Am. J. Trop. Med. Hyg., 75(6), 2006, pp. 1127–1134

PHYLOGENETIC AND GEOGRAPHICAL RELATIONSHIPS OF HANTAVIRUS STRAINS IN EASTERN AND WESTERN PARAGUAY

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Abstract. Recently, we reported the discovery of several potential rodent reservoirs of hantaviruses in western (*Holochilus chacarius*) and eastern Paraguay (*Akodon montensis, Oligoryzomys chacoensis,* and *O. nigripes*). Comparisons of the hantavirus S- and M-segments amplified from these four rodents revealed significant differences from each another and from other South American hantaviruses. The ALP strain from the semiarid Chaco ecoregion clustered with Leguna Negra and Rio Mamore (LN/RM), whereas the BMJ-ÑEB strain from the more humid lower Chaco ecoregion formed a clade with Oran and Bermejo. The other two strains, AAI and IP37/38, were distinct from known hantaviruses. With respect to the S-segment sequence, AAI from eastern Paraguay formed a clade with ALP/LN/RM, but its M-segment clustered with Pergamino and Maciel, suggesting a possible reassortment. AAI was found in areas experiencing rapid land cover fragmentation and change within the Interior Atlantic Forest. IP37/38 did not show any strong association with any of the known hantavirus strains.

INTRODUCTION

Hantaviruses are enzootic viruses that can maintain persistent infections in their natural hosts without apparent disease symptoms in these rodents.[1] More than 20 different hantavirus species are recognized, and each is predominantly associated with one rodent species or a few very closely related species.[2,3] In nature, transmission of hantavirus between rodents within a species is thought to be primarily through aggressive behavior and exposure to saliva and excreta.[4–6] Laboratory studies of virus transmission among rodents of the same species show that an infected rodent can transmit virus horizontally to another rodent within the same cage or through infected bedding.[7–11] However, to our knowledge it has not been shown that hantavirus can be transmitted between different rodent species in the laboratory, which suggests that the coevolution of each virus with its rodent host may be the reason that observations of reassortments in nature are rare[12,13] and are difficult to obtain in the laboratory.[14,15] However, it also plausible that the geographic and habitat restrictions of these rodents may simply limit overlap and interactions among hantavirus strains and their rodent reservoirs.[16] Very little is known about potential routes of transmission of hantavirus from rodent to human outside of inhalation of aerosolized virus from rodent excreta, which is thought to be the main route.[17,18] In Argentina, person to person transmission has been reported.[19] Give the distribution of hantaviruses and that human cases are most often linked to outbreaks, it would seem that additional factors, perhaps environmental, contribute to effective transmission. When transmitted to humans, several members of the genus cause deadly illnesses such as hemorrhagic fever with renal syndrome (HFRS)[20] and hantavirus pulmonary syndrome (HPS).[21] Old World hantaviruses cause HFRS in Asia and Europe, whereas New World hantaviruses cause HPS in the Americas. The pattern of disease follows the geographical distribution of the habitat preference of the rodent.

Since the initial discovery in 1993 of Sin Nombre (SN) virus, an etiologic agent of HPS in the United States,[22] several new hantaviruses and hantavirus sequences have been identified from Sigmodontinae throughout the Americas.[22,23] In contrast with North America, studies of hantaviruses in Latin America have revealed an increasingly complex picture of their ecology. Strikingly, numerous rodent species have been identified to harbor unique strains of *Hantavirus* in Argentina,[24,25] Bolivia,[26] Brazil,[27] Chile,[28] Costa Rica,[29] Panama,[30] Peru,[31] and Venezuela.[32] In Paraguay, *Calomys laucha* was discovered as the rodent reservoir for Laguna Negra (LN) virus, which was responsible for the outbreak of HPS in the western region of Paraguay or Chaco.[33] We have recently reported additional rodent species with serological evidence of a hantavirus infection in Paraguay[34] in *Akodon azarae, A. montensis, Bibimys chacoensis, Graomys griseoflavus, Holochilus chacarius, Nectomys squamipes, Oligoryzomys chacoensis, O. fornesi, O. nigripes*, and an unidentified *Oryzomys* species. The discovery of hantaviruses in rodents in three areas of eastern Paraguay was surprising, because cases of HPS have only been reported in western Paraguay. However, HPS cases have been confirmed in eastern Paraguay in 2005 and 2006, suggesting the rodents in this area can transmit a HPS to humans.

To map the phylogenetic and geographical relationships of these hantaviruses and their rodent hosts in Paraguay, we amplified hantaviral cDNA from the lung tissues of four species, including *H. chacarius* collected in the Chaco region in western Paraguay and *A. montensis, O. chacoensis,* and *O. nigripes* collected in eastern Paraguay. Hantaviruses contain three negative-sense single-stranded RNA genome segments L, M, and S, which encode the RNA-dependent RNA polymerase, the G1 and G2 envelope glycoproteins, and the nucleocapsid protein, respectively. Phylogenetic relationships were reconstructed from S- and M-segment sequences. Herein, we present the deduced amino acid homologies and compare them with those from all available North and South American hantavirus sequences. Furthermore, we report phylogenetic relationships of the sequences that were amplified and cloned from the S- and M-segments within the context of the genetic diversity of the known Paraguayan and South American hantaviruses. To assist with the phylogenetic analy-

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sis, cDNA was made from the M-segment of Rio Mamore (RM) virus from Bolivia[26] and Cano Delgadito (CD) virus,[32] which have not been previously reported. The phylogenetic and geographical relationships of these Paraguayan hantavirus sequences shed light into their evolution in Paraguay and suggest that host switching and reassortment along with Paraguay's geographic features may have played a role in their evolution and distribution.

