that the virus and rodent have evolved together. Each hantavirus variant is focal in distribution. Prevalence of antibody is high in some regions, and low or absent in others, even when the same species of rodent is found in both places. Rodents are not infected at birth; they acquire the virus from other rodents. Once infected, the animals develop antibody, but many, or maybe all, infected rodents remain infected. Because of rapid turnover of the rodent population (as older antibody-carrying animals die and nonimmune animals are born), antibody prevalence can vary greatly, from 0% to 50%, with prevalence often lower than 20%.

The studies also have raised new questions. Why and by what route are animals infected? The articles in this issue suggest that contamination of wounds when animals fight may be an important route of infection, while allowing for the possibility of secondary mechanisms, such as venereal transmission or close association during communal nesting. Although textbooks state that people become infected by inhaling aerosols created by the rodents, we need better information. If the animals create infectious aerosols, why are only a small proportion of susceptible animals infected, even in enclosed colonies of the rodents (3)? Why is human infection so rare, even among forest workers and mammalogists (4)? Are some animals supersecretors of virus? Are individual virus-carrying rodents infectious for life or only periodically? If periodically, what makes them shed and stop shedding virus?

Classic pathogenesis studies are required to answer these questions. Two technologic advances—reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA)—have revolutionized science but at the same time lured us away from other time-tested methods. Before the RT-PCR technique was developed, virologists measured infectious virus by isolating it in cell cultures or laboratory animals. Although we need information about the infectivity of rodent blood, urine, throat secretions, feces, and organs, few investigators attempt to isolate infectious hantaviruses. The reasons are obvious. Virus isolation is tedious, technically difficult, and (without biosafety level three or four facilities) dangerous. Because detecting nucleic acid by RT-PCR is safe and easily accomplished, most investigators are satisfied with that method. Safe isolation of hantaviruses in the Americas will require additional investment in physical biocontainment facilities for university and government laboratories.

Many compelling reasons exist for isolating hantaviruses. How else will serotype specificity, animal models, pathogenic potential, replication, transmission, and other phenotypic properties be studied? I cannot emphasize too strongly the importance of propagating and preserving this biologic material. To isolate hantaviruses from rodent tissues will require new approaches. The technical difficulty now in

isolating hantaviruses is analogous to that encountered 30 years ago with dengue viruses. The solution for dengue was to use a natural host, the mosquito (5). Colonized, virus-free rodent reservoir hosts of hantaviruses could possibly provide the increased sensitivity to infection needed to facilitate routine isolation of hantaviruses. This approach has been successful on a limited scale in Europe (6).

Only two serologic methods—ELISA and, to a lesser extent, immunofluorescence assay—are used on a large scale in the Americas for testing rodent sera for hantavirus antibody. CDC developed a recombinant antigen product of the small RNA segment of SNV (7), which was produced in large quantity and distributed gratis to state health departments and to collaborators in both North and South America for use in ELISA. The antigen is broadly cross-reactive and entirely safe. Before the advent of ELISA, classic virologists surveyed for antibody with one test and then confirmed a portion of the antibody-positive and -negative serum specimens with a different test, often the neutralization test. Such confirmatory tests are desirable in the studies of rodents for hantavirus antibody but are rarely done. The neutralization test, used to a limited extent (8), is not practical for study of large numbers of rodent serum specimens or for work with biosafety level four agents. An alternate to this test is the hemagglutination-inhibition test. Asian and European hantaviruses agglutinate goose cells (9,10); it should be feasible, therefore, to develop the hemagglutination-inhibition test for American hantaviruses.

What will it take to cure or prevent HPS? The only proposed antihantavirus drug is ribavirin, which although still under trial, has not been efficacious in treating HPS (11). Supportive emergency care can save lives, but diagnosis must be made early, clinical expertise is concentrated in only a few medical centers, and HPS cases are often dispersed. Approaches to hantavirus human vaccines have been developed (12), but such vaccines are probably not commercially feasible in the Americas, which have a low incidence of disease caused by focal genotypes. Rodent control does not seem practical because of the immense geographic range of hantaviruses. Ongoing rodent studies may eventually determine whether a wildlife vaccine analogous to that used successfully for rabies will be practical (13).

For the immediate future, we must depend on education to prevent human exposure. To design an effective education program, we must know much more about the rodent reservoir and the mode of virus transmission, both among rodents and to people. The publication of these ecologic studies in a medical journal represents important changes in the medical and ecologic sciences. These studies have required collaboration of ecologists, epidemiologists, and virologists. Their successful continuation, as well as the conception, design, and conduct of future studies, requires the spirit of innovation best achieved in a multidisciplinary atmosphere, as well as a long-term commitment to collect data for several years.

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Navigational Instinct: A Reason Not to Live Trap Deer Mice in Residences

To the Editor: Although the rodent that most often invades homes in North America is the house mouse, *Mus musculus*, the deer mouse, *Peromyscus maniculatus*, principal vertebrate host of Sin Nombre virus (SNV) (1), also invades homes (2), particularly in rural areas. Barring deer mice from human habitations would prevent domiciliary acquisition of SNV. Current recommendations (3) are to prevent wild rodents from entering homes or to snap trap (kill) them should they enter.

