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THE COMPLEX ECOLOGY OF HANTAVIRUS IN PARAGUAY

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Abstract. Following an outbreak of hantavirus pulmonary syndrome (HPS) in the Paraguayan Chaco in 1995, *Calomys laucha* was identified as the rodent host for the hantavirus associated with these cases. To explore the possibility of additional hantaviruses in Paraguay, we collected 636 mammals from 10 of the 17 departments. Plasma from 27 animals in Alto Paraguay and Boquerón in the Chaco and Ñeembucú and Itapúa in the eastern region had antibody to Andes virus antigens. Of these 27, five individuals (among four species) were positive for hantavirus RNA. Sera were collected from indigenous people in eastern Paraguay to ascertain whether persons were being infected with hantavirus outside of the Chaco. Seventeen percent were antibody-positive. These results suggest that several different hantaviruses are co-circulating in Paraguay, and that HPS cases occurring in eastern Paraguay may result from exposure to hantaviruses that are distinct from those in the Chaco.

INTRODUCTION

Hantaviruses cause two serious and often fatal human diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS).[1] Natural reservoirs of hantaviruses are wild rodents within the Order Rodentia, Family Muridae. Hantaviruses are carried by the three rodent subfamilies: Murinae, Arvicolinae, and Sigmodontinae, which form three phylogenetic clades. Transmission to humans occurs through inhalation of aerosolized animal excreta.[2,3] Each hantavirus is predominantly associated with a specific rodent host that is indigenous to a geographic area;[4] however, occasional spillover infection in related rodent species has been reported.[5] The geographic and ecologic restrictions of the rodents separate hantaviruses into two phylogenetically distinct groups, one in Eurasia (Old World) and the other in the Americas (New World). The two groups are further distinguished by the illnesses associated with their infection in humans. Hantavirus pulmonary syndrome caused by the New World hantaviruses is characterized by acute cardiopulmonary syndrome, while HFRS caused by the Old World viruses is characterized by fever, renal failure, and in severe cases, hemorrhagic manifestation. There are also hantaviruses in the Old World and New World that are not known to cause disease in humans.

Following the HPS outbreak in the southwestern United States in 1993,[6] clusters of HPS cases have been reported in western Paraguay or the Chaco,[7] Argentina,[8] Chile,[9] Bolivia,[10] Brazil,[11] and Panama.[12] Field studies have identified hantaviruses in rodents in each of these regions. The vast majority of North and South American indigenous hantaviruses are associated with wild rodents of the Subfamily Sigmodontinae. Several distinct hantavirus genotypes have been described in South America, such as Rio Mamore virus (RMV) from *Oligoryzomys microtis* (Bolivia),[13] Andes virus (ANDV) from *O. longicaudatus* (Argentina),[8] Laguna Negra virus (LNV) from *Calomys laucha* (Paraguay),[14] and Cano Delgatito virus (CDV) from *Sigmodon alstoni* (Venezuela).[15] Additional strains of ANDV have been identified from *Akodon azarae, Necromys benegactus, Oligoryzomys flavescens, O. chacoensis,* and *O. longicaudatus* in Argentina[16] and from *O. longicaudatus* in Chile.[17]

In 1995, one of the largest outbreaks of HPS occurred in the Chaco region of western Paraguay in two agricultural Mennonite communities.[7] Although the LNV of the Paraguayan Chaco was identified as the hantavirus associated with these HPS cases and its reservoir identified, there has been no additional information about the possibility of additional hantavirus reservoir(s) in other areas of Paraguay. The complex ecology of hantavirus in Argentina led us to propose the hypothesis that a very similar complexity of hantavirus existed in neighboring Paraguay, particularly since these countries share one of the largest ecosystems in the world, the Interior Atlantic Forest. The Atlantic Forest formerly covered large portions of southern Brazil, northeastern Argentina, and eastern Paraguay. As such, it was one of the largest ecosystems in the New World, and one of the most outstanding examples of the Earth's most diverse habitats. To explore the possibility of additional species or strains of hantavirus(es) in Paraguay, we analyzed a comprehensive collection of sigmodontine rodents and other small mammals from the major biomes of the entire country. Furthermore, since HPS cases have not yet been documented in the eastern region, we were interested in whether any of the rural populations with limited access to health care in the eastern region of Paraguay might be exposed to hantavirus. Therefore, we chose to examine a group of individuals with high risk for exposure to hantavirus, the Ache community of Caazapa (Ava'i district).