MATERIALS AND METHODS

Rodent samples, RNA isolation, and nested reverse transcriptase-polymerase chain reaction. As reported previously,[34] rodent samples were obtained from an inventory of small mammals of Paraguay. Five RNA-positive rodent lung tissues were provided by the Museum of Texas Tech University (TTU). We isolated RNA from *H. chacarius* (TK62276), which harbored the Alto Paraguay strain (ALP) in western Paraguay. In eastern Paraguay in the department of Ñeembucu, we isolated RNA from *O. chacoensis* (TK64399), which harbored the Bermejo-Ñeembucu strain (BMJ-ÑEB), and in the department of Itapúa, we isolated RNA from *A. montensis* (TK65816), which harbored the Ape Aime-Itapúa strain (AAI) and from two *O. nigripes* (TK65937, TK65938), which harbored the Itapúa strains (IP37 strain; IP38 strain). TK numbers are the catalog numbers for the individual rodents in the TTU frozen tissue collections. Hereafter, we will refer to each of these strains identified by their location rather than by their strain identification.

Partial M- and S-genome segments were amplified by nested reverse transcriptase-polymerase chain reaction (RT-PCR).[35] We amplified complete open reading frame of the S-segment of ALP, ÑEB, IP37, IP38, and 1038 nt from AAI. The regions cloned for each strain were 1944 nt of ALP (TK62276, aligned to LN from 28 to 1881), 1887 nt of BMJ-ÑEB (TK64399, aligned to LN from 28 to 1881), 1038 nt of AAI (TK65816 aligned to LN from 28 to 1065), 1853 nt of IP37 (TK65937, aligned to LN from 28 to 1881), and 1853 nt of IP38 (TK65938, aligned to LN from 28 to 1881). For the M-segment, identical regions were cloned for each sample, 439 nt from the G1 region (1236-1674 nt based on LN virus), and 575 nt from the G2 region of the M genome segment (2234-2808 nt based on LN virus). These regions were chosen because they are the most commonly reported sequences in GenBank and thus this region allows for the maximal number of comparisons. Briefly, 0.1 g of lung tissue was ground in a 1.5-mL microfuge tube containing 1 mL of Trizol (Invitrogen, Bethesda, MD) with a disposable tissue grinder (Fisher, Atlanta, GA). Total RNA was extracted from Vero E6 cells, infected with CD or RM virus in a T25 flask, with 2 mL of Trizol. The extracted RNA pellet was diluted with 10 μL of RNase-free distilled water and subjected to RT-PCR with S- and M-segment outer generic primers (available on request) using the One-Step RT-PCR kit (Invitrogen, San Diego, CA). Degenerate S-segment outer generic primers were selected from a consensus region chosen from an alignment of seven different American hantaviruses as reported previously.[34] Degenerate M-segment outer generic primers to the G1 and G2 regions of the M segment were selected from the conserved region identified by an alignment of Andes (AND) virus, Bayou (BAY) virus, Blue River (BR) virus, LN virus, Lechiguanas (LEC) virus, Oran (ORN) virus, and Sin Nombre (SN) virus (strain CC107) using Vector NTI software package, version 7.0 (Informax, Bethesda, MD). Two microliters of each amplicon from the first RT-PCR was further amplified by PCR, using the PCR Core Kit (Roche, Indianapolis, IN), with the generic inner primers that were specific for the S, G1, and G2 regions of the M-segment. The initial and second rounds of amplification were performed for one cycle of 45 minutes at 45°C, followed by 35 cycles at 94°C for 30 seconds; at 50°C for 30 seconds; and at 72°C for 45 seconds. PCR was conducted for 35 cycles at 94°C for 30 seconds and at 72°C for 30 seconds. Amplicons were separated in 1.2% agarose gels in Tris-Acetate-EDTA (TAE) buffer and visualized by ethidium bromide staining.

Cloning, nucleotide sequencing, and genetic analysis. The cDNA from each sample was extracted from an agarose gel, purified (Bio101, La Jolla, CA), and ligated into pGEM-T (Promega, Madison, WI). T7 and SP6 primers were used to sequence three clones from each amplicon in both directions using the BigDye 3.0 terminator sequencing system (ABI, Foster City, CA) as described by the manufacturer. Nucleotide and deduced amino acids sequences of the S- and M-segment were obtained for all available American hantaviruses from GenBank (Table 1). Sequence alignments and comparisons were performed with the AlignX programs from the Vector NTI software package, version 7.0. Phylogenetic analysis was performed using the maximum likelihood program from PAUP software version 4.0 b10 (Sinauer Associates, Sunderland, MA). These analyses excluded the primer regions for PCR amplification. Phylogenetic trees were obtained using the heuristic search method with transversions weighted four times transitions. Gaps were treated as missing data or as a fifth character. Bootstrap confidence limits were obtained by 1,000 heuristic search repetitions.

