VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 5, Number 1, 2005

Research Paper

Newly Recognized Hantaviruses Associated with Hantavirus Pulmonary Syndrome in Northern Brazil: Partial Genetic Characterization of Viruses and Serologic Implication of Likely Reservoirs

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ABSTRACT

Following the occurrence of the first laboratory-confirmed cases of hantavirus pulmonary syndrome (HPS) in Maranhão State, Brazil, rodents were trapped and rodent materials screened by ELISA for antibodies to Sin Nombre and Andes hantaviruses. Antibody-positive samples were tested by RT-PCR, amplified products were sequenced, and phylogenetic trees were constructed for comparison with known hantaviruses. From 104 rodent blood samples collected (40 *Bolomys lasiurus*, 52 *Holochilus sciureus*, 12 *Oligoryzomys fornesi*, and one *Proechimys guyannensis*), 21 (20.2%) were antibody-positive (one *B. lasiurus*, five *O. fornesi*, and 15 *H. sciureus*). Hantavirus RNA was amplified by PCR from two *O. fornesi* and four *H. sciureus*. Viral sequencing identified two hantavirus genotypes. The genotype recovered from *O. fornesi*, is designated herein as Anajatuba (ANAJ) and the genotype recovered from *H. sciureus* is designated Rio Mearim (RIME). Phylogenetic analysis of a 643-nucleotide region of the N segment showed both viruses to be most closely related (94–96% nucleotide homology) to Río Mamoré virus, a virus associated with *Oligoryzomys microtis* in Bolivia and Peru, but not found in northern Brazil. *O. fornesi* was frequently captured in and around human dwellings. *H. sciureus*, is a semi-aquatic rodent captured only in remote areas rarely frequented by humans. Key Words: Hantaviruses—HPS—Anajatuba virus—*Oligoryzomys fornesi*—Rio Mearim virus—*Holochilus sciureus*—Phylogenetic characterization—Brazil. Vector-Borne Zoonotic Dis. 5, 11–19.

INTRODUCTION

THE FAMILY *Bunyaviridae* comprises more than 300 serologically distinct viruses in five genera, including the genus *Hantavirus*. Hantaviruses, like other bunyaviruses have three negative-stranded RNA segments. The large (L), medium (M), and small (S) segments are responsible for encoding the viral polymerase, the G1 and G2 glycoproteins of the envelope, and the nucleocapsid protein, respectively (Schmaljohn 1996). Hantaviruses are hosted in

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nature by rodents of the family Muridae. Three distinct groups of hantaviruses have been recognized. Those associated with the murid subfamily Murinae are responsible for a clinical syndrome called hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia. A second group of viruses is associated with the subfamily Sigmodontinae and causes hantavirus pulmonary syndrome (HPS) in the Americas. A third group is associated with the subfamily Arvicolinae throughout the northern hemisphere. At least one arvicoline hantavirus, Puumala virus, is associated with a mild form of HFRS in Europe (Schmaljohn and Hjelle 1997).

Since the recognition of Sin Nombre virus and its association with HPS in the USA (Duchin et al. 1994, Nichol et al. 1993), more than 25 hantaviruses or hantavirus genotypes have been named in North (Ksiazek et al. 1997, Morzunov et al. 1995, Rollin et al. 1995, Rowe et al. 1995), Central (Vincent et al. 2000), and South America (Johnson et al. 1999, Levis et al. 1998, Padula et al. 2000, Powers et al. 1999), many of them associated with HPS.

The first recognized HPS cases in Brazil occurred in November 1993 in Juquitiba, São Paulo State, and were associated with Juquitiba virus (Iversson et al. 1994). Two additional viral lineages (Araraquara and Castelo dos Sonhos) have been associated with HPS cases in southern and southeastern Brazil (Johnson et al. 1999). Currently, 502 HPS cases have been reported to the Brazilian Ministry of Health; more than 90% of these cases were from southern and southeastern Brazil (M.R. Elkhoury and E.D. Santos, personal communication). Several suspected cases and three laboratory confirmed cases have been reported from Anajatuba, Maranhão State (Mendes et al. 2001). Here, we report the description of two novel hantavirus genotypes recognized in the Brazilian Amazon region and the ecologic studies implicating their most likely rodent reservoirs.