MATERIALS AND METHODS

Collecting of small mammals. The rodents used in the study were obtained from an inventory of small mammals performed in Paraguay from May 1996 to May 1997 and directed by one of the co-authors (RDO) and Michael R. Willig (Texas Tech University, Lubbock, TX). Specimens from the rodent Subfamily Sigmodontinae were selected for the study, along with one other Murid subfamily, three other rodent families and four other mammalian orders. The samples were from 17 sites in 10 departments, representing most habitats of the country. These sites (Appendix 1) encompassed six of the seven major biomes[18,19] and the two main ecologic regions of Paraguay, the Oriental and the Chaco, which are situated east and west of the Rio Paraguay, respectively.[20]

Rodents were captured using Sherman live traps (Sherman Trap Company, Tallahassee, FL), and processed in field camps as standard museum specimens. The following standard measurements were collected from each individual captured: weight, total length, and tail, ear, and hind foot lengths.

The reproductive condition of each individual was also recorded. Liver, lung, heart, kidney, and muscle tissues were removed, stored immediately in liquid nitrogen, and transported to the Museum of Texas Tech University, where the samples were stored at -80°C.

Immunofluorescent antibody (IFA) assay. During the collecting of small mammals, no blood samples were taken. Therefore, to screen rodents for the presence of antibody to hantavirus antigens, plasma was extracted from tissues that were placed into a 1.5-mL microfuge tube, weighed, and then soaked into 15 parts of phosphate-buffered saline (PBS) overnight at 4°C. Sixteen-fold diluted (w/v) plasma was centrifuged at 4,000 rpm in a cold room and the supernatant was used for the IFA assay as described previously.[21] To detect antibody, ANDV-infected Vero E6 cells were grown on a spotted glass slide and fixed with pure acetone, followed by irradiation with ^{60}Co for four hours in a container packed with dry ice. Forty microliters of diluted plasma from each rodent tissue was added to each well of the antigen slide and incubated for 30 minutes at 37°C in a moist chamber. Slides were washed twice with PBS for five minutes and rinsed with distilled water. Slides were air-dried inside of a biological safety cabinet, 25 μL of fluorescein isothiocyanate (FITC)–labeled anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was added, incubated for 30 minutes, and washed as described earlier. Slides were mounted with mounting media (90% glycerol in PBS buffer) and observed under a fluorescent microscope (Axioscope; Zeiss, Oberkochen, Germany).

Immunoblot assay. The 28 serum samples collected from the Ache were tested for the presence of antibodies to hantavirus using a commercially produced strip immunoblot assay (SIA; Chiron, Emeryville, CA).[22] The 1997 SIA test contained immobilized membrane-bound antigens from Sin Nombre (SNV), Puumala (PUUV), and Seoul (SEOV) viruses. The SIA antigens included bacterial-expressed and purified recombinant N (rN) proteins from SNV, SEOV, and PUUV, as well as synthetic peptides from SNV (G1 pep and N pep). To reduce nonspecific binding of antibodies, serum samples (1:50) were pre-incubated for one hour with 30 mL of a bacterial lysate in specimen diluent as described previously.[23] Subsequent analysis of the sera with the SIA was performed exactly as specified by Chiron. Strips were interpreted within one hour after development. Intensities of the bands were ranked from ± to 4+ as compared with the IgG internal control bands. Samples with reactivities of 1+ to both N pep and rN were considered antibody positive for SNV or SNV-like hantaviruses. Samples with no reactivities to N pep and 1+ reactivities to rN were considered indeterminate. Samples considered positive or indeterminate were examined further by Western blot analysis with the Rio Mamore (RMV) N protein[23] and the plaque reduction neutralization test (PRNT).

