PREVALENCE OF HANTAVIRUS IN FOUR SPECIES OF *NEOTOMA* FROM ARIZONA AND UTAH

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Sin Nombre virus (SNV), a hantavirus, can cause severe respiratory illness and death in humans. The primary carrier is the deer mouse, *Peromyscus maniculatus,* but other species of rodents may be infected with the virus. We screened four species of woodrats (*Neotoma*) for hantavirus using both enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) tests. Woodrats were collected from a variety of habitats in Arizona and Utah. Only *Neotoma lepida* tested serologically positive in an ELISA assay for hantavirus and also contained SNV RNA. Moreover, across three distinct populations of *N. lepida,* individuals that tested serum positive were restricted to one population near Jericho, Utah. The prevalence of hantavirus in this population was 27% (4 of 15).

Key words: *Neotoma,* woodrat, Sin Nombre virus, hantavirus, zoonosis

The emergence of previously unknown zoonotic diseases has caused concern among mammalogists as to how common and in which species of mammals such disease causing agents occur (Childs et al., 1995). One such agent that has elicited much attention is Sin Nombre virus (SNV), a hantavirus belonging to the family Bunyaviridae. Sin Nombre, discovered in 1993, causes severe pulmonary failure with a high mortality in humans (Duchin et al., 1994; Nichol et al., 1993). Transmission of hantaviruses from rodent to human is thought to occur via inhalation of virus in aerosols from rodent urine, saliva, and feces (Tsai et al., 1987). However, this route of transmission had not been firmly established for SNV.

Several species of rodents have been identified as chronic carriers of hantaviruses. The primary reservoir of SNV is the deer mouse, *Peromyscus maniculatus,* which is abundant across most of the United States. Prevalence of SNV in populations of *P. maniculatus* ranges from 30.4% in the four corners region of the southwestern United States to 8.5% in other areas (Kaufman et al., 1994). Congeners such as *P. truei* and *P. boylii,* and species from other families of rodents have Sin Nombre virus RNA present in their tissues, which is suggestive of chronic infections (Childs et al., 1994). However, the incidence of SNV is lower in these species than in *P. maniculatus* (Childs et al., 1994).

We report on the prevalence of SNV in four species of *Neotoma* from seven sites in the southwest. Prior to our study, all four species had been tested for SNV, but only *N. albigula* tested positive for antibodies against SNV (Childs et al., 1994). In these individuals, viral RNA could not be obtained from the blood suggesting that *Neotoma* were not chronic carriers of SNV (Childs et al., 1994). Therefore, we expected to find only individuals with antibody titers to SNV in *N. albigula.* Contrary to this prediction, we found infections of SNV in one population of *N. lepida* and no other species. These animals contained both antibody against SNV as

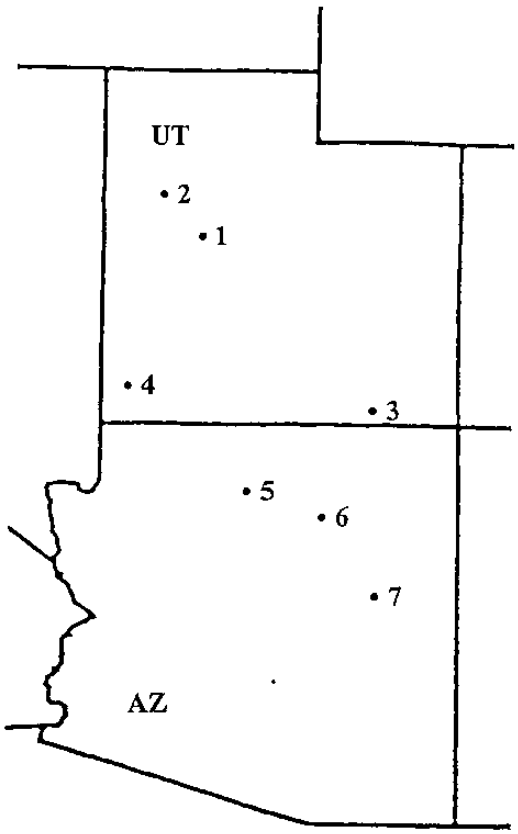

FIG. 1.—Locations of trapping sites of *Neotoma* in Arizona and Utah; numbers of sites correspond with descriptions in Table 1.

well as viral RNA. Our findings suggest that SNV may still be emerging in *Neotoma* and demonstrate the importance of screening for hantavirus at the individual level prior to the establishment of a laboratory colony.