MATERIALS AND METHODS

Study sites

Anajatuba (3°16′S; 44°37′W; population 22,968), is about 112 Km south of São Luís, capital of the state of Maranhão (Fig. 1). Anajatuba is in the western region of the floodplain of the Maranhão River (the "Baixada Maranhense"). The climate is tropical, with high temperatures ranging from 26°C to 32°C. The rainy season (January through July) brings 2,000–2,500 mm of rainfall per year.

Much of this vast floodplain is inundated during the rainy season. In the dry season, waters recede, exposing vast grassy fields that are used for cattle grazing and cultivation of manioc, rice, sugar cane, and corn. Hook-and-line fishing is an important occupation in the lowlands. Many inhabitants live under poor conditions, occupying mud huts along the margins of the fields.

Our studies were conducted near the homes of reported HPS case patients in the communities of Quebra (3°14.159′S, 44°33.902′W; population approximately 600) and São Jerônimo (3°17.053′S, 44°32.671′W, population approximately 80). The two communities are within sight of each other across a marsh, and are located approximately 10 km south, by road, from the town of Anajatuba.

Rodent trapping

From September 11th to the 21st, 2000, we placed Sherman (8 × 9 × 23 cm, H.B. Sherman Trap Company, Tallahassee, FL) and Tomahawk (14 × 14 × 40 cm, Tomahawk Trap Company, Tomahawk, WI) live-capture traps in lines of 10–20 traps in major representative habitats in the vicinity of case homes. Traps were placed in the evening and baited with peanut butter and rolled oats. Early each morning, captured animals were collected and returned to a field laboratory for processing. Trapping and sampling of rodents followed standard safety procedures (Mills et al. 1995). Briefly, animals were anesthetized prior to taking a 0.5–1.0-mL sample of blood from the retro-orbital capillary sinus using a capillary tube. The deeply anesthetized rodents were weighed and measured and then sacrificed prior to collecting samples of spleen, kidney, liver, lung, and heart. All samples were quickly frozen in liquid nitrogen in the field and later transferred to mechanical freezers (−70°C) for storage until they were tested. Field identifica-