Plaque reduction neutralization test. To test for the presence of neutralizing antibody among the antibody-positive human serum specimens, a PRNT was performed as described previously.[21] Briefly, serum samples were diluted twofold dilution in Earle's minimum essential medium (EMEM) containing 10% fetal bovine serum and antibiotics (penicillin/streptomycin [10 μg/ml]). An aliquot of ANDV was added and left overnight at 4°C. The initial serum dilution was 1:10. The next morning, virus-serum mixtures were applied to confluent monolayers of Vero E6 cells (CRL 1586; American Type Culture Collection, Manassas, VA) grown in six-well tissue culture plates (ICN, Costa Mesa, CA), and adsorbed for 90 minutes at 37°C in a CO_2 incubator. Plates were overlaid with EMEM agarose medium containing 10% fetal bovine serum (FBS), sodium glutamate, and antibiotics (penicillin/streptomycin [10 μg/ml]) and then incubated for seven days at 37°C in a CO_2 incubator. To visualize virus plaques, plates were incubated for seven days and then stained with a second agarose overlay containing 5% FBS, 5% neutral red, glutamate, and antibiotics (penicillin/streptomycin [10 μg/ml]). Plaques were counted each day for three days.

Antigen test. Tissues of the collected small mammals were cryosectioned to 5 μm, mounted onto a glass slide, and then fixed with ice-cold pure acetone for 10 minutes. Fixed tissues were examined by IFA assay as described previously.[21] Briefly, 50 μL of diluted HPS patient sera (Chiron) was added to each tissue mounted slide glass and incubated for 30 minutes at 37°C in the moist chamber. Slides were washed twice with PBS for five minutes and rinsed with distilled water. Slides were air-dried inside a biologic safety cabinet, and 40 μL of diluted FITC-labeled anti-human IgG (Kirkegaard and Perry Laboratories, Inc.) was added. Slides were incubated and washed as before. Slides were mounted with mounting media and observed under a fluorescent microscope (Axioscope; Zeiss).

Extraction of RNA from rodent specimens and nested reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNA from antibody-positive and antigen-suspected rodent tissues were extracted and amplified by a nested RT-PCR as described previously.[21] Briefly, 0.1 grams of tissue was ground in a 1.5-mL microfuge tube containing 1 mL of Trizol (Invitrogen, Bethesda, MD) using a disposable tissue grinder (Fisher Scientific, Atlanta, GA) and following manufacture's protocol for extraction of total RNA. Extracted RNA was diluted with 10 μL of RNase-free distilled water, and subjected to RT-PCR with small (S)–segment generic outer primers using a one-step RT-PCR kit (Invitrogen, San Diego, CA). The S-segment generic primers were selected from a consensus region among seven different American hantaviruses with the following GenBank accession numbers: Andes, AF004660;[8] Bayou, L36929;[24] Laguna Negra, AF005727;[14] Mule Shoe, U54575;[25] Prairie vole, U19303 (Hjelle BL and others, unpublished data); SNV CC107, L33683;[26] and SNV NMR11, L37904.[27] The outer primers used were HTS F538, 5′-GAAGADGTCAAYGGBAT-3′ and HTS R1163, 5′-TGDATYCCCATWGAYTGWGT-3′, and they yielded a 662-basepair product. The inner primers used were HTS NF691, 5′-AGYCCWGTYATGGGDGT-VAT-3′ and HTS NR1061, 5′-GTRTTTCKCATRTCCT-GVAG-3′ and they yielded a 371-basepair product. Two microliters of amplicon was amplified by a nested PCR with S-segment inner primers using the PCR core kit (Roche, Penzberg, Germany). The RT-PCR program was one cycle of 45 minutes at 45°C, followed by 35 cycles at 30 seconds at 94°C, 30 seconds at 50°C, and 45 seconds at 72°C. The nested PCR program was 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 45 seconds at 72°C. Amplicons were analyzed by electrophoresis in 1.2% agarose gels in Tris-acetate-EDTA buffer.