RESULTS

Sequence similarity of the G1 and G2 regions of the M-segment. Nucleotide sequence and amino acid similarities of the G1 (Table 2) and G2 (Table 3) regions for the five Paraguayan hantavirus sequences from the four different Paraguayan rodent species (Alto Paraguay [ALP], Bermejo-Ñeembucú [BMJ-ÑEB], Ape Aime-Itapúa [AAI], Itapúa37 [IP37], and Itapúa38 [IP38]) were compared with all North and South American hantavirus sequences available from GenBank (Table 1). IP37 and IP38 sequences were amplified from RNA extracted from lung tissues of *O. nigripes*, whereas each of the others came from unique rodent species (*H. chacarius*: ALP, *O. chacoensis*: ÑEB, *A. montensis*: AAI strain). LN virus showed 74–81% nucleotide (nt) and 88–93% amino acid (aa) sequence similarity to the five sequences from the G1 segment (Table 2). The nucleotide sequence and amino acid sequence similarity between IP37 and IP38 viruses were 98% and 99%, respectively. This is not surprising given that both were obtained from *O. nigripes* collected in the same locale in Itapúa. The other hantavirus sequence identified in Itapúa, AAI from *A. montenis*, was distinct from IP37 and IP38 (77% nt; 89–90% aa). Nucleotide and deduced amino acid sequence similarities between hantaviruses from Paraguay and other South American hantaviruses showed a wide range in nucleotide (71–88%) and amino acid (82–99%) simi-

TABLE 1
North and South American hantavirus sequences analyzed in this study

Virus	Abbreviation	Source of virus	Origin	GenBank accession number	References
Sin Nombre CC107	SN	*Peromyscus maniculatis*	S.W. United States	L33474 [M], L33683 [S]	45
New York 1	NY	*Peromyscus leucopus*	E. United States	U36802 [M], U29210 [S]	46, 47
Blue River	BR	*Peromyscus leucopus*	Cen. United States	AF030551 [M]	
Black Creek Canal	BCC	*Sigmodon hispidus*	S.E. United States	L39950 [M], L39949 [S]	49
Bayou	BAY	*Human*	S.E. United States	L36930 [M], L36929 [S]	50
Mule Shoe	MUL	*Sigmodon spp*	S. United States	U54575 [S]	51
El Moro Canyon	EMC	*Reithrodontomys megalotis*	S.W. United States	U26828 [M], U11427 [S]	53
Rio Segundo	RS	*Reithrodontomys mexicanus*	Costa Rica	U18100 [S]	
Laguna Negra	LN	*Calomys laucha*	Paraguay	AF005728 [M], AF005727 [S]	33
Rio Mamore	RM	*Oligoryzomys microtis*	Bolivia	AY953443 [M], AY953445 [M], U52136 [S]	26
Cano Delgadito	CD	*Sigmodon alstoni*	Venezuela	AY953442 [M], AY953444 [M], AF000140 [S]	32
Maciel	MAC	*Necromys benefactus*	Argentina	AF028027 [M], AF482716 [S]	24, 36
Pergamino	PRG	*Akodon azarae*	Argentina	AF028028 [M], AF482717 [S]	24, 36
Bermejo	BMJ	*Oligoryzomys chacoensis*	Argentina	AF028025 [M], AF482713 [S]	24, 36
Lechiguanas	LEC	*Oligoryzomys flavescens*	Argentina	AF028022 [M], AF482714 [S]	24, 36
Andes Chile-9717869	CHL	*Oligoryzomys longicaudatus*	Chile	AF291703 [M], AF291702 [S]	54
Andes AH-1	AH1	*Human*	Argentina	AF324901 [M], AF324902 [S]	55
Oran ("Andes Norte")	ORN	*Oligoryzomys longicaudatus*	Argentina	AF028023 [M], AF482715 [S]	24, 36
Andes	AND	*Human*	Argentina	AF004660 [S]	56
Castelo dos Sonho	CAS	*Human*	Brazil	AF307326 [M]	57
Araraquara	ARA	*Human*	Brazil	AF307327 [M]	57
Alto Paraguay (TK62276)	ALP	*Holochilus chacoensis*	W. Paraguay	AY515597 [M], AY515602 [M], [S]	
Bermejo-Neembucu (TK64399)	BMJ-ÑEB	*Oligoryzomys chacoensis*	E. Paraguay	AY515598 [M], AY515603 [M], [S]	
Ape Aime Itapua (TK65816)	AAI	*Akodon montensis*	E. Paraguay	AY515599 [M], AY515604 [M], [S]	
Itapua 37 (TK65937)	IP37	*Oligoryzomys nigripes*	E. Paraguay	AY515600 [M], AY515605 [M], [S]	
Itapua 38 (TK65938)	IP38	*Oligoryzomys nigripes*	E. Paraguay	AY515601 [M], AY515606 [M], [S]	

larity (Table 2). In contrast, nucleotide and deduced amino acid sequence similarities of the hantavirus sequences from Paraguay with the North American hantaviruses were 72–75% (nt) and 81–88% (aa). For comparison, the nucleotide and deduced amino acid sequence similarities between North American hantaviruses were 72–79% and 84–97%, respectively.