To conduct longitudinal studies of hantaviruses in southeastern Colorado on a former cattle ranch now returning to its natural condition as short-grass prairie, we often stay in an old bunkhouse, used by many research groups at irregular intervals. The house, furnished with beds and full kitchen facilities, is well maintained but has openings through which mice can pass to and from the outside. For safety and cleanliness, we removed mice we found inside the house, but between April 1996 and April 1998, we live trapped and released them rather than snap trapping them. Before release the rodents were identified to species; were measured and assessed regarding general appearance and health, sexual preparedness, and presence of wounds; were bled for antibody tests; and were ear-tagged. Nineteen deer mice and one pinyon mouse (a *P. truei*, which did not return) were examined and tagged. At first, we simply released these animals approximately 50 m from the house, but when we realized that they were returning, we released them at increasing distances (50 m to 1,500 m) from the house; the distances were measured by pace counts by at least two investigators.

Three deer mice had been captured multiple times in our test grid (as far as 250 m from the house) before they were first captured in the house. Once captured in the house, however, they were not captured in traps of the grid (i.e., outside the house). The mean distance traversed by the five deer mice that returned to the house was at least 394 m; one mouse returned after being released 500 m and 1,000 m, then 750 m, and 1,200 m from the house at consecutive daily trapping sessions of 3 days. Sometime within the subsequent 6 weeks, this mouse returned to the house from the 1,000-m release point and then from 750 m and 1,200 m away on consecutive days within our 3-day trapping period. Each of the mice returning to the house did so within 24 hours of release, two as few as 6 hours after release from 500 m and 750 m away. Nine mice were captured once; six of eight mice captured twice were captured at least once more; one was captured 10 times, one 7 times, one 6 times, one 4 times, and two 3 times. Equal numbers of male and female, adult and juvenile mice were captured in the house, but only adult mice (5 of 5) returned to the house. Returning deer mice maintained or gained weight between captures and grew in length at approximately the same rate as deer mice captured in the test grid.

Some rodents have been documented to move similar distances (e.g., 1,200 m), but they took more than 2 weeks to complete the trek (4). Homing ability, site fidelity, and navigational proficiency of rodents are well documented (5,6). Teferi and Millar (7) studied the homing ability of deer mice in Alberta, Canada; 50% of deer mice in that study returned to their home sites (a short-grass prairie habitat). The mice traveled 650 m to 1,980 m (mean 1,500 m) and had to cross a river and pass optimal habitat patches to reach their home sites. Deer mice with previous homing experience were more successful in returning home (100%) than inexperienced mice (60%) and faster in doing so (8). Teferi and Millar (7) suggest that these deer mice were able to navigate in a direct route to their home sites. We released mice in locations where they had no direct route to the house; they had to follow a winding road, climb over rocky outcroppings nearly 17 m high, or otherwise surmount obstacles and dangers, such as predators (7).

None of the mice we captured had immunoglobulin G (IgG) antibody to SNV. However, infected deer mice released and then returning to a house or uninfected deer mice released, infected, and then returning to a house would increase the likelihood of human contact with an SNV-infected mouse. The risk would be the same for other hantaviruses infecting other peridomestic rodents. Against current recommendations that rodents in homes be snap trapped, some homeowners live trap and release them outside their homes. Our data strongly support snap trapping mice in homes and provide evidence that released wild mice return and may place the residents at risk.

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Bartonella quintana in Body Lice Collected from Homeless Persons in Russia

To the Editor: Lice are obligate blood-feeding insects; three lice species (*Pediculus humanus* var *capitatis*, *P. humanus* var *corporis*, and *Phtirus pubis*) have been connected with humans throughout history. The body louse (*P. humanus corporis*) is the vector for three infectious diseases: epidemic typhus caused by *R. prowazekii*, trench fever caused by *B. quintana*, and relapsing fever caused by *Borrelia recurrentis* (1-3). Infestation with the body louse is associated with cold weather, poverty, and poor hygiene. In Russia, louse-transmitted diseases have caused more deaths than any other infectious disease in recent centuries (4). During the last decade, pediculosis (infestation with *P. humanus*) has increased markedly throughout the world (5,6), especially in developing countries and in areas (e.g., Eastern Europe, Russia) that have undergone vast social and economic changes. The incidence of pediculosis in Russia is approximately 220 to 300 cases per 100,000 inhabitants (7). Social and economic upheavals in the former Soviet Union have increased the number of homeless people, among whom pediculosis is highly prevalent (6).

A disease of the past, epidemic typhus, has reemerged as a public health concern after a 1996 outbreak in Burundi, the largest outbreak of the disease since World War II (5,8). During World War II, a huge typhus epidemic caused illness in more than 20,000,000 people in Russia. *R. prowazekii* infection can persist in a latent form in convalescent typhus patients, remanifesting itself in a recrudescent form (Brill-Zinsser disease) in patients under stress (1). Sporadic cases of Brill-Zinsser disease are reported every year in all regions of the former Soviet Union (9) and because most of the population has no immunity to *R. prowazekii,* the risk for a typhus outbreak is increased. In a recent outbreak in the Lipetsk region, 360 km from Moscow, 24 louse-infested, febrile patients in an unheated psychiatric institution had serologically diagnosed typhus (10).

The great epidemics of trench fever in Europe took place during World War I (2). However, recently a large outbreak of trench fever associated with epidemic typhus has been reported in Burundi (5). Sporadic cases of *B. quintana* infection have occurred during the last decade in Europe and the United States, mainly in HIV-infected patients, the homeless, and persons with chronic alcoholism; the infection has manifested itself as trench fever, bacteremia, bacillary angiomatosis, or endocarditis (11-16). Relapsing fever has not been reported in Russia for more than 50 years, despite a high prevalence after the 1917 revolution and during World War II (17).