TABLE 1

Summary of antibody to hantavirus and antigen (RNA) detected among rodents by each department in Paraguay

Location	No. examined	%	Antibody		Viral RNA	
			No. positive	%	No. positive	%
Alto Paraguay	103	16.2	4	3.9	1	1.0
Boquerón	75	11.8	1	1.3	0	0
Presidente Hayes	50	7.9	0	0	0	0
Caazapá	10	1.6	0	0	0	0
Canindeyú	47	7.4	0	0	0	0
Concepción	11	1.7	0	0	0	0
Cordillera	1	0.2	0	0	0	0
Itapúa	238	37.4	18	7.6	3	1.3
Ñeembucú	91	14.3	4	4.4	1	1.1
Paraguarí	10	1.6	0	0	0	0
Total	636	100	27	4.2	5	0.8

RESULTS

Serologic survey of the rodents for antibodies to hantavirus. A total of 636 small mammals were evaluated from 17 sites in 10 departments in Paraguay, including all three departments in western Paraguay and seven of the 14 departments in eastern Paraguay (Appendix 1). As shown in Table 1, 228 (35.8%) specimens were collected in three departments of the Chaco region in western Paraguay and 408 (64.2%) specimens from seven departments in eastern Paraguay.

Plasma was extracted from lung tissue from each rodent. We detected 27 (4.2%) specimens of 10 species of Sigmodontine rodents that were positive by the IFA assay for antibody to ANDV antigens among four departments in Paraguay. In the Chaco region, four (3.9%) of 103 specimens in Alto Paraguay were antibody positive, as was one (1.3%) of 75 specimens in Boquerón. In eastern Paraguay, 18 (7.6%) of 238 specimens in Itapúa and four (4.4%) of 91 specimens in Ñeembucú were antibody positive.

The 636 animals sampled represent five mammalian Orders: Didelphimorphia (15 individuals), Xenarthra (2), Chiroptera (3), Lagomorpha (2), and Rodentia (614) (Tables 2–4). No specimens belonging to Didelphimorphia, Xenarthra, Chiroptera, or Lagomorpha had antibody to hantavirus antigen (Table 2). The 614 specimens that belonged to the Rodentia represent four families: Caviidae (19), Ctenomyidae (11), Echimyidae (4), and Muridae (580). Among the 576 specimens that belonged to Family Muridae, two subfamilies are represented: Murinae (six individuals among two species) and Sigmodontinae (570 individuals among 28 species within 14 genera). None of the individuals from the Families Caviidae, Ctenomyidae, or Echimyidae or from the Subfamily Murinae were seropositive (Table 3). Twenty-seven of the 570 sigmodontine specimens were seropositive (Table 4). Among 28 Sigmodontine species sampled, we detected hantavirus antibody-positive specimens in 10 species. One (9.1%) of 11 *A. azarae* had an IFA assay antibody titer of 512; 11 (8.3%) of 133 *A. montensis* had titers from ranging from 32 to 2,048; one (20%) of five *Bibimys chacoensis* had a titer of 1,024; three (4.3%) of 70 *Graomys griseoflavus* had titers ranging from 32 to 64; three (6.7%) of 45 *H. chacarius* had titers from ranging from 32 to 256; two (14.3%) of 14 *Nectomys squamipes* had titers of 128; one (16.7%) of six *O. chacoensis* had a titer of 128, one (7.7%) of 13 *O. fornesi* had a titer of 4,096; two (5.9%) of 34 *O. nigripes* had titers of 128 and 256; and two (6.1%) of 33 *Oryzomys* species had titers of 512 and 2,048. Twenty animals had tissues that showed an indeterminate reaction.