Nucleotide sequence comparisons of the G2 region among the five hantavirus strains from Paraguay showed a slightly higher similarity than those of the G1 region (77–82% nt and 93–98% aa; Table 3). The nucleotide sequence similarity and amino acid sequence similarities between IP37 and IP38 were 99% and 100%, respectively, clearly supporting the idea that these sequences were from one virus circulating in the same rodent species, *O. nigripes*. The amino acid similarities of AAI with these two strains were 94%, which suggests this sequence is from a distinct strain of hantavirus. Nucleotide and deduced amino acid sequence similarities of hantaviruses from Paraguay to other South American hantaviruses were 75–91% (nt) and 84–99% (aa), respectively. Nucleotide and

TABLE 2
Sequence similarity of a G1 region within the M-segment among American hantaviruses

	Paraguayan						South American						North American					
	ALP	ÑEB	AAI	IP37	IP38	LN	RM	CHL	AH1	ORN	LEC	CD	BR	EMC	NY	SN	BAY	BCC
ALP	–	75	75	76	76	81	76	77	76	77	76	73	73	72	72	73	72	73
BMJ ÑEB	89	–	78	80	79	74	76	78	78	82	88	71	75	72	73	76	75	74
AAI	90	90	–	77	77	75	74	78	77	78	78	74	75	72	72	72	73	74
IP37	92	94	90	–	98	75	76	78	78	80	77	76	75	73	74	74	74	76
IP38	91	93	89	99	–	75	76	78	78	78	77	75	74	73	74	73	73	76
LN	93	88	90	89	88	–	78	73	74	76	75	74	75	73	77	75	72	75
RM	95	90	88	92	91	94	–	77	78	77	74	74	75	74	75	74	71	72
CHL	90	93	90	92	92	87	89	–	93	76	77	74	72	70	71	72	73	72
AH1	90	94	90	93	92	88	90	99	–	76	79	74	72	70	73	73	73	72
ORN	89	98	90	94	93	88	90	92	92	–	80	73	74	71	72	72	73	74
LEC	89	99	90	94	93	88	90	93	94	96	–	71	75	71	73	74	75	73
CD	86	82	83	85	84	84	86	82	83	82	82	–	74	73	72	73	75	72
BR	86	87	85	88	87	88	87	86	87	86	87	86	–	72	78	79	73	78
EMC	84	81	82	81	82	83	83	82	83	79	80	82	87	–	72	76	73	73
NY	86	85	87	86	85	86	86	86	86	84	85	84	93	88	–	77	74	76
SN	86	86	86	88	87	87	86	88	88	85	86	86	94	87	97	–	74	77
BAY	86	84	82	86	86	86	85	84	85	84	83	88	86	86	87	88	–	76
BCC	84	84	84	86	85	84	83	83	84	84	83	84	86	84	89	88	90	–

TABLE 3
Sequence similarity of a G2 region within the M-segment among American hantaviruses

	Paraguayan						South American										North American					
	ALP	ÑEB	AAI	IP37	IP38	LN	RM	CHL	AH1	ORN	LEC	MAC	PRG	BMJ	ARA	CAS	CD	BAY	BCC	BR	EMC	NY	SN
ALP	–	77	78	79	80	79	81	79	79	79	77	78	78	76	77	78	76	76	76	76	77	78	75
BMJ ÑEB	94	–	79	82	82	78	79	82	82	83	89	80	82	91	81	81	76	75	76	78	74	75	79
AAI	93	94	–	78	79	80	77	78	78	79	81	80	84	80	79	79	75	76	77	76	76	76	78
IP37	96	98	94	–	99	78	78	81	80	82	80	77	82	82	79	81	75	74	76	80	77	79	77
IP38	96	98	94	100	–	78	78	80	80	82	80	76	82	83	79	81	75	75	76	80	76	79	77
LN	94	94	92	93	93	–	81	80	79	79	78	78	82	78	77	81	75	76	77	78	75	75	78
RM	95	94	91	94	94	96	–	79	80	80	79	76	79	78	77	79	72	77	77	78	75	77	73
CHL	94	98	93	98	98	93	94	–	94	81	80	80	79	80	82	80	75	76	77	79	74	79	77
AH1	94	98	93	98	98	93	93	99	–	79	80	81	78	79	81	81	73	77	76	77	74	78	76
ORN	94	99	94	98	98	94	94	98	98	–	82	80	79	83	80	82	73	75	76	77	76	75	76
LEC	95	99	94	99	99	94	95	99	98	99	–	79	81	88	80	80	75	75	75	78	75	77	80
MAC	93	93	95	93	93	93	92	92	93	93	93	–	78	80	79	80	75	75	76	77	74	72	75
PRG	94	96	97	96	96	93	93	95	95	96	96	94	–	81	79	80	75	73	76	80	76	77	76
BMJ	92	99	93	98	98	93	94	98	97	98	99	92	95	–	80	80	75	74	77	78	75	75	77
ARA	96	95	94	95	95	91	91	95	94	95	96	93	95	95	–	81	74	76	76	77	75	75	75
CAS	96	98	94	97	97	94	94	97	97	98	98	93	96	97	95	–	76	75	78	80	76	77	78
CD	87	84	86	85	85	84	83	84	85	84	85	87	86	84	85	86	–	72	75	75	76	74	76
BAY	88	87	86	87	87	87	87	87	87	87	87	87	87	86	85	88	84	–	80	76	76	76	76
BCC	86	86	85	86	86	87	86	85	85	85	86	86	85	85	85	86	82	93	–	78	78	76	75
BR	90	92	90	91	91	89	89	91	91	92	92	89	93	91	89	92	86	90	88	–	77	81	83
EMC	87	87	87	87	87	87	86	86	87	87	87	87	88	86	88	86	84	87	86	87	–	73	74
NY	90	90	90	90	90	88	88	89	89	90	90	87	91	89	87	91	86	88	86	96	86	–	81
SN	92	91	89	91	91	89	89	90	91	91	91	89	92	90	88	92	87	91	89	98	88	97	–