We studied the presence of typhus, trench fever, and relapsing fever agents in body lice

collected from homeless persons in Moscow. The lice were collected at the Moscow Municipal Disinfection Center, where the homeless wash and delouse themselves, as well as disinfect or change their clothes. Only participants who gave informed consent were included. Lice were collected from the participant's clothing (from the inner surface and seams of t-shirts, shirts, and sweaters); 3 to 25 lice were found on each volunteer. Lice were collected from May to October 1996 (214 samples) and from June to September 1997 (54 samples).

From June to September 1997, 300 homeless male attendees were examined; 57 (19%) had body lice or louse eggs (three had only eggs) on their clothing. Lice were identified as *P. humanus corporis,* according to standard taxonomic keys (6,18). Lice from each person were split into pools of three to eight insects, and DNA was extracted from each pool and tested for *R. prowazekii, B. quintana, and B. recurrentis* by polymerase chain reaction (PCR) analysis. Primers used for PCR analysis and conditions for DNA amplification have been described (5,19-22). Uninfected, laboratory-reared lice served as negative controls, and DNA of *R. rickettsii, B. elizabethae,* and *B. burgdorferi* were used as positive controls.

Results of each amplification were resolved in 1% agarose gels (type LE; Sigma-Aldrich Chimie, St. Quentin Fallavier, France) and were visualized under UV light after ethidium bromide staining. The sizes of amplicons were determined by comparison with the DNA molecular weight marker VI (Boehringer, Mannheim, Germany). To confirm the identity of amplicons, their nucleotide base sequence was determined by using an AmpliCycle sequencing kit (Perkin-Elmer Corp., Foster City, CA) according to the manufacturer's instructions.

PCRs incorporating rickettsia- and borrelia-specific primers did not yield products from any DNA extracts derived from the louse samples. Positive controls in both reactions yielded bands of the expected size. Thus, louse samples were not infected with *R. prowazekii* or *B. recurrentis*. Initial screening with PCR incorporating nonspecific primer pairs for *Bartonella* species yielded products of the estimated amplicon size of approximately 1,200 bp for 33 (12.3%) of the 268 louse samples. These results were confirmed by PCR incorporating primers (CS.443p–CS.979n) specific for the *gltA* gene. Products of this reaction were characterized by base-sequence determination. All 33 *Bartonella*-positive samples yielded a partial gltA sequence identical to that of *B. quintana* (22). Persons infested with infected lice were younger than 30 years to older than 60 years of age.

A recent report indicates that 11% of the homeless in Russia are infested with lice (23); in our limited study, we observed a prevalence as high as 19%. With widespread louse infestation and overcrowding, a single case of Brill-Zinsser disease can cause an outbreak of epidemic typhus. A patient more than 50 years of age with Brill-Zinsser disease was the suspected primary source of typhus infection during the 1997 Lipetsk outbreak. Presence of lice in the hospital permitted disease dissemination (10). Although our data showed that none of the 268 louse pools were infected with *R. prowazekii*, the serious threat of an outbreak requires continued surveillance. No samples were found to contain *B. recurrentis* DNA, yet dissemination of body lice could also cause relapsing fever to reemerge.

Interest in bartonellosis has recently increased, particularly in association with HIV infection, because *Bartonella* species can cause bacteremia in the immunocompromised (15). Recent investigations have demonstrated that *B. quintana* cause bacillary angiomatosis, lymphadenopathy (16), endocarditis (24), and infections of the central nervous system (25,26) in healthy persons. Recent reports of *B. quintana* infection outbreaks in the United States (14,27), Africa (5), and Europe (11,13,28) suggest either greater awareness or a reemergence of this infection. Persons who are homeless or alcoholic are particularly at risk (11-13,27,29). In all recently reported cases, the role of a possible arthropod vector has remained unclear (30,31), although lice exposure, together with homelessness, is a risk factor for *B. quintana*-induced bacillary angiomatosis (15). The fact that 12.3% of studied lice samples were *B. quintana*-positive confirms the role of this arthropod vector in the contemporary life cycle of the agent. A similar prevalence of *B. quintana* in body lice was reported in Burundi (5) and has been observed in France (D. Raoult, unpub. data). On the basis of data from our study, Moscow should be considered an area at high risk for an outbreak of bartonellosis.

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Tick-Transmitted Infections in Transvaal: Consider *Rickettsia africae*

To the Editor: We report a case of African tick-bite fever (ATBF) in a 54-year-old French hunter returning to France on 21 April 1997, after a 15-day visit to Transvaal, South Africa. While

traveling in the veld, the hunter removed (but did not keep) two ticks from his left leg. Two days later, he observed eschars at the bite sites. Within 5 days, he had high fever (39.5°C) and headache and decided to fly back to France, where he was admitted to the Infectious Diseases Department in the Hotel Dieu Hospital in Clermont-Ferrand. The patient's clinical symptoms were persistent fever, severe headache, and two inflammatory eschars on the left leg. Laboratory results were normal. On 22 April, an acute-phase serum sample and eschar biopsy were sent to our laboratory. The patient was treated with 200 mg per day doxycycline for 10 days. His symptoms resolved. A second serum sample was collected on 13 May.