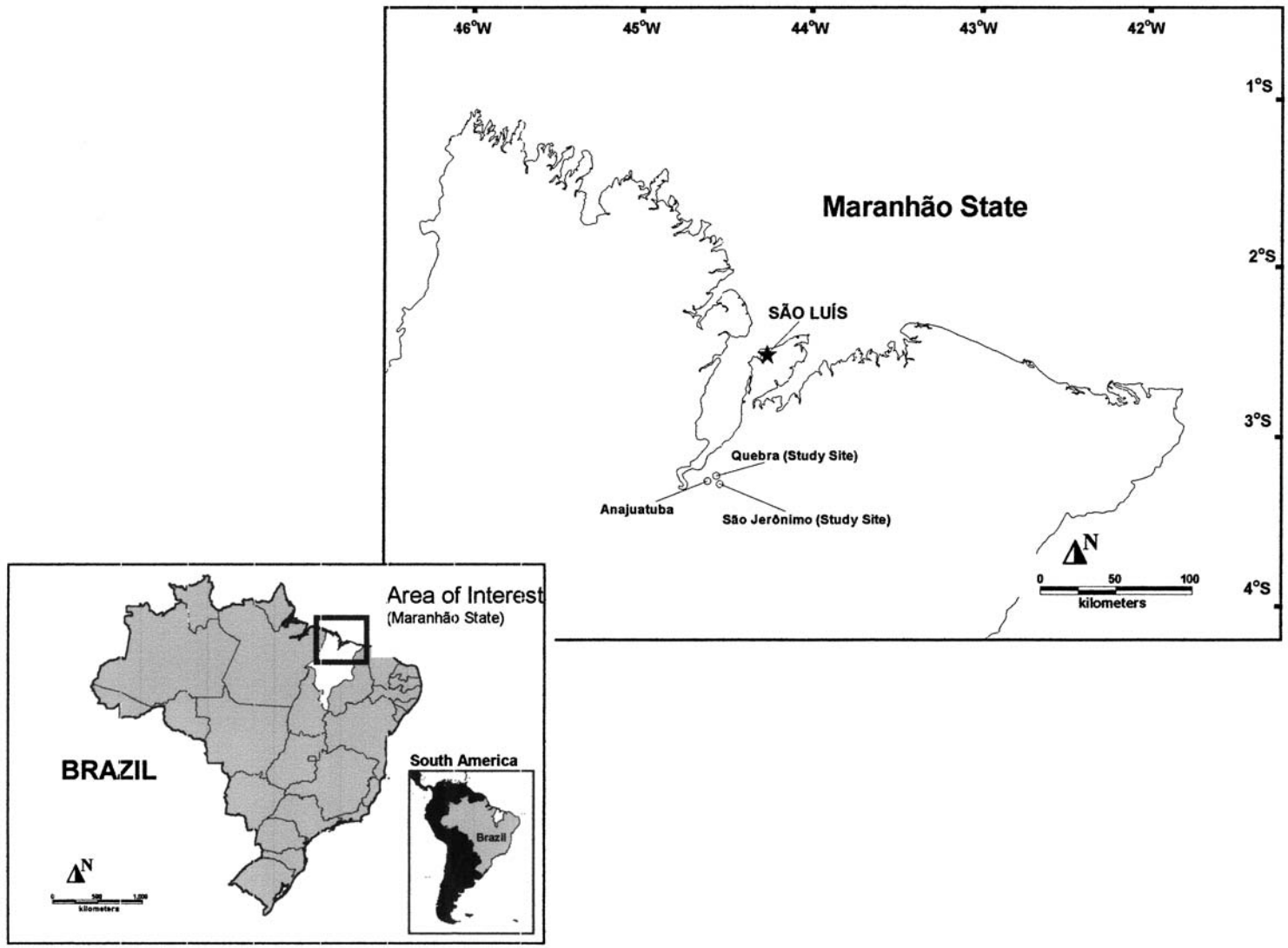

FIG. 1. Map of northern Maranhão State, Brazil, depicting the location of sampling locations near the town of Anajatuba.

tion of rodent species was confirmed, and voucher specimens were archived by the Museum of Southwestern Biology (University of New Mexico, Albuquerque, NM).

Serologic testing

Whole blood samples from the rodents were tested by enzyme-linked immunoassay (ELISA) for antibodies reactive with nucleocapsid antigen of Sin Nombre (SN) and Andes (AND) viruses, using standard techniques (Feldmann et al. 1993, Padula et al. 2000).

Molecular analyses

Total RNA was extracted from lung tissues of antibody-positive rodents using the guanidinium isothiocyanate–acid phenol extraction procedure (Padula et al. 2000). For RNA purification, the RNAid kit (Bio 101) was used following the manufacturer's recommendations. One-step RT-PCR was performed followed by a second round of nested or heminested PCR. Specific oligonucleotide primers of the S fragment and partial M segments of AND virus were used for amplification and sequencing (Della Valle et al. 2002, Padula et al. 2000).

Amplification products were separated on agarose gels, gel-purified, and sequenced in both directions by the fluorescent sequencing technique (dRhodamine Terminator Cycle Sequencing kit, Applied Biosystem) using an ABI 377 automatic sequencer.

For nucleotide and amino acid sequence analysis, one fragment of the N coding region of the S segment (nts 50–954), numbered in the antigenome-sense sequence relative to AND virus, was amplified.

Phylogenetic analyses

Analysis of nucleotide and deduced amino acid sequence differences among hantavirus N amplimers was performed using NALIGN and PALIGN programs of the PCGENE 6.8 software from Intelligenetics Inc (Mountain View, CA).