deduced amino acid sequence similarities between hantaviruses from Paraguay and North America were 74–80% and 85–92%. Nucleotide and deduced amino acids sequence similarity among North American hantaviruses ranged from 73–83% to 85–98%, respectively.

Sequence similarity of S-segment. Nucleotide sequence similarity of the S-segment among the five sequences ranged from 76% to 82%, whereas the level of amino acid sequence similarity ranged from 86% to 95% (Table 4). As noted for G1 and G2, the nucleotide and amino acid sequence similarities between IP37 and IP38 were 99% and 100%, respectively. The nucleotide and deduced amino acid sequence similarities of the hantaviruses from Paraguay to other South American hantaviruses were 69–93% and 82–99%, respectively. Nucleotide and deduced amino acid sequence similarities of Paraguayan hantaviruses to the North American hantaviruses were 68–78% and 80–88%, respectively, whereas the similarities among North American hantaviruses were 63–81% and 81–93%.

Phylogenetic relationships of Paraguayan hantaviruses to other American hantaviruses. The phylogenetic relationships among the five newly identified Paraguayan hantavirus M sequences and the previously characterized North and South American hantaviruses were examined using a maximum

TABLE 4
Sequence similarity of the S-segment among American hantaviruses

	Paraguayan						South American									North American				
Virus	ALP	ÑEB	AAI	IP37	IP38	LN	RM	ORN	AH1	CHL	BMJ	LEC	MAC	PRG	CD	BAY	BCC	MUL	RS	SN
ALP	–	79	78	79	79	79	82	78	79	79	78	79	77	78	69	77	77	76	71	76
BMJ-ÑEB	88	–	76	82	82	81	80	87	83	83	93	90	82	82	69	77	78	77	69	76
AAI	89	86	–	77	78	77	78	76	77	77	75	77	77	76	75	77	76	75	73	75
IP37	89	95	87	–	99	79	79	84	82	82	83	83	80	81	72	78	77	76	71	76
IP38	89	95	87	100	–	79	80	84	83	82	83	83	80	82	73	78	77	76	71	77
LN	92	89	86	90	90	–	82	79	79	79	79	80	78	78	66	78	77	76	68	75
RM	96	90	89	90	90	93	–	81	80	81	80	80	78	79	68	77	77	77	68	76
ORN	89	98	86	96	97	90	91	–	84	84	87	87	82	83	70	78	76	76	68	76
AH1	89	96	87	95	95	90	91	97	–	94	84	84	81	82	72	77	76	77	70	77
CHL	89	96	87	95	95	90	91	96	100	–	84	84	81	82	69	77	76	76	69	76
BMJ	89	99	87	96	96	90	90	99	97	96	–	92	82	82	70	77	78	77	67	77
LEC	89	99	87	95	96	90	91	98	96	96	100	–	82	83	72	77	78	77	68	77
MAC	89	94	86	93	93	88	89	95	94	94	94	94	–	83	66	76	77	76	66	77
PRG	89	95	85	93	93	90	90	96	95	95	96	96	96	–	68	77	76	76	68	75
CD	83	83	82	83	84	82	83	83	84	84	84	84	82	82	–	75	73	74	74	74
BAY	88	88	86	87	88	87	88	89	88	88	89	88	88	88	81	–	81	80	68	76
BCC	87	86	84	85	86	86	87	86	86	87	86	86	86	86	80	92	–	81	67	75
MUL	86	84	82	84	84	85	86	85	86	86	85	85	86	86	80	93	90	–	63	76
RS	81	82	80	81	81	81	82	82	82	82	82	82	82	81	79	84	82	81	–	69
SN	84	86	85	86	86	85	84	86	86	86	87	86	85	85	82	86	83	82	83	–