Microimmunofluorescence was performed as previously described (1). Although the acute-phase serologic results were negative, the convalescent-phase serum exhibited anti–*R. africae* and anti–*R. conorii* titers of 16 for immunoglobulin (Ig) G and 8 for IgM. Sera were adsorbed with *R. conorii* and *R. africae* antigens (2), and serologic testing and Western blot analysis (1) were performed on the resultant supernatants. Cross-adsorption of the convalescent-phase serum caused the homologous and heterologous antibodies to disappear when adsorption was performed with *R. africae* antigens; only homologous antibodies disappeared when adsorption was performed with *R. conorii*. Western immunoblot, performed with the same adsorbed serum, indicated *R. africae* infection by demonstrating a specific reactivity pattern with *R. africae*–specific antigens in the 110-kDa to 145-kDa region (2). An inoculation eschar biopsy specimen was injected into human embryonic lung fibroblasts, according to the centrifugation shell-vial technique (3). After 6 days' incubation at 32°C, a Gimenez staining of methanol-fixed human embryonic lung fibroblasts showed rickettsialike bacilli. The strain was identified by direct immunofluorescence performed on the cells with an anti–*R. africae* monoclonal antibody (4). Moreover, DNA was extracted from the ground eschar biopsy specimen and from 200 µL of shell-vial supernatant, by using a QIAmp Tissue kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. These extracts were used as templates with primers complementary to a portion of the coding sequence of the rOmpA encoding gene in a polymerase chain reaction (PCR) assay (5), and the base sequences of the resulting PCR products were determined (5). The sequence obtained by both methods was the same as the *R. africae* sequence in Genbank (100% similarity).

Since first described in Africa in 1910, tick-transmitted rickettsioses have been imputed to a single rickettsial species, *Rickettsia conorii*, although two distinct clinical illnesses have been observed (6): an urban form in patients in contact with dogs and their ticks (*Rhipicephalus* spp.) characterized by fever, headache, myalgia, cutaneous rash, and a lesion at the site of the tick bite (7), and a rural form in patients in contact with cattle or game and their ticks (*Amblyomma* spp.) characterized by mild signs and frequent lack of rash (8).

Although *R. africae* was initially isolated from *Amblyomma* cattle ticks in 1973, the first evidence of its pathogenic role in humans was seen in 1992 in a patient who, after a tick bite, had fever, an inoculation eschar, regional lymphadenopathy, but no cutaneous rash (9). Since then, an additional 20 cases of *R. africae*–related infections have been reported in travelers returning from Zimbabwe and South Africa (2,10).

R. conorii has long been considered the only African spotted fever group rickettsia, responsible for both Mediterranean spotted fever and ATBF. Since the first case was described (9), most of the 20 reported cases of ATBF occurred as outbreaks (2,10) in Europeans returning from Zimbabwe and South Africa. The occurrence of concomitant ATBF cases is unusual since Mediterranean spotted fever is generally sporadic and is likely related to the biologic characteristics of the recognized vector of *R. africae*, *Amblyomma* spp. ticks. While both are nonnidicolous ticks, *Amblyomma* spp. and *Rhipicephalus* spp. exhibit very different host-seeking behavior (11). *Amblyomma* spp. are ticks of cattle and wild ungulates, are not host-specific, and can readily feed on humans; they are "hunter ticks" and exhibit an "attack strategy" (in response to stimuli they specifically converge on nearby hosts). *Rhipicephalus* spp. are dog ticks and vectors of *R. conorii*; very host-specific, they exhibit an "ambush strategy" (they are passive and remain quiescent in their habitat until a vertebrate host passes). Up to 72% of *A. hebraeum* are infected with *Rickettsia*-like organisms, in particular *R. africae* (12); *Amblyomma* spp. are widely distributed in rural

areas in sub-Saharan Africa (13) and prevalence of *A. hebraeum* ticks, incidence of ATBF cases, and prevalence of *R. africae* antibodies have been strongly linked (14). Rural Africans are also commonly infected with *R. africae*, usually at a young age (14). In Zimbabwe, Kelly et al. (15) demonstrated that 55% of the tested human sera had antibodies against *R. africae*.

ATBF usually has specific clinical features: shorter incubation period than for Mediterranean spotted fever, multiple inoculation eschars (related to the host-seeking behavior and host-specificity of *Amblyomma* spp. ticks, which are "attack ticks" [15]), regional lymphadenopathies, frequent lack of cutaneous rash or a pale vesicular eruption, and absence of complications (2). Although only 22 proven cases have been described so far (including the present case), ATBF has been recognized as a commonly encountered disease in southern Africa since 1900 (8,16). Epidemiologic and clinical features indicate that several cases previously diagnosed on the basis of serology results only as *R. conorii*-caused may have been caused by *R. africae*.

Given the serologic cross-reactivity among spotted fever group rickettsiae, microimmunofluorescence, the easiest serologic method, may not be sufficient for the etiologic diagnosis of a rickettsial spotted fever. A definitive diagnosis of ATBF requires either additional serologic procedures, such as cross-adsorption or Western blot, or the use of PCR or culture. As for PCR, rOmpA-amplification possesses sufficient sequence heterogeneity among the spotted fever group rickettsiae to be used as an identification tool (5). The centrifugation-shell vial-cell culture (3), used routinely in our laboratory, reliably isolates strictly intracellular bacteria, including rickettsia, from blood and tissue specimens, especially eschar biopsies (the specimen of choice for isolation procedures or genomic detection). We noted cross-reactions between *R. africae* and *R. conorii*. Cross-adsorption between anti–*R. africae* and anti–*R. conorii* antibodies and Western blots confirmed that the antibodies we detected were directed specifically at *R. africae*. Furthermore, both PCR and cell culture confirmed the diagnosis of *R. africae* infection.

ATBF appears to be an important emerging disease in visitors to rural areas of southern Africa. *R. africae* should be considered a potential pathogen in patients returning from such areas who have fever, headache, multiple inoculation eschars, or regional lymphadenopathy after a tick bite.