MATERIALS AND METHODS

Study area.—We trapped woodrats at four sites in Utah and three in Arizona (Fig. 1, Table 1). At sites in Jericho and Dugway, Utah, vegetation was dominated by juniper (*Juniperus osteosperma*) and big sage (*Artemisia tridentata*). These sites rarely burn because the soil, primarily sand, does not support extensive underbrush. Thus, junipers occurring there were quite large with lower interdigitating branches that covered several meters of the ground around the tree. Woodrat middens were located most often at the bases of such trees. At the site in Castle Valley, Utah, the dominant shrub was *J. osteosperma,* but junipers did not attain the large sizes of those at Dugway and Jericho. Beavertail cactus (*Opuntia*) was common. Woodrat middens typically were found either in beavertail cactus or constructed of beavertail cactus clades, but they also occurred at the bases of junipers. At the site in Beaver Dam, Utah, dominant species of plants were creosote bush (*Larrea tridentata*), desert almond (*Prunus fasciculata*), and Joshua tree (*Yucca brevifolia*). Woodrat middens were found under desert almonds and yucca and in rock ledges.

Across all study sites in Arizona, *J. monosperma* was the dominant tree. The vegetation at Wupatki, Arizona was Great Basin ecotone (Dial, 1988). Middens were located primarily in rock crevices. Vegetation at Padre Canyon, Arizona was mixed woodland-shrub (Howe, 1976). Middens were found in rock crevices and under shrubs such as *Atriplex canescens* and *Berberis fremonti.* At Lone Pine, Arizona the vegetation was similar to Padre Canyon but less diverse. Middens were located in rock crevices and woodpiles in open woodlands.

Animal capture and viral assessments.—Animals were trapped with Sherman and Tomahawk live traps baited with peanut butter and oats. Cotton batting for bedding also was placed in traps. Two to three traps were placed at middens of woodrats that appeared to have recent activity of woodrats such as fresh plant clippings or feces. Traps were opened after 1600 h to avoid trapping diurnal mammals and checked after sunrise. Trapping was conducted from 21 March to 31 May 1996. Animals were transported to the animal facility at the University of Utah where they were kept in quarantine closets at the animal facility. Captive rats were provided with water, rabbit chow, and occasionally, apples and carrots. About 30 animals were housed per quarantine closet.

Initial blood samples were taken shortly after animals were taken into captivity. Animals were anesthetized with Metafane (methoxyflurane) and blood was drawn by clipping the distal end of the tail and palpating the tail if necessary to initiate blood flow. Blood samples were kept on dry ice or at −20°C until analysis. We delayed collection of blood samples from nursing females until young were weaned (3–4 weeks

TABLE 1.—*Trapping locations, types of habitats, dates on which trapping occurred, and number of each species trapped at each site. Site numbers refer to locations in Fig. 1.*

Site	Latitude and longitude	Type of habitat	Elevation (m)	Dates	Number of animals
1. Jericho, Juab Co., Utah	39°57'N, 112°22'W	Great Basin	1935	3 April, 31 May	15 *N. lepida*
2. Dugway, Tooele Co., Utah	40°19'N, 112°57'W	Great Basin	1676	1, 15, 20 May	11 *N. lepida*
3. Castle Valley, San Jaun Co., Utah	38°38'N, 109°18'W	Great Basin	1829	23, 24 May	20 *N. albigula*
4. Beaver Dam, Grand Co., Utah	37°06'N, 113°58'W	Mojave	914	8–12 April	19 *N.lepida*
5. Wupatki, Coconino Co., Arizona	35°30'N, 111°27'W	Great Basin	1676	20–24 March	17 *N. stephensi*
					4 *N. albigula*
6. Padre Canyon, Coconino Co., Arizona	35°08'N, 111°17'W	Great Basin	1768	8–9 May	2 *N. mexicana*
					1 *N. albigula*
7. Lone Pine, Navajo Co., Arizona	34°21'N, 110°05'W	Pinyon-Juniper	1798	10 May	5 *N. mexicana*