Survey of hantavirus antigens from the tissues of the rodents captured in Paraguay. We could not detect hantavirus antigen in the lung tissues of specimens by the IFA assay, but viral RNA was detected in several samples. To survey the antibody-positive specimens for viral RNA, we used a nested RT-PCR using S-segment specific generic primer pairs as described in the Materials and Methods. Total RNA was extracted from lung tissue form each rodent, subjected to the RT-PCR, and the amplicon was screened by size by agarose gel electrophoresis. We detected five hantaviral RNA-positive rodents; three from Itapúa and one from Ñeembucú in eastern Paraguay, and one from Alto Paraguay in western Paraguay (Table 1). These RNA-positive animals included one (0.8%) of 133 *A. montensis*, one (2.2%) of 45 *H. chacarius,* two (5.9%) of 34 *O. nigripes*, and one (16.7%) of six *O. chacoensis* (Table 4). The RT-PCR products were sequenced to confirm authenticity.

Relationship of animal sex to hantavirus prevalence. Among 580 animals from the Family Muridae, 21 (6.9%) of 304 male specimens and six (2.2%) of 276 female specimens showed seropositivity (Table 5). Five (1.6%) of the 304 male specimens had hantavirus RNA in their lung tissues, but we could not detect Viral RNA in any female specimens.

Serologic analysis of human sera from eastern Paraguay. A participation rate of 19.4% (28 of 144) was obtained from the residents of an Ache community in 1997 as part of a Primary Health Care project sponsored by the Japanese International Cooperation Agency. The ages in the study ranged from 12 to 70 years with an average of 32.6 years. The sample analyzed came from 14 males and 14 females. In the SIA, five of the 28 samples showed strong reactivity against the SNV N pep and SNV rN, as well as the PUUV N protein antigen. Two of the

TABLE 2

Summary of Paraguayan small mammals outside the order Rodentia that were surveyed for antibody to hantavirus and viral RNA

Order	Species	No.	%	No. antibody positive (%)	No. RNA positive (%)
Didelphimorphia	*Marmosa pusilla*	4	0.6	0	0
	Monodelphis sorex	11	1.7	0	0
Xenarthra	*Tolypeutes matacus*	2	0.3	0	0
Chiroptera	*Molossus molossus*	1	0.2	0	0
	Platyrrhinus lineatus	1	0.2	0	0
	Sturnira lilium	1	0.2	0	0
Lagomorpha	*Sylvilagus brasiliensis*	2	0.3	0	0

TABLE 3

Summary of Paraguayan small mammals in the order Rodentia surveyed and found negative for antibody to hantavirus and viral RNA

Family/subfamily	Species	No.	%
Caviidae	*Dolichotis salinicola*	5	0.8
	Galea musteloides	14	2.2
Ctenomyidae	*Ctenomys pilarensis*	11	1.7
Echymyidae Echimyidae	*Euryzygomatomys*	1	0.2
	Proechimys	1	0.2
	Thrichomys apereoides	2	0.3
Muridae/Murinae	*Mus musculus*	5	0.8
	Rattus rattus	1	0.2
Muridae/Sigmodontinae	*Akodon nigrita*	38	6.0
	A. toba	31	4.9
	Andalgalomys pearsoni	12	1.9
	Bolomys lasiurus	27	4.2
	Calomys callosus	34	5.3
	C. laucha	5	0.8
	C. tener	7	1.1
	C. sp.	7	1.1
	Oecomys mamorae	3	0.5
	Oligoryzomys sp.	8	1.3
	O. fornesi	2	0.3
	Oryzomys angouya	1	0.2
	O. megacephalus	2	0.3
	O. nitidus	2	0.3
	O. subflavus	6	0.9
	Oxymycterus delator	3	0.5
	Pseudoryzomys simplex	5	0.8
	Scapteromys tumidus	13	2.0