likelihood model. The resulting cladogram (Figure 1) showed the BMJ-ÑEB strain from *O. chacoensis* formed a subclade with Lechiguanas virus (LEC) from *O. flavescens* and Bermejo virus (BMJ) from *O. chacoensis*, which were both discovered in Argentina. AAI from *A. montensis* also fell within the clade that encompassed BMJ-ÑEB and all of the reported Argentinean and Chilean hantaviruses, and Chile-9717869, and AH-1, LEC, and ORN. IP37 and IP38 strains from *O. nigripes* formed a separate clade within the South American hantaviruses. The ALP strain from *H. chacarius*, a rodent captured in the northern part of the Chaco, formed a clade with the LN and RM viruses, which were originally isolated from *C. laucha* in the Chaco and *O. microtis* in Bolivia. Together they form a third South American clade in this phylogenetic tree. The M-segment sequence of AAI formed a strong subclade with Pergamino (PRG).

The phylogenetic tree based on the S-segment (Figure 2) showed that the BMJ-ÑEB sequence made a strong subclade with the BMJ virus and LEC and was part of a larger clade with other viruses from Argentina: Andes and ORN viruses. ALP formed a clade with LN and RM as observed for the M-segment. Strikingly, AAI was also part of this clade, in contrast to the M-segment cladogram that showed a strong association with PRG. Finally, as shown in the M-segment cladogram, the S-segments of IP37 and 38 were not closely associated with other viruses. El Moro Canyon (ELMC) virus from *Reithrodontomys megalotis* (southwestern United States) and the Rio Segundo (RIOS) virus from *R. mexicanus* (Costa Rica) formed one clade. SN and NY viruses formed a clade, distinct from a group of BAY, BCC, and MUL viruses, and a group of EMC and RS viruses.

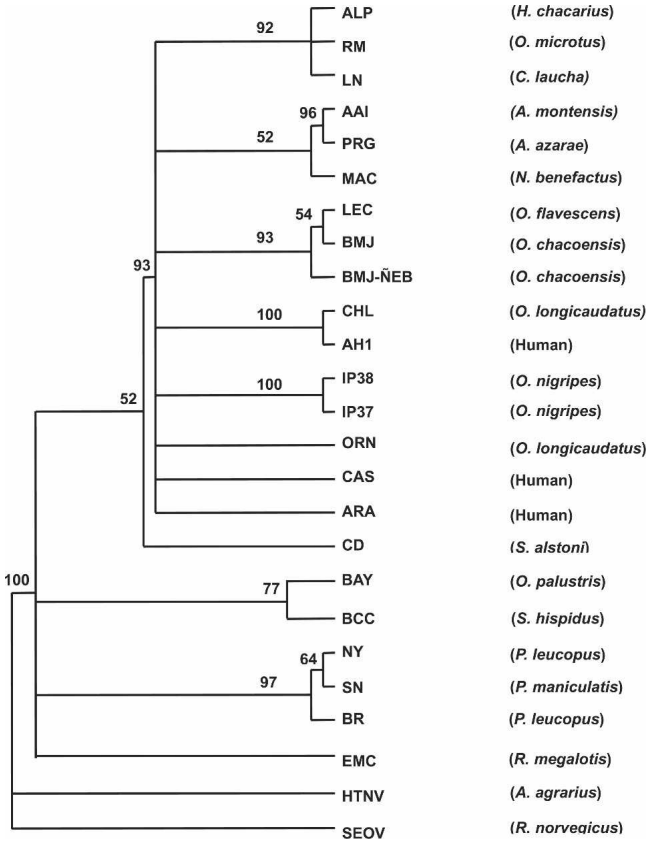

FIGURE 1. Phylogenetic tree based on maximum likelihood analysis of 438 and 575 nucleotides from G1 and G2, respectively. Nucleotide sequences were analyzed by maximum likelihood analysis of PAUP version 4 b10, using heuristic search option and weighting transversions four times transitions. Bootstrap values of > 50%, obtained from 1,000 replicates of the analysis, are shown at the appropriate branch points. See Table 1 for the definition of the abbreviations used to identify each sequence.