Multiple sequence alignment and comparison of nucleotide and deduced amino acid sequences were performed using CLUSTAL W, in the PCGENE 6.8 software.

Maximum parsimony (MP) and Neighbor-Joining (NJ) analysis of nucleotide and putative amino acid sequence viruses were carried out using PHYLIP version 3.57c (Felsenstein1985). Programs DNAPARS and PROTPARS were used to obtain maximum parsimony trees for nucleotide and protein sequences, respectively. Program DNADIST was used to obtain genetic distance estimates, weighting 3:1 transversion to transition. Sequence distances were obtained using program PROTDIST with dayhoff substitution matrix. Program FITCH was used to fit distances to maximum parsimony trees.

The following published S segment sequences were included in the analysis: New York virus (NY; GenBank accession no. U36801), Sin Nombre virus (SN; L25784), Bayou virus (BAY; L36929), Black Creek Canal virus (BCC; L39949), Andes (AND) Cent Buenos Aires virus strain Hu39694 (Af482711), AND Nort Oran virus strain Ol22996 (Af028024), AND Cent Lechiguanas virus strain Of22819 (Af482714), Laguna Negra virus (LN) ; Af005727), AND Sout virus strain AH-1 (AF324902), Maciel virus (MAC) strain Bo13796 (Af0482716), AND Nort Bermejo virus strain Oc22531 (Af482713), Pergamino virus (PRN; 482717), Castelo dos Sonhos virus (CAS; AF307324), Araraquara virus (ARA; AF307325), Caño Delgadito virus (CANO; Af000140), Río Segundo virus (RIOS; U18100), Río Mamoré Bolivia virus (RIOM Bolivia; U52136), Río Mamoré Peru (HTN-007 Peru; Af133254) AND Cent Plata, Rio Mearim virus (RM), Anajatuba virus (ANAJ).

RESULTS

From September 11th to the 21st, 2000, we conducted small-mammal trapping (1400 trap nights: 1040 at São Jerônimo and 360 at Quebra) capturing and sampling 104 rodents in major peridomestic, agricultural, and sylvatic habitats (Tables 1 and 2). Ten (9.6%) animals were trapped in Quebra and 94 (90.4%) in São Jerônimo. Forty (38.0%) were *Bolomys lasiurus*, 51 (49.0%) were *Holochilus sciureus*, 12 (11.5%) were *Oligoryzomys fornesi*, and one (1.0%) was *Proechimys guyannensis*.

Results of ELISAs using AND and SN virus antigens were concordant. Of the 104 blood

Table 1. Numbers of Hantavirus Antibody–Positive Rodents (Total Number Captured in Parentheses) by Species in Two Localities Near Anajatuba, Maranhão State, Brazil

Locality	*Species*	*No. Ab+ (total)*	*Prevalence (%)*
Quebra	*Bolomys lasiurus*	0 (7)	0.0
	Oligoryzomys fornesi	2 (2)	100.0
	Proechimys guyannensis	0 (1)	0.0
Quebra total		2 (10)	20.0
São Jerônimo	*Bolomys lasiurus*	1 (33)	3.0
	Holochilus sciureus	15 (51)	29.4
	Oligoryzomys formesi	3 (10)	30.0
São Jerônimo total		19 (94)	20.2
Grand total		21 (104)	20.2

samples tested by ELISA, 21 (20%) had antibody reactive with both AND and SN virus antigens (Table 1). Antibody-positive rodents by species were: one *B. lasiurus* (2.5% antibody prevalence), 15 *H. sciureus* (29.4%), and five *O. fornesi* (41.7%). Although the overall antibody prevalence was the same between the two sites (20%), the number of antibody positive rodents was much greater at São Jerônimo, owing to the greater effort and greater capture success for *O. fornesi* and *H. sciureus*. The only two *O. fornesi* captured at Quebra were antibody-positive (Table 1). *H. sciureus* was largely restricted to marshy habitats where it was numerically dominant (Table 2). *H. sciureus* was not captured in peridomestic environs. *O. fornesi* was most dominant in dry pasture and peridomestic habitats, but was also captured with *H. sciureus* in the inundated banana plantation (Table 2).