samples showed an indeterminate pattern. The seropositive and indeterminate samples were analyzed further by Western blot using N antigen from a South American hantavirus (RMV). All five seropositive samples showed strong cross-reactivity with the RMV N. To further confirm the seropositive specimens and to clarify the antibody status of the indeterminate specimens, selected samples were used in a PRNT for ANDV. All five seropositive samples showed neutralizing antibody titers to ANDV (≥1:20) by the PRNT (Table 6). The two indeterminate samples had no neutralizing antibodies to ANDV, and were therefore considered negative for antibodies to hantavirus. The results of the SIA, Western blot, and PRNT were concordant. The three tests reflected a seroprevalence of 17.9% (5 of 28) in the Ache community. The youngest seropositive individual was 12 years of age (mean age = 41 years, range = 12–70) and the 12-year-old was the only seropositive female.

DISCUSSION

Since the discovery of SNV in the southwestern United States in 1993, numerous indigenous hantaviruses have been identified in the Americas.[1] All of these newly discovered hantaviruses have been associated with the *Sigmodontinae*. Sigmodontine rodents exhibit an impressively high genetic diversity[28] that mirrors the genetic diversity of their hantaviruses. There are four major sigmodontine tribes in South America: Phyllotini, Akodontini, Sigmodontini, and Oryzomyini. From the Phyllotine rodents, Laguna Negra virus was isolated from *C. laucha*.[14] From the Akodontine rodents, Maciel and Pergamino virus were isolated from *N. benefactus* and *A. azarae* in Argentina.[16] From the Oryzomyine rodents, Andes-related viruses were isolated from *O. chacoensis, O. flavescens,* and *O. longicaudatus* in Argentina and Chile,[17] and Rio Mamore virus was isolated from *O. microtis* in Bolivia.[13] From Sigmodontine rodents, Cano Delgadito virus was isolated from *S. alstoni* in Venezuela.[15]

In our survey, we identified 27 antibody-positive rodents from 10 different species with antibody titers ranging from 32 to 4,096: *A. azarae, A. montensis, B. chacoensis, G. griseoflavus, H. chacarius, N. squamipes, O. chacoensis, O. fornesi, O. nigripes,* and *Oryzomys* sp. Of these animals, four species had at least one individual positive for viral RNA, one belonging to the tribe Akodontini (*A. montensis*), and three to the Oryzomyini (*O, nigripes, O. chacoensis,* and *H. chacarius*). Among those species that were positive for viral RNA, *O. chacoensis* had been identified as the host of Bermejo or Andes Nort hantavirus,[16] but *O. nigripes, A. montensis,* and *H. chacoensis* were not known to carry hantavirus.

The distribution of *A. montensis* extends from southern Brazil into Paraguay, Uruguay, and northeastern Argentina. This is a common species in forests and forest-grassland ecotones. In Paraguay, we encountered it at nearly all of our sites east of the Rio Paraguay. The distribution of *O. nigripes* includes eastern Paraguay and northern Argentina. In Paraguay, this species primarily inhabits forests and areas of second growth, and can also live at high densities in freshly cleared fields. Similar to *Akodon montensis*, this species was encountered at most sites in eastern Paraguay. *Oligoryzomys chacoensis* is found in drier habitats in westcentral Brazil, southeastern Bolivia, western Paraguay, and northern Argentina. This rodent species is common in thorn scrub and dry grassland, and in Paraguay we encountered it in many western (Chaco) sites, as well as in three Chaco-like sites just east

TABLE 4

Summary of Paraguayan small mammals in the order Rodentia surveyed and found positive for antibody to hantavirus and/or viral RNA