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Extended-Spectrum Beta-Lactamase-Producing *Salmonella* Enteritidis in Trinidad and Tobago

To the Editor: *Salmonella* Enteritidis, a predominantly localized pathogen of the human gastrointestinal tract, can become invasive in very young, very old, malnourished, and immunocompromised patients. In recent years, *S.* Enteritidis has emerged as a major intestinal pathogen in Trinidad and Tobago (population 1.2 million); in 1997, *S.* Enteritidis caused 79 (66%) of 119 culture-confirmed salmonella infections, in contrast to 18 (18%) of 99, 48 (47%) of 102, and 107 (61%) of 178 in 1994, 1995, and 1996, respectively. Increased incidence of *S.* Enteritidis infections has been reported worldwide (1,2). Of 216 human *S.* Enteritidis isolates tested for antimicrobial susceptibility between 1994 and 1996 in Trinidad, none were resistant to cephalosporins, aminoglycosides, ampicillin, trimethoprim-sulphamethoxazole, chloramphenicol, and norfloxacin/ciprofloxacin by the Kirby-Bauer disk diffusion method, which uses the National Committee for Clinical Laboratory Standards (NCCLS) breakpoints (3).

Here we report an unusual isolate of *S.* Enteritidis resistant to all penicillins and cephalosporins—including third-generation cephalosporins, gentamicin, tobramicin, and trimethoprim-sulphamethoxazole—by the Kirby-Bauer disk diffusion method. Amoxicillin-clavulanate and piperacillin-tazobactam disks gave zone sizes of 15 mm and 19 mm, respectively, which are classified as intermediate in the NCCLS guidelines. This isolate was recovered from the blood culture of a febrile, nonneutropenic patient with multiple myeloma on two occasions 24 hours apart in March 1998. The isolate was sensitive only to ofloxacin and imipenem. Admitted to the hospital with compressed fracture of the spine for physiotherapy in December 1997, the patient had several febrile episodes and received several courses of multiple empirically prescribed antibiotics (cefotaxime, gentamicin, and piperacillin). The patient had not traveled abroad during the previous 6 months.

Because cephalosporin resistance in salmonellae has not been reported before in the Caribbean, we investigated the mechanism behind this third-generation cephalosporin resistance further. Using amoxicillin-clavulanate in combination with ceftazidime, ceftriaxone, and aztreonam, we performed the double disk synergy test to determine whether this strain was an extended-spectrum beta-lactamase producer as described elsewhere (3); augmentation of the zone at the junction of amoxicillin-clavulanate and aztreonam/ceftriaxone/ceftazidime zones confirmed that indeed it was.

In the past few years, third-generation cephalosporin resistance in *S.* Enteritidis has been described in Europe (4), the United States (5), Turkey (6), India (7,8), and Argentina (9). Few reports exist of extended-spectrum beta-lactamase–mediated third-generation cephalosporin resistance in *Salmonella* spp. To our knowledge, this is the first report of this type of resistance among *S.* Enteritidis in the Caribbean. This patient was treated with ciprofloxacin for 1 week; subsequent blood cultures were negative.

This unusual isolate highlights the need to establish an antimicrobial resistance surveillance network for Salmonella isolates, including *S.* Enteritidis, to monitor the trends and new types of resistance mechanisms in the Caribbean. An epidemiologic study of *S.* Enteritidis infections is being planned to describe the extent of the problem and to define risk factors and vehicles of human infections in three Caribbean countries, including Trinidad and Tobago.

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New *emm* (M Protein Gene) Sequences of Group A Streptococci Isolated from Malaysian Patients

To the Editor: We analyzed the M-type–specific *emm* gene sequences of 24 random *Streptococcus pyogenes* isolates from sterile- and nonsterile-site clinical specimens of Malaysian patients. In contrast to isolates in the United States, which rarely have new *emm* sequences, 6 of these 24 Malaysian isolates had new *emm* gene sequences, which suggests a large reservoir of group A streptococci expressing new M-type specificities in Malaysia.

The M protein is a surface-exposed principal virulence factor of group A streptococci (GAS) and a potential vaccine candidate. The hypervariable M-type–specific N-terminal portion of the M molecule extends from the cell wall and evokes protective antibodies. Approximately 75 M antigenic types of GAS are recognized, and several provisional types have been proposed (1). Formulation of a universally effective vaccine is complicated by the M-type–specific nature of protective anti-GAS antibodies, temporal and geographic variations in GAS M-type prevalence (2), and lack of information on GAS M types from areas where rheumatic fever and rheumatic heart disease, sequelae of GAS pharyngitis, are endemic (3). The lack of specific M-typing antisera is also a limiting factor in determining type distribution. Recently, Beall and colleagues (4,5) demonstrated that sequence analysis of the hypervariable portion of the *emm* gene encoding M-type specificity (*emm* typing) was an alternative when M-typing antisera were not available.

Attempts to type selected Malaysian strains of GAS by M protein status have yielded poor results. Fewer than 16% of strains were typable with standard M-typing antisera (6). The existence of new M types in Southeast Asia was suggested as an explanation. To investigate this possibility, we subjected 27 selected strains (6 from blood, 15 from pharyngitis, 3 from pus, and 3 pharyngeal carrier cultures) collected between January 1994 and December 1996 to *emm* typing. Initial isolation, serogrouping, T typing, and determination of opacity factor production were performed in Kuala Lumpur, by standard techniques, commercial media, reagents, and antisera (7). Strains were transported to the Centers for Disease Control and Prevention in Atlanta, Georgia, USA, for *emm* typing, where serogrouping, T typing, and opacity factor determinations were repeated, and *emm* typing was performed (4,5). DNA sequences were subjected to homology searches against all known *emm* sequences by Genetics Computer Group Software, Version 9. (Most sequences in this database were found in strains isolated from patients living in Europe and North America.)

