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Genetic characterization and phylogeny of a hantavirus from Western Mexico

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Abstract

Hantaviruses can cause two serious illnesses when transmitted from their rodent reservoirs to humans; hantavirus pulmonary syndrome (HPS) in the New World and hemorrhagic fever with renal syndrome in the Old World. Cases of HPS were first recognized in the Americas in small, focal outbreaks in rural populations in the Southwestern USA in 1993. Since that time, outbreaks as well as sporadic cases of HPS have been recognized throughout the Americas. Remarkably, HPS cases have not been reported in Mexico. Mexico is one of the most biodiverse regions in the world and this is reflected in the species diversity of the peromyscine, sigmodontine and oryzomyine rodents; all potential hosts of hantaviruses. Hence, we collected and surveyed several rodent species in Western Mexico and identified three previously unrecognized rodents with antibodies to hantaviral antigens: *Oryzomys couesi*, *Sigmodon mascotensis* and *Baiomys musculus*. The S and M segments cloned from *O. couesi* and *S. mascotensis,* referred to herein as Playa de Oro (ORO) virus, showed strongest similarity to Bayou and Catacamas viruses with 92/93% and 92/92% similarity based on S/M amino acid sequences, respectively. This and phylogenetic analysis of the M and S segments suggests that ORO virus is a unique genotype within *Hantavirus*.

Keywords: Hantavirus; Playa de Oro (ORO); *Oryzomys couesi*; *Sigmodon mascotensis*; Seroprevalence; Colima; Mexico; Phylogenetics

1. Introduction

Hantaviruses are rodent-borne viruses that cause a mild to severe hemorrhagic fever with renal syndrome (HFRS) throughout the Eurasian continent (Lee and van der Groen, 1989) and a usually severe, and often fatal, hantavirus pulmonary syndrome (HPS) in the Americas (Schmaljohn and Hjelle, 1997). Transmission of hantaviruses