Family/subfamily	Species	No. examined	Percent of total screened	Antibody positive (%)	RNA positive (%)
Muridae/Sigmodontinae	*Akodon azarae*	11	1.7	1 (9.1)	0
	A. montensis	133	20.9	11 (8.3)	1 (0.8)
	Bibimys chacoensis	5	0.8	1 (20.0)	0
	Graomys griseoflavus	70	11.0	3 (4.3)	0
	Holochilus chacarius	45	7.1	3 (6.7)	1 (2.2)
	Nectomys squamipes	14	2.2	2 (14.3)	0
	Oligoryzomys chacoensis	6	0.9	1 (16.7)	1 (16.7)
	O. fornesi	13	2.0	1 (7.7)	0
	O. nigripes	34	5.3	2 (5.9)	2 (5.9)
	O. sp.	33	5.2	2 (6.1)	0
	Total	364	57.1	27 (4.2)	5 (0.8)

TABLE 5
Summary of antibody to hantavirus and antigen (RNA) detected among family Muridae rodent species by sex

Genus	Species	Total no. antibody positive/total no. tested (%)			Total no. RNA positive/total no. tested (%)		
		Male	Female	Total	Male	Female	Total
Akodon	*azarae*	1/3 (33.3%)	0/8	1/11 (9.1%)	0/3	0/8	0/11
	montensis	9/74 (12.2%)	2/59 (3.4%)	11/133 (8.3%)	1/74 (1.4%)	0/59	1/133 (0.8%)
Bibimys	*chacoensis*	1/5 (20%)	0/0	1/5 (20%)	0/5	0/0	0/5
Graomys	*griseoflavus*	1/33 (3.0%)	2/37 (5.4%)	3/70 (4.3%)	0/33	0/37	0/70
Holochilus	*chacarius*	3/20 (15.0%)	0/25	3/45 (6.7%)	1/20 (5.0%)	0/25	1/45 (2.2%)
Nectomys	*squamipes*	1/9 (11.1%)	1/5 (20%)	2/14 (14.3%)	0/9	0/5	0/14
Oligoryzomys	*chacoensis*	1/5 (20%)	0/1	1/6 (16.7%)	1/5 (20%)	0/1	1/6 (16.7%)
	fornesi	1/8 (12.5%)	0/5	1/13 (7.7%)	0/8	0/5	0/13
	nigripes	2/13 (15.4%)	0/21	2/34 (5.9%)	2/13 (15.4%)	0/21	2/34 (5.9%)
Oryzomys	unknown	1/15 (6.7%)	1/18 (5.6%)	2/33 (6.1%)	0/15	0/18	0/33
Total		21/304 (6.9%)	6/276 (2.2%)	27/580 (4.7%)	5/304 (1.6%)	0/276	5/580 (0.9%)

of the Rio Paraguay. These rodents can become agricultural pests in rice fields and storage bins. *Holochilus chacarius* is found in Paraguay and northeastern Argentina, generally in wet, seasonally wet, or semiaquatic habitats. In Paraguay, we encountered it primarily in wet or transitional Chaco sites, but also occasionally farther east, in areas along watercourses. This species can cause extensive damage to rice, banana, sugarcane, and other crops.

No clinical HPS cases have been reported in the eastern region of Paraguay, and our report is the first that documents infection with hantavirus in this region. We surveyed 28 Ache (indigenous) persons living in Canindeyú. These people live in housing constructed of bamboo, leaves, and wood, and are periodically nomadic hunters and gatherers in a large wooded region. Five seropositive persons had neutralizing antibody in their sera, which suggests that they were infected at an earlier time. Neutralizing antibody appears in the convalescent phase after the acute phase of infection. Until about 35 years ago, the Ache were entirely nomadic,[29] and although they now have permanent housing in settlements, they still engage in periodic hunting trips of 1–2 weeks within the Mbaracayú Biosphere Reserve, a large forested area within their original range in the department of Canindeyú. This would suggest that the seropositive individuals we identified reflect hantavirus infections that have occurred within this region of Canindeyú. In the Chaco, *C. laucha* is the primary reservoir of LNV, but only recently has been found to occur in eastern Paraguay, including Canindeyú. It remains to be determined whether the Ache have been exposed to LNV, or to one of the hantaviruses associated with Oryzomyine rodents.[14]

The South American continent presents a unique and challenging opportunity to study the relationships of hantaviruses with their natural rodent hosts. More extensive serologic studies of Paraguayan hantaviruses and comparative sequence analysis with Paraguayan HPS patients should help clarify the distribution of these viruses and their role (if any) as an etiologic agent for human hantavirus disease.