Comparative sequence analysis

Samples from seropositive rodents were examined for viral genetic material by nested and hemi-nested RT-PCR. The single antibody-positive specimen from *B. lasiurus* was PCR-negative. cDNA from four *H. sciureus* and two *O. fornesi* were successfully amplified from RNA extracted from lung tissue.

At least partial sequence comparisons were made among these samples and compared with previously characterized hantaviruses (Fig. 2, Table 3). Percentage identities of both the nucleotide and the amino acid sequences of a coding region of the nucleoprotein of the S segment (Table 3) were compared with published sequences of other hantaviruses. The sequencing of the RT-PCR materials identified two viral genotypes, one from *H. sciureus* (designated Rio Mearim (RIME) virus) and one from *O. fornesi* (designated Anajatuba (ANAJ) virus).

For the S segment, N region, RIME virus sequences were most similar to HTN-007 Peru and RIOM Bolivia sequences (82.6% and 81.8% at the nucleotide level and 95.3% and 94.3% at the amino acid level, respectively). In the same comparison, ANAJ virus is even more similar to HTN-007 Peru and RIOM Bolivia (85.6% and

Table 2. Habitat Associations of Rodents Captured Near Anajatuba, Maranahão State, Brazil

Habitat	Bolomys lasiurus	Holochilus sciureus	Oligoryzomys fornesi	Proechimys guyannensis	*Total*
Inundated banana trees	—	25	4	—	29
Moist floodplain	1	1	—	—	2
Ipomoea marsh	2	5	—	—	7
Sugar cane	5	3	—	—	8
Fence line	9	—	—	—	9
Grassy marsh	14	15	—	—	29
Peridomestic	8	—	3	1	12
Fenced pasture	1	1	5	—	7
Total	40	51	12	1	104

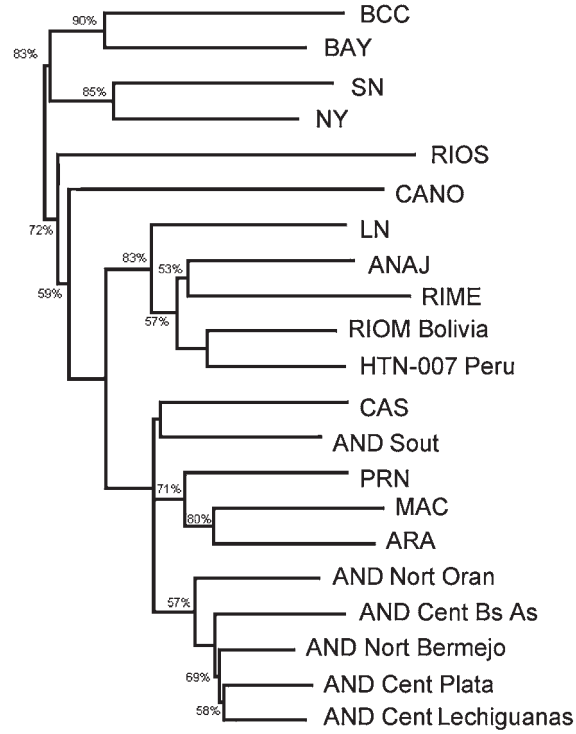

FIG. 2. Phylogenetic tree derived from maximum parsimony analysis comparing two recently discovered Brazilian hantavirus genotypes (designated Rio Mearim and Anajatuba) with sequences from other American hantaviruses. Analysis is based on a 643-nucleotide region of the S segment (nucleotides 57–699). The lengths of the lines are proportional to genetic distances. The values next to the branches indicate the bootstrapping confidence limits (in percentages) from 500 replicates. SN, Sin Nombre; LN, Laguna Negra; AND, Andes; NY, New York; BAY, Bayou; BCC, Black Creek Canal; ANAJ, Anajatuba; RIME, Rio Mearim; PRN, Pergamino; CAS, Castelo dos Sonhos; ARA, Araraquara; MAC, Maciel; RIOS, Río Segundo; CANO, Caño Delgadito; RIOM, Río Mamoré; HTN-007, Río Mamoré variant, Peru.