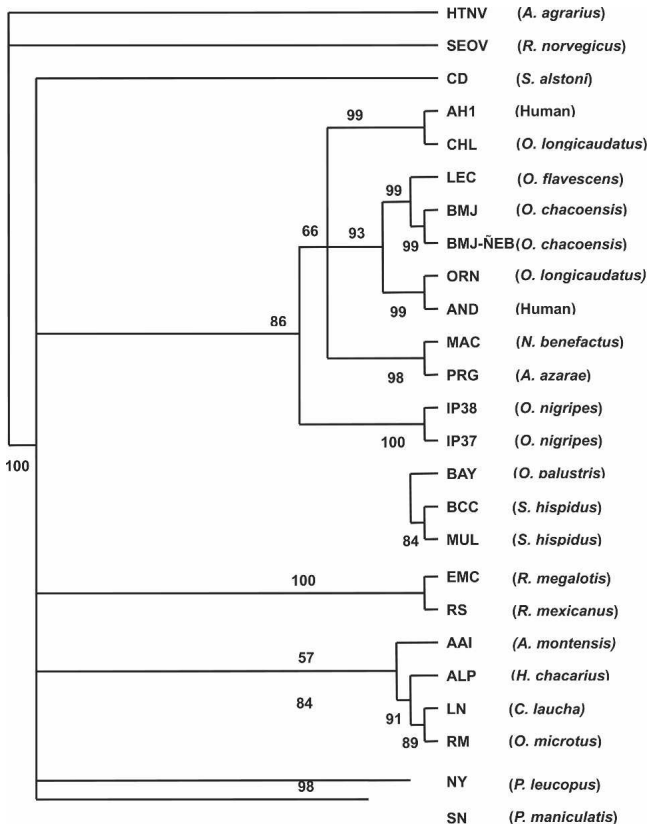

FIGURE 2. Phylogenetic tree based on maximum likelihood analysis of the partial S-segment. Nucleotide sequences were analyzed by maximum likelihood analysis of PAUP version 4 b10, using heuristic search option and weighting transversions four times transitions. Bootstrap values of > 50%, obtained from 1,000 replicates of the analysis, are shown at the appropriate branch points. See Table 1 for the definition of the abbreviations used to identify each sequence.

DISCUSSION

Considering the general paradigm of "one rodent host species—one hantavirus" in hantavirus evolution, the South American hantaviruses are phylogenetically intriguing. For example, numerous strains of hantaviruses have been have been isolated or identified from various *Oligoryzomys* species by a number of laboratories. In Argentina alone, there are at least six distinct hantavirus strains, Andes virus, Oran or Andes Norte virus, Lechiguanas virus, Bermejo virus, Maciel virus, and Pergamino virus, which form a single clade that also includes the hantaviruses from Chile.[24,36,37]

In nature, hantaviruses maintain persistent infections in one or a few closely related rodent species, which reside within a specific geographical region. The ecological constraints of the rodents' habitat have played a primary role in *Hantavirus* evolution.[2] Phylogenetic analysis of the relation-

ships among hantaviruses and their rodent hosts suggests that hantaviruses have a long-standing co-evolutionary history with their predominant rodent carriers.[38,39] Therefore, the existing relationships among hantaviruses are most likely derived from adaptation to the distinct genetic environment of their rodent hosts, which evolved from a complicated history of co-speciation events[40] and the geographic constraints of the landscape that have influenced rodent migration patterns and habitats.

The diversity of sigmodontine rodents in South America is one of the most impressive examples of broad adaptive radiation of an evolutionary lineage after invading a previously unoccupied region.[41] Moreover, this phylogenetic diversity is also reflected as ecological diversity, and a complex biogeographic history within continental South America.[42] It is therefore not surprising that South American hantaviruses seem to be highly diverse and biogeographically complex, because they follow their rodent hosts through speciation events and changing landscapes.

We show herein that the South American hantaviruses, including those we identified from Paraguay, share 87–92% and 92–99% amino acid sequence homology in the G1 and G2 region and that their phylograms were concordant as noted previously.[2,37] In contrast to the M-segment, amino acid sequence homologies of the S-segment among South American hantaviruses varied from 82% to 99%. The greater homology of the M-segment compared with the S-segment among South American hantaviruses may suggest that the South American Sigmodontinae rodents share similar receptors for viral entry. In the development of effective diagnostics for hantaviruses, these ranges should be considered. In our studies, we obtained a greater sensitivity in the detection of antibody in rodent blood when we use both antigens.[34]

The BMJ-ÑEB strain, which we identified from *O. chacoensis* in eastern Pargauay in Ñeembucu, seems to be a strain of the BMJ virus that was identified from the same rodent species in Argentina. Nucleotide sequence differences of the G2 region of the M- and complete ORF region of the S-segments of BMJ-ÑEB and BMJ was 9% and 7%, respectively, whereas their amino acid difference was 1% in both segments. BMJ was originally isolated from the northern part of Argentina in Oran, which borders with the southern part of Bolivia. The location of these two strains suggests that the habitat of *O. chacoensis* and this strain of hantavirus extends along the Argentine–Paraguayan border in the Gran Chaco and into parts of eastern Paraguay (Figure 3). The other three hantavirus strains, ALP, AAI, and IP37/IP38, were identified from rodent species that have not been detected previously; *H. chacarius*, *A. montensis*, and *O. nigripes.*[34] IP37/IP38 from *O. nigripes* did not form a clade with any of the known American hantaviruses. The ALP strain from *H. chacarius*, which was collected from the northern Paraguayan Chaco, formed a clade with RM virus (Bolivia) and LN virus (Paraguayan Chaco). The amino acid sequence differences in G1 and G2 regions of the M-segment of the ALP strain with RM and LN were 5% and 7%, respectively, and the amino acid difference of the S-segment of ALP strain to RM and LN was 5% and 6%, respectively, which would suggest they are all the same strain. Nucleotide and amino acid sequence homology of ALP was higher to RM than LN. Thus, one might conclude that ALP, RM, and LN are all closely related species based on both M- and S-segments. The AAI strain from *A. montensis*