postpartum). If initial results of the enzyme-linked immunosorbent assay (ELISA) were negative for all animals in a quarantine closet, secondary blood samples were taken ≥30 days after the initial samples. When an animal in a quarantine closet tested sero-positive for hantavirus in the initial blood sample, we removed that animal from the closet and bled all remaining animals 30 days after their last exposure to the sero-positive animal. Sero-positive animals also were screened for SNV using reverse transcriptase PCR (RT PCR).

We performed ELISA for detection of anti-hantavirus antibody. Wells of polyvinyl chloride microtiter plates (Dynatech Laboratories Chantilly, VA) were coated overnight at 41°C with recombinant nucleocapsid antigen (SNV recombinant antigen—Feldmann et al., 1993) diluted 1:2,000 in phosphate buffered saline (PBS, pH 7.4). A negative recombinant antigen also was coated to plates and used as a control. After incubation, unbound antigen was removed from wells by washing three times with wash buffer (PBS pH 7.4, 0.5% between 20, and 0.01% thimerosal). Heat-inactivated sera of woodrats were diluted 1:100 in serum-dilution buffer (PBS, pH 7.4, 0.5% between 20, 0.01% thimerosal, and 5% skim milk) added to the antigen coated wells and incubated 37.1°C for 60 min. After incubation with serum, wells were washed three times with wash buffer and incubated with the specific secondary or tertiary antibody at 37.1°C for 60 min. Antibodies included horseradish peroxidase (HRP) labeled goat anti-*Peromyscus leucopus,* HRP labeled goat anti-rat (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), HRP labeled goat anti-mouse (Southern Biotech, Birmingham, AL), unlabeled rabbit anti-*Perognathus,* and unlabeled rabbit anti-*Spermophilus.* Plates were washed three times with wash buffer and incubated at 37.1°C 30 min with 100 microliters of ABTS Microwell Peroxidase Substrate Solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The A405 was recorded with a Ceres 900 enzyme-immunosorbent assay workstation (Bio-Tek Instruments, Inc., Winooski, VT). Values >3 *SD* of the six negative control wells were considered positive. In addition, all serum samples with titers <1:400 were considered negative.

Viral RNA was extracted from tissues and blood of woodrats containing antibody to the

ELISA using nested RT-PCR and DNA sequencing of viral RNA. Total RNA was extracted from tissue and blood clots using an RNA extraction kit (Bio 101, La Jolla, CA) as per manufacturer's protocol. Briefly, tissues were solubilized in guanidine thiocyanate solution, then RNA was extracted with acid phenol-chloroform, and purified using an RNA glass-binding matrix. Because of the potential hazards involved in working with infected tissues, all steps in isolation and purification of total RNA from tissues and blood were performed in a laminar flow hood in a BSL-3 facility.

The RNA was reverse transcribed and amplified using modified primers and protocols described by Nichol et al. (1993) and Spiropoulou et al. (1994). The first round RT-PCR resulted in a PCR product of 337 base pairs (bp). Five percent of the first round PCR reaction was used as a template for the second round nested PCR reaction that generated a PCR product of 280 bp. The PCR products were separated by electrophoresis through a 1% agarose gel containing ethidium bromide. The DNA was viewed using UV light, and the band at 280 bp was removed and purified using a DNA purification kit (Bio 101, La Jolla, CA) following manufacturer's protocols. The DNA was sequenced by dideoxynucleotide chain termination.