85.5% at the nucleotide level, and 97.0% and 95.7% at the amino acid level, respectively).

DISCUSSION

The evidence linking the ANAJ and RIME viral lineages to human disease is and will remain circumstantial until viral sequences are obtained from human case patients for comparison to the sequences from host rodents. Given that successful amplification of hantaviral RNA from human case patients is generally restricted to autopsy tissues from fatal cases, it may be several years before the definitive link to human disease can be established. Nevertheless, the implication of two potential host species allows the development of specific risk-reduction education programs designed to minimize human contact with these species.

This is the first identification of New World hantaviruses in northern Brazil. Previously, Seoul virus was isolated from domestic rats in Belém (LeDuc et al. 1985), and antibody reactive with Seoul and Puumala viruses was detected among inhabitants of Manaus (Vasconcelos et al. 1992). With the recognition of circulating New World hantaviruses in the Brazilian Amazon region, the interpretation of this serological reactivity should be reconsidered.

The designation of species within the genus *Hantavirus* is a matter of much discussion. We do not claim that the novel genotypes we have described should be given the status of viral species. That will require further genetic characterization or the development of specific cross-neutralization assays for hantaviruses. Nevertheless we emphasize that both viruses occupy distinct niches from any known hantaviruses. The closest relatives identified by our phylogenetic analyses are Río Mamoré and Laguna Negra viruses. The HTN-007 isolate was described as a variant of Río Mamoré virus by the authors who characterized it (Powers et al. 1999). Río Mamoré virus is hosted by *Oligoryzomys microtis* (Hjelle et al. 1996), and Laguna Negra virus is hosted by *Calomys laucha*. Neither of these species occurs in Northern Brazil (Eisenberg and Redford 1999).

The possible association of an *Oligoryzomys* species with HPS in Northern Brazil is not unexpected. Species of this genus have been implicated as hosts for six other hantaviruses in South and Central America, and at least five of these viruses are associated with HPS (Della Valle et al. 2002, Levis et al. 1998, Padula et al. 2002). Nevertheless, the host species involved are different for each place and (with one possible exception) for each virus. This pattern follows from the hypothesis that the association between hantaviruses and sigmodontine rodents in the New World is ancient, and the viruses have co-speciated with their hosts (Plyusnin and Morzunov 2001).