Of the 27 cultures analyzed, 24 were GAS, 2 were group G streptococci, and 1 was nongroupable Streptococcus. Ten of the 24 GAS strains were standard *emm* types *emm*3, *emm*12, *emm*22, *emm*60, and *emm*76 (encoding the classic M types M3, M12, M22, M60, and M76, respectively); 4 were the provisional *emm* types *pt180*, *pt2841*, and *pt5757*; and 3 were previously identified *emm* sequence types *st64/14* and *st2034* (GenBank accession numbers X72932 and U74320, respectively). The *st2034* sequence, originally identified in children from Papua New Guinea, has also been found in Brazil, California, and Hawaii (B. Beall, R. Facklam, unpub. data). One GAS had a sequence previously found in group G streptococci (*emmLG6*, accession number U25741). Finally, 6 were of five new *emm* sequence types discovered in this study

(*st4529*, *st4547*, *st4532*, *st4545*, and *st3018,* with accession numbers AF060368, AF052426, AF077666, AF077668, and AF077669, respectively). The newly found group A *st4545* sequence was more similar to various group G streptococcal *emm* sequences than to known group A *emm* sequences. One group G isolate had a previously found group G 5' *emm* sequence (*stLG6*, accession number U25741). The nongroupable Streptococcus had an *emm* sequence previously associated with group L Streptococcus (Beall and Facklam, unpub. data). These results demonstrate the usefulness of *emm* typing in areas where specific M-typing antisera are not available.

Identifying 6 (25%) of 24 GAS with new *emm* types provides further evidence of new M serotypes of GAS in Malaysia. The deduced amino acid sequences of the mature hypervariable N termini of ST4529, ST4532, ST4547, and ST3018 ranged from 43% to 82% identity to M proteins of known sequence (data not shown). The M nontypability of these isolates suggests unique serologic specificity. ST4547, ST4532, and ST3018 had 70% to 82% identity over the first 55-variable-region amino acids, with their closest matching known M proteins (M70, M27, and M22, respectively), but whether antibodies against any of these proteins would cross-protect against strains expressing these M proteins is unknown. Even though the M70 protein is 70% identical over its first 50 variable N terminal amino acids to the M33 protein, antibodies against the M70 and M33 proteins do not cross-protect, which suggests that no cross-protection would occur. The new deduced M protein with the lowest similarity to any known M protein was ST4529, whose closest match had a 43% identity over the N-terminal 55 residues of the M5 protein. *st4529* likely encodes a new serospecifically unique M protein.

These findings potentially affect vaccine development. Although new *emm* sequences were identified in a survey in the United States (5), the percentage of new strains with new *emm* sequences was much lower (6%) than was found with these Malaysian isolates. *emm* typing of a larger number of strains from rheumatic fever–and rheumatic heart disease–endemic areas is required to deduce amino acid sequences for the development of a suitable M protein–based vaccine.

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Mutant Chemokine Receptor (CCR-5) and Its Relevance to HIV Infection in Arabs

To the Editor: Approximately 10% of HIV-infected patients may remain AIDS-free for a long time; moreover, some persons do not become infected with HIV despite multiple high-risk sexual exposures (1,2). Factors responsible for this relative resistance to infection and disease include cytotoxic T cells, neutralizing antibodies, high concentrations of certain chemokines (e.g.,

RANTES, MIP-1), human leukocyte antigen haplotype, mannose-binding protein, and tumor necrosis factor alpha, C4, and TAP polymorphism (2-4). One of the chemokine receptors, CCR-5, which along with CD4 acts as co-receptor for HIV entry into macrophages, provides upon mutation a genetic restriction to HIV infection in homozygous persons and control of disease progression in heterozygous persons (5,6). Thus a 32bp deletion in the open reading frame of the region encoding the second extracellular loop of this receptor causes synthesis of a highly truncated protein that fails to express on the cell surface, leading to loss of HIV-1 co-receptor activity.

Studies in healthy Caucasian Europeans and North Americans show that approximately 1% of the population are homozygous for this deletion (Δ32), whereas 15% to 20% are heterozygous (5-9); surprisingly, a higher percentage (up to 20%) of persons at high risk for HIV but HIV-negative are homozygous for this deletion. However, no such mutation is seen in Japanese, Native Americans, Chinese, Africans, and Tamil Indians, which suggests that in these groups either resistance to HIV infection is not present or factors other than mutated CCR-5 are in operation. African-Americans and Hispanics show a low rate of mutation, possibly because of intermarriage with Caucasians (4). The low frequency of CCR-5 mutation in Arabs with close contacts with Turks in the Eastern Province of Saudi Arabia may also be due to intermarriage. However, certain persons with mutated CCR-5 can become HIV-infected (10); in such cases other chemokine receptors (e.g., CXCR-4, CCR-2, and CCR-3) are believed to facilitate infection.