Results

Across all study sites and species of *Neotoma,* we trapped 94 individuals (Table 1). All except one were tested for hantavirus. Only four individuals tested sero-positive in the ELISA; all four were *N. lepida* from the population at Jericho, Utah. Antibody reactivity was discovered during the initial blood sampling. Based on RT-PCR analysis of the blood, all sero-positive individuals maintained active infections. No woodrats in the colony that were initially sero-negative converted 30 days after removal of sero-positive animals.

Analysis of the sequence of viral RNA detected in the blood of infected animals by RT-PCR and direct sequencing of the PCR product demonstrated that SNV was present in the woodrats. The M segment sequenced from viral RNA in *N. lepida* was 99% similar to an isolate from Las Vegas, Nevada; 86% similar an isolate from New Mexico and 85% similar to an isolate from the central Sierra Nevadas, California.

The only wild-caught animal not tested for hantavirus was a female that gave birth in captivity to a litter of four. At ca. 4 weeks postpartum and prior to blood sampling, the mother and three of the young died. The surviving offspring tested positive for hantavirus. This offspring had consumed part of its mother's dead body, and we could not determine whether transmission of SNV to this individual was vertical (in utero) or horizontal (due to consumption of infected tissue).

Discussion

The recent emergence of SNV in southwestern United States has elicited concern among mammalogists, especially those establishing rodent colonies (Childs et al., 1995; Mills et al., 1995). Because of the absence of information on the dynamics of SNV and its potential rodent hosts, combined with the high morbidity and mortality associated with SNV, prudence and testing have been suggested for mammalogists working with species that have not been extensively screened for SNV (Mills et al., 1995). In establishing a colony of four species of *Neotoma,* we followed these precautions and tested all individuals, even though existing data implicated only one species of woodrat, *N. albigula,* as susceptible to SNV.

Our results underscore our limited understanding of the ecology of SNV. Contrary to our predictions, we found no evidence of SNV in populations of *N. albigula.* However, we did find the highest incidence (26.7%, 4 of 15) of SNV ever documented for *Neotoma* and in a species, *N. lepida,* not known to contract SNV. Occurrence of SNV in *N. lepida* was limited to only one of the three populations surveyed.

Surprisingly, *N. lepida* that were serologically positive for SNV antibody also contained SNV RNA, and this sequence of RNA was most similar to SNV sequence

found in *P. maniculatus* from the Great Basin. This result suggests that the virus was transmitted between *N. lepida* and *P. maniculatus*. This finding is contrary to the conventional wisdom that persistent infections of specific hantaviruses can only occur in closely related hosts (i.e., within the same genus) and infections of unrelated hosts are incidental ones that do not result in a persistent infection. For example, when humans are infected with SNV, they develop disease symptoms and if they survive, they clear the virus within 2 weeks and are not infected persistently. In our case, the virus infecting *N. lepida* was a persistent infection because rodents had viral RNA in their blood for ≥2 months after capture. The significance of persistent infections in *N. lepida* in the transmission and maintenance of SNV in nature remains unknown.

The disjunct distribution of SNV in populations of *N. lepida* is intriguing. We propose two hypotheses to account for this pattern. First, it is possible that SNV is still emerging in the other populations of *N. lepida* tested. Thus, our small sample sizes of 10–20 individuals/population have a limited ability to detect SNV at very low frequencies, which would be expected during the incipient stage of emergence (May, 1993). In contrast, there may be some abiotic or biotic factor specific to the population of woodrats at Jericho that makes it more susceptible to SNV. Potential factors include demography of rodent populations, availability of resources, or climate.

Transmission of SNV among woodrats is speculative at this point. Many species of *Peromyscus*, including *P. maniculatus* occupy active middens of woodrats. At all study sites, we inadvertently trapped *Peromyscus* at middens. Thus, it is possible that woodrats contract SNV from deer mice living in their middens in the same way that humans contract it from feces and urine of deer mice living in their homes. Woodrats also may become infected through more intimate contact with deer mice such as bites. The woodrat born in captivity that tested positive for hantavirus could have contracted it either in utero or later from its mother. The nature of interactions between SNV, woodrats, and deer mice inhabiting their middens warrants further attention.

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