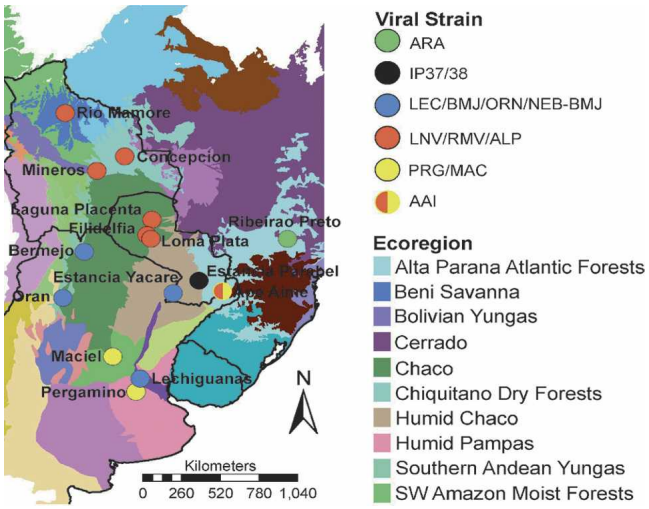

FIGURE 3. Distribution of hantavirus strains in Paraguay and surrounding countries. Symbols indicate viral strain occurring at each geographic location. Map also shows the distribution of viral strains relative to major South American ecoregions.[44]

clustered with the PRG strain from *A. azarae* in Argentina when comparing the M-segment, but when comparing the S-segment, it clustered with ALP, RM, and LN viruses. Natural genetic reassortment of genomic RNA segments has been reported within the SN virus among *Peromyscus maniculatis*[12,16] and within the DOB virus among two different rodent host species, *Apodemus flavicollis* and *A. agrarius.*[43] In an *in vitro* study with mixed infection using AND and SN viruses, genetic reassortants were isolated with the large (L) and small (S) segments of SNV and M-segment of ANDV.[15] Last, phylogenetic analyses of CD virus from *Sigmodon alstoni* in Venezuela has shown that its M- and S-segment clusters with different clades within the cladogram of North and South American hantaviruses. Further efforts in sequencing of the L-segments from these hantaviruses will help to substantiate the reassorment relationships of the virus strains of Paraguay.

Although there is no entirely consistent relationship between the virus strains that we and others have identified, and the ecoregions in which they occur, there are some apparent biogeographic patterns of note (Figure 3). The group of viruses that include ALP, LN, and RM occur within the Gran Chaco ecoregion, which is characterized by semiarid climate pattern. Several habitats occur in the region, with savanna and thorn forest dominating. Quebracho woodlands, more open than thorn forests, are characterized by thorny bushes, shrubs, and cactuses among scattered trees. The group of viral strains associated with the BMJ-ÑEB strain in Argentina (ORN and BMJ) also occur in the Chaco ecoregion, but this group of interrelated viral strains occurs in the Lower Chaco, a humid subregion of the Gran Chaco. The Lower Chaco encompasses a variety of landscapes, creating a transition zone between the arid Upper Chaco ecoregion to the west and the humid subtropical forests to the east. The region encompasses the flooded savannas of southwestern Paraguay, including the site where the BMJ-ÑEB strain was identified. The third related group of hantaviruses, including the AAI and PRG strains, were found in rodents that exist in very different ecoregions. The AAI strain, identified at Ape Aime in *Akodon montensis* in Itapúa, occurs in the Upper Paraná

Atlantic Forest biome. This biome is characterized by a humid continental climate, and is dominated by semi-deciduous broadleaf forest. In contrast, the rodent that harbors the PRG strain, *Akodon azarae*, lives in a region dominated by the Espinal, a semiarid temperate grassland ecoregion. Although these two regions are distinct ecologically, they are both experiencing rapid anthropogenic land cover change and are increasingly characterized by fragmentation and habitat disturbance. Comparable and full length sequence information on all genomic segments for each viral isolate is especially important for ascertaining both the degree of reassortment among segments and the geographic diffusion of viral strains. While we have been able to detect a potential reassortment event between the M- and S-segments and have been able to determine relationships suggesting complex geographical differentiation within South America, our ability to test appropriate hypotheses quantitatively is limited by the available sequence data. Future efforts will focus on isolation of Paraguayan hantaviruses, and obtaining full length clones so that comprehensive phylogenetic comparisons among segments and among isolates can be made.

Received January 4, 2006. Accepted for publication July 5, 2006.

Acknowledgments: The authors thank Robert J. Baker and Heath Garner of the Museum of Texas Tech University for approving and facilitating loans of rodent tissues; the Secretaría del Ambiente (Paraguay) for permits to collect and export rodents and tissues; and the field crew, led by Ismael Mora, for dedication to their work, regardless of circumstances.

Financial support: This work was supported by a grant from the Fogarty International Center 1 R01 TW006986-01 to CBJ under the NIH-NSF Ecology of Infectious Diseases initiative. In addition, this work was supported by a grant from the NIH to B.M., S06 GM08136, project 2-S2. Rodents and tissues were collected under NSF Grants DEB-9400926, DEB-9741543, and DEB-9741134 to R.D.O. and Michael R. Willig.

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