HIV infection in Saudi Arabia (population 18 million) is uncommon; the World Health Organization has so far (1985 to 1997) documented 334 cases of AIDS in this region (11). We, therefore, studied for the first time the mutation of CCR-5 in Arabs residing in Saudi Arabia. DNA was isolated from the peripheral blood mononuclear cells of 105 male blood donors not infected with HIV and nine HIV-infected patients (seven male and two female). The latter were divided into three groups, according to published criteria (2): four persons whose infection did not progress over the long term and who showed only modest decline of CD4 count after several years of infection, one person whose infection progressed normally, and four persons whose infection progressed rapidly and CD4 count fell below 100/μl within 2 years. Primers flanking 32 nucleotide deletion of CCR-5 were used to generate wild type (W) and deleted (Δ32) fragments of 189 bp and 157 bp, respectively (5). Amplification was done in a Perkin-Elmer thermal cycler 9,600, by a 20 μl reaction mixture that contained 0.25mM of dNTPs, 20 pM of each primer ('5-CAAAAAGAAGGTCTTCATTACACC-3,5-CCTGTGCCTCTTCTTCTCATTTCG-3'), and 0.5 units of Taq polymerase in 1x reaction buffer. All reagents were obtained from Pharmacia (Sweden). The amplified product was separated on 2% agarose at 120 V for 45 min and examined under UV light. Of the uninfected blood donors, 104 (99%) were homozygous for the wild type, and 1 (0.96) was heterozygous for the mutation. None of the HIV-infected patients had the mutation. Thus, the mutation is present, albeit infrequently, in Arabs. A review of 68 HIV-infected patients in our files showed that, as in Caucasians, infection progressed rapidly in 8%, did not progress over the long term in 6%, and progressed normally in 86% (2). Therefore, other hitherto unknown protective factors must be operative in Arabs.

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Meeting Summaries

Workshop on the Potential Role of Infectious Agents in Cardiovascular Disease and Atherosclerosis

Cardiovascular and cerebrovascular disease currently exact a substantial human and economic toll in the established market economies and are expected to become increasingly more prevalent in developing countries. The noncommunicable nature of coronary artery disease, myocardial infarction, stroke, and atherosclerotic plaques has now been questioned; *Chlamydia pneumoniae*, human cytomegalovirus, periodontal disease, *Helicobacter pylori*, and herpes simplex virus-1 have all been associated, to some extent, with these conditions. The strongest evidence links *C. pneumoniae*, then human cytomegalovirus, to coronary artery disease, but direct causation has not been established. Nevertheless, large antibiotic trials employing broad spectrum macrolides are now in progress, intended to treat *C. pneumoniae* infection in symptomatic cardiac atherosclerosis. In the future, if even a portion of vascular disease can be prevented with antimicrobial drugs, vaccines, and health education, the public health impact of these findings will be imposing.

To address this global public health issue, the National Center for Infectious Diseases and the National Center for Chronic Disease Prevention and Health Promotion, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, cosponsored a multidisciplinary workshop of invited national and international researchers, clinicians, and state public health officials August 31–September 1, 1998. The workshop had two primary goals: 1) define the basic science and clinical applied research studies required to verify or refute the associations, within the context of other cardiovascular risk factors (emphasizing *C. pneumoniae* and human cytomegalovirus); 2) outline a preliminary public health strategy (noting the potential involvement of CDC) that will correctly address the role of infection in cardiovascular disease prevention and treatment.

Discussions of reviewed and new data reaffirmed that evidence of causality between any organism and atherothrombotic disease is sufficient neither to establish antibiotic treatment guidelines nor to generate a public health strategy that incorporates infection into the cardiovascular disease paradigm. To change the chronic course of disease, treatment must be specifically directed at the responsible agent and applied to the appropriate stage of pathogenesis in the population at risk. Thus, the pathobiologic interaction between infection, inflammation and free radicals, lipids, genetics, and other cardiovascular risk factors must be clarified with more sensitive, specific, and standardized tools. Reagants and methods must uniformly differentiate past exposure from active infection; they must examine how acute, recurrent, and persistent infection could initiate or aggravate atherosclerosis and acute arterial thrombotic events. Expansion of animal and in vitro models will add valuable information to human treatment trials and to epidemiologic studies that address the multifactorial nature of cardiovascular disease and focus on groups at risk. Laboratory innovations are needed to maximize interpretation of results and evaluate the impact of antimicrobial and non-antimicrobial interventions. Only in this way can a public health plan based upon sound scientific evidence be developed.

The following were among the recommendations of the workshop: 1) the basic epidemiology of *C. pneumoniae* should be defined, including the prevalence of past and active infection, risk factors for infection and the interaction of these determinants with traditional cardiovascular and cerebrovascular disease risk factors; 2) sensitive and specific diagnostic assays (both new and revised) for laboratory, animal, and clinical studies should be standardized to assess the true long- and short-term effects of infection and intervention; 3) pathobiologically relevant animal and in vitro models should be used to investigate the pathogenesis of single and multiple agents at different stages in the disease process, enhance the standardization of tools, and evaluate interventions; 4) activities should be linked to judicious antimicrobial use—examining baseline knowledge and use of antibiotics in cardiovascular disease, monitoring changes in disease incidence and prevalence with increasing antibiotic prescription, and investigating the effects of treatment on other microbes; 5) a close alliance should be formed between CDC, the National Institutes of Health, and investigators from diverse fields to advance these objectives in a timely fashion, accumulat-

ing high quality scientific evidence that will define the true role of infections and inflammation in atherosclerotic disease and the appropriateness of antimicrobial therapy.

For more information on the conference, contact Siobhán O'Connor, Centers for Disease Control and Prevention, 1600 Clifton Rd., NE, Mailstop C12, Atlanta, GA 30333, USA; tel: 404-639-1454; fax: 404-639-3039; e-mail: sbo5@cdc.gov.