TABLE 3. NUCLEOTIDE AND AMINO ACID SEQUENCE HOMOLOGIES BETWEEN PARTIAL S SEGMENTS OF AMERICAN HANTAVIRUSES

	Rio Mearim	*Anajatuba*	*Rio Mamore Bolivia*	*Laguna Negra*	*AND Nort Oran*	*AND Sout*	*AND Nort Bermejo*	*AND Cent Lec*	*AND Cent BsAs*	*Pergamino*	*Sin Nombre*	*New York*	*Bayou*	*Black Creek*	*Araraquara*	*Castelo dos Sonhos*	*HTN007*	*Rio Segundo*	*Maciel*	*Cano Delgadito*
Rio Mearim	—	83.4	81.8	78.9	77.2	79.3	77.4	76.8	77.8	76.6	74.7	75.3	74.9	74.2	78.4	79.5	82.6	71.7	75.4	73.9
Anajatuba	95.7	—	85.5	80.1	79.9	79.1	79.8	80.1	79.3	78.7	75.4	76.5	76.8	76.5	80.6	81.8	85.6	72.8	77.4	74.1
Rio Mamore Bolivia	94.3	95.7	—	83.1	80.7	78.9	79.8	80.6	78.8	78.9	75.3	76.2	77.1	76.4	82.3	81.3	87.1	73.6	77.2	74.9
Laguna Negra	90.3	91	91.7	—	78.9	78.8	79	78.8	79.2	77.7	75.4	75	76.4	76.4	81.7	80.4	82	73.6	76.6	73.3
AND Nort	87.3	87.7	88.4	87	—	83.8	87.3	87.2	87.9	83.7	76.6	76.6	77.5	75.4	84.3	84.9	80.8	74	82.4	76
AND Sout	87.3	88.7	88.7	87.7	95.7	—	84.3	84.8	83.9	82.1	75.7	76.2	75.4	75.4	85.1	85.5	79	73.2	80.8	76.9
And Nort Bermejo	87.3	87.4	88	86.7	98.3	95.7	—	91.7	88.3	82	76.8	75.5	76.1	77.8	83.2	86.5	79.9	73.4	82.2	75.9
AND Cent Lec	87	87	88.4	86.4	98	95.4	99.7	—	87.2	82.4	76.1	76.1	75.6	77.7	82.6	87.1	80.8	73.3	82.1	76.8
AND Cent BsAs	87.3	87.7	88.4	87	98	96	99.7	99.3	—	82.7	75	75.8	75.4	74.7	82.9	82.6	78.8	73.5	82.1	75.1
Pergamino	87.3	87.7	87.7	86.4	97.7	93.7	94.4	94	94.7	—	75.7	76.5	76.6	75	85.5	83.2	77.7	73.2	83	73.8
Sin Nombre	83	82.4	82.4	82.4	84.7	83.7	85.4	85.1	85.1	83.4	—	83.8	76.2	74.5	79.2	79.8	74.6	74.8	76.9	75.1
New York	83.7	84.1	84.1	84.1	84.7	85.1	84.7	84.4	85.1	84.4	93	—	77	74.8	81.7	78.9	75.8	72.4	75.4	75.6
Bayou	84.7	84.4	85.1	84.1	86.1	85.4	85.7	85.4	86.1	85.7	84.7	84.7	—	81.1	78.9	80.7	77.8	74.3	75	74.4
Black Creek	83	82.4	83.7	82.1	83.4	83.1	83.1	82.7	83.3	82.4	81.4	82.1	91.7	—	78.5	78.7	76.4	71.8	75.3	72.6
Araraquara	89.7[a]	90.2[a]	90.6[a]	90.6[a]	96.3[a]	96.3[a]	95.3[a]	95.3[a]	95.8[a]	95.8[a]	89.2[a]	92.1[a]	88.3[a]	86.4[a]	—	81.8	80.7	77.6	86.2	77.9
Castelo dos Sonhos	91.1[a]	91.1[a]	91.6[a]	91.6[a]	97.2[a]	98.1[a]	98.1[a]	98.1[a]	97.7[a]	96.3[a]	91.1[a]	92.1[a]	89.7[a]	87.8[a]	94.4[a]	—	81.2	79.6	82.4	77.6
HTN007	95.3	97	97.7	91.7	88.4	89.4	88	87.7	88.4	88	82.7	84.4	85.7	83.7	90.6[a]	91.6[a]	—	73.7	77.8	75
Rio Segundo	77.7	77.1	78.7	77.7	79.1	79.1	79.7	80.1	79.4	78.7	81.7	80.7	81.4	79.1	85.9[a]	90.2[a]	78.4	—	72.4	72.5
Maciel	86	86.7	86.7	84.1	93	92.4	92.7	92.4	93	94.7	82.7	84.1	84.7	82.7	98.1[a]	95.3[a]	87	79.1	—	72.4
Cano Delgadito	81.3	80.1	81.1	80.7	82.1	82.1	82.4	82.7	82.4	80.7	81.4	81.4	79.4	77.7	86.9[a]	88.3[a]	81.1	76.7	79.7	—

Values above dashes are nucleotide sequence identities and those below dashes are amino acid sequence identities.

N, correspond to nucleotides 50 to 954; NA, not available.

[a]Only nucleotides 57 to 669 were compared.