TABLE 6
Antibody titers to hantavirus in indigenous people in eastern Paraguay by SIA and PRNT*

No.	SIA						PRNT
	SNV G1 pep	SNV N pep	SNV N rec	PUUV N rec	SEOV N rec	RMV N rec	ANDV
A2	±	2+	3+	1+	±	2+	1:20
A4	±	4+	4+	3+	±	4+	1:16
A8	±	3+	4+	3+	±	4+	1:64
A9	±	±	1+	±	±	±	—
A10	±	±	1+	±	±	±	—
A26	±	3+	4+	2+	±	4+	1:32
A34	±	3+	4+	3+	±	4+	1:40

* SIA = strip immunoblot assay; PRNT = plaque reduction neutralization test; SNV = Sin Nombre virus; PUUV = Puumala virus; SEOV = Seoul virus; RMV = Rio Mamore virus; ANDV = Andes virus; pep = peptide; rec = recombinant.

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Appendix 1

Sampling localities from which specimens were used in this project*

04	Estancia Sombrero. Depto. Cordillera. 25°03′S, 56°40′W. October 10–20, 1995; February 13–17, 1997.
13	Parque Nacional Serranía San Luis. Depto. Concepción. 22°40′S, 57°21′W. April 8–19, 1996; December 6–13, 1996.
14	Estancia Yacaré. Depto. Ñeembucú. 26°38′S, 58°08′W. May 9–20, 1996; January 6–13, 1997.
15	Reserva Natural del Bosque Mbaracayú (now Mbaracayú Biosphere Reserve). Depto. Canindeyú. 24°08′S, 55°32′W. May 29–June 9, 1996; November 23–30, 1996.
16	Estancia Loma Porá. Depto. Pte. Hayes. 23°30′S, 57°33′W. June 19–30, 1996; January 21–25, 1997.
17	Laguna Placenta. Depto. Alto Paraguay. 21°17′S, 59°33′W. July 8–18, 1996; April 18–25, 1997.
18	Estancia Samaklay. Depto. Pte. Hayes. 23°29′S, 59°48′W. July 27–August 5, 1996; February 25–March 4, 1997.
19	Pedro P. Peña. Depto. Boquerón. 22°27′S, 62°21′W. August 16–25, 1996.
20	Parque Cué. Deptos. Alto Paraguay and Boquerón. 20°05′S, 61°47′W. September 2–9, 1996; May 21–28, 1997.
21	Itabó. Depto. Canindeyú. 24°27′S, 54°40′W. September 24–October 3, 1996; February 1–5, 1997.
22	Estancia Golondrina. Depto. Caazapá. 24°34′S, 55°29′W. October 11–13; November 1–5, 1996.
23	Parque Nacional Ybycuí. Depto. Paraguarí. 26°05′S, 56°51′W. November 12–16, 1996.
24	Parque Nacional Teniente Agripino Enciso. Depto. Boquerón. 21°03′S, 61°45′W. March 16–25, 1997.
25	Palmar de las Islas. Depto. Alto Paraguay. 19°38′S, 60°37′W. May 4– 11, 1997.
26	Ape Aimé. Depto. Itapúa. 26°32′S, 54°50′W. July 4–13, 1998.
27	Estancia San José. Depto. Ñeembucú. 27°10′S, 58°24′W. July 23–August 1, 1998.
28	Estancia Parabel. Depto. Itapua. 26°10.85′S, 55°30.95′W. August 22–September 2, 1998.

*Locality number and name are given, followed by the department, coordinates, and dates of sampling. Locality numbers correspond to those listed in Willig and others,[19] in which localities are also mapped.