Workshop on Risks Associated with Transmissible Spongiform Encephalopathies (TSEs)

A workshop on the evaluation of risks posed by TSEs was held June 8-9, 1998, at the University of Maryland College Park, under the auspices of the Joint Institute for Food Safety and Applied Nutrition (JIFSAN), a cooperative venture of the University of Maryland and the U.S. Food and Drug Administration. The workshop, attended by 300 representatives from 17 countries, evolved in response to the need voiced by governmental agencies, industry groups, international organizations, and academic experts to evaluate TSE risks related to source materials, processing, and end products for animal and human use. Support for the workshop came from government agencies, industry groups, international organizations, and JIFSAN.

One goal of the workshop was to develop a framework of guiding principles for evaluating TSE risks. An initial draft outline of critical elements applicable to evaluations of TSE risk was presented. The next steps are to provide definitions for the critical elements and to develop an annotated outline that explains the relevance of each key element. A set of information tools (under development) to facilitate risk evaluation and access to TSE knowledge was demonstrated at the workshop.

The workshop 1) identified research needs and reviewed risks associated with sources of raw materials (material collection and procurement are primary control points for ensuring low risk of TSE transmission); 2) focused on inactivation schemes for TSE agents, which may be the most reliable way of reducing the level of TSE agent; 3) examined use of several categories of end products: foods, pharmaceuticals, cosmetics, blood and blood products, biologics, and tissues of animal and human origin; and 4) proposed strategies for TSE risk evaluation.

Representatives of governments, private organizations, and industry groups presented their approaches to the evaluation of TSE risks and found common themes: 1) zero risk does not exist; 2) decisions concerning public health issues must often be made in the absence of complete information; 3) risk analysis may be useful in understanding the key elements of a problem or situation; 4) the risk evaluation process must be responsive to rapidly changing information and new scientific data; 5) the risk evaluation process should be transparent, involving all partners (general public, regulated industry, government); 6) disagreements still exist concerning the appropriateness of models, assumptions, and methods; and 7) although both qualitative and quantitative approaches to TSE risk evaluation have merit, no single approach is applicable to all situations.

Workshop documents, including illustrated transcripts of the presentations, a draft outline of critical elements in TSE risk evaluations, observations on human and animal issues, and workshop overviews, are available at http://www.life.umd.edu/jifsan/tse.html).

USDA Report on Potential for International Travelers to Transmit Foreign Animal Diseases to U.S. Livestock or Poultry

The U.S. Department of Agriculture has published a new report—The Potential for International Travelers to Transmit Foreign Animal Diseases to U.S. Livestock or Poultry—which explores the issue of animal disease transmission by human travelers. Millions of people travel internationally each year, for both pleasure and business, and thus may be at risk for being infected by animals and causing infections in animals.

The report highlights and summarizes what is currently known about the potential for human infection and human-to-animal transmission of foreign animal diseases. It focuses on the International Office of Epizootics List A diseases. These transmissible animal diseases, which may spread rapidly and have serious

socioeconomic or public health consequence, are important in the international trade of animals and animal products. These diseases include avian influenza, Newcastle disease, Rift Valley fever, foot and mouth disease, swine vesicular disease, vesicular stomatitis, classical swine fever, African horse sickness, African swine fever, bluetongue, contagious bovine pleuropneumonia, lumpy skin disease, peste des petits ruminants, rinderpest, and sheep and goat pox.

The report includes in-depth discussion on the qualitative risk of human-to-animal transmission, by both biologic and mechanical transmission modes, and assigns a qualitative risk rating to each List A disease. Because many of these diseases can survive outside the host for extended periods of time, mechanical transmission is entirely possible. The report concludes that humans can transmit Office of Epizootics List A diseases to animals in the United States. Risk of either biologic or mechanical transmission was found to be negligible to none for most List A diseases. However, the risk of mechanical human-to-animal transmission is high for two diseases (Newcastle disease, swine vesicular disease), moderate for three diseases (avian influenza, foot and mouth disease, and African swine fever), and low, but not negligible, for one disease (vesicular stomatitis).

The full report can be found at www.aphis.usda.gov/vs/ceah/cei/travrisk.pdf.

Second Annual Conference on Vaccine Research, March 1999

The Second Annual Conference on Vaccine Research: Basic Science, Product Development, and Clinical and Field Studies will be held March 28-30, 1999, in Bethesda, Maryland. Sponsored by the Centers for Disease Control and Prevention, National Foundation for Infectious Diseases (NFID), National Institute of Allergy and Infectious Diseases, Center for Biologics Evaluation and Research of the Food and Drug Administration, U.S. Department of Agriculture, World Health Organization, Children's Vaccine Initiative, International Society for Vaccines, and Albert B. Sabin Vaccine Institute, the conference will focus on scientific data from diverse disciplines involved in vaccine research and development and on associated technologies for disease control through immunization.

To obtain program announcements and forms for registration, hotel reservation, and abstract submission, send name, address, telephone, fax, and e-mail address to: NFID, Attention: Kip Kantelo, Suite 750, 4733 Bethesda Ave., Bethesda, MD 20814-5228, USA; fax: 301-907-0878; tel.: 301-656-0003; e-mail: kkantelo@aol.com. Program information is available at: http://www.nfid.org/conferences/.

International Scientific Forum on Home Hygiene

Experts in health and hygiene announce the inauguration of the International Scientific Forum on Home Hygiene (IFH). A nonprofit organization, IFH comprises scientists and professionals in hygiene policy and research. It was formed to raise awareness of the role of domestic hygiene in the prevention of home-acquired infections. Through its international activities, IFH focuses on home hygiene in situations where infection risk exists: food, personal cleanliness, and community medical care.

For more information on the organization, its aims, and scientific research on home hygiene, access their web site: http://www.ifh-homehygiene.org.