Given the habitat associations of the two reservoir species identified in this study, it is most likely that *O. fornesi* is most frequently associated with hantavirus transmission to humans. *O. fornesi* were captured in and around human habitations and other structures, including a small brick factory. This close co-existence with humans would facilitate the transfer of virus to humans via contamination of food, clothing, or utensils with virus-laden urine or feces, production of infectious aerosols in closed spaces, or direct contact between rodents and humans. *H. sciureus* is a semi-aquatic species that was captured primarily in wet or marshy habitats rarely visited by humans.

It is possible that *O. fornesi* plays an important role in the maintenance of Anajatuba virus in the Amazon region, similar to what has been observed for other *Oligoryzomys* species in Argentina (Padula et al. 2000), Peru (Powers et al. 1999), and Panama (Vincent et al. 2000). The high prevalence of hantavirus-specific antibody in *O. fornesi* (24%), combined with its peridomestic affinities, suggest that it may represent a significant risk of infection to humans.

Hantavirus transmission to humans has occurred and continues to occur frequently in the Anajatuba area. The prevalence of hantavirus antibody detected by IgG-ELISA was 18% in inhabitants of Quebra and São Jeronimo (PFC Vasconcelos and EST Rosa, unpublished data). Additionally, in a 2-year study following 292 initially antibody-negative people in Quebra and São Jeronimo, five people became hantavirus antibody-positive. All of these persons denied any HPS-like illness during the study period. Finally, three clinical HPS cases (all fatal) have occurred in the Anajatuba area since our rodent sampling was conducted in September 2000 (W.S. Mendes, unpublished data).

Given the very high antibody prevalence and incidence of hantavirus infection in humans in the Anajatuba area, it is clear that transmission of at least one hantavirus from rodents to humans is a frequent event. The documented peridomestic affinities of *O. fornesi* would suggest that this species is responsible for the majority of the transmission to humans. Nevertheless, a very low proportion of cases of hantavirus infection in the Anajatuba area appear to result in frank disease. This may be due to differences in human host genetics, or the existence of predisposing underlying conditions. Alternatively, it may indicate the circulation of two different hantaviruses in Anajatuba, one considerably more virulent than the other.

This is the first study to implicate a member of the genus *Holochilus* as a reservoir for a hantavirus. Additional epidemiologic, clinical, and molecular studies will be necessary to clearly establish which rodent host species are responsible for HPS in Anajatuba and for the unusually high prevalence of hantavirus antibody.

The co-circulation of two hantavirus genotypes in the rodent host assemblage of an HPS-endemic area is not unprecedented. In Panama, Vincent et al. (2000) documented the simultaneous circulation of Choclo virus (HPS associated) and Calabazo virus (not associated with HPS) and an increased prevalence of antibody to hantaviruses in localities where HPS cases were diagnosed. These authors suggested that Calabazo virus could play a role in the increased antibody prevalence observed among inhabitants. Similar patterns were observed in Argentina and Paraguay (Padula et al. 2000).

ACKNOWLEDGMENTS

We thank Dr. Jorge Salazar of the University of New Mexico, Albuquerque, NM, for identification of the rodents captured at Anajatuba. We are grateful to Dr. Nelson Aragão, Chief of Department of Health in Anajatuba municipality and Drs. Henrique Santos, Maria de Lourdes Raposo, and Luiz Fernando Ferreira of the Maranhão State Health Department, São Luiz, Maranhão state, for their logistical support during the studies at Anajatuba. Special thanks to Dr. Jarbas Barbosa da Silva, Jr., Director of the CENEPI/FUNASA for financial support and facilities during the development of the studies. We thank Dr. Kent Wagoner for graphics, Patty Yu for technical assistance, Emily Jentes for statistical analyses, and Serena Reeder and Darin Carroll for review and valuable comments on the manuscript. Partial data (serology) were presented at the Fifth International Conference on HFRS, HPS, and hantaviruses,

June 13–16, 2001, sponsored by Mérieux Foundation, Annecy, France. This study had financial support of Project VIGISUS, CENEPI/Instituto Evandro Chagas–FUNASA, CNPq (grant 302770/02-0)-Brazil, INEI "Dr. Carlos G. Malbrán"–Argentina (grant OPS/OMS 127/PG/02-03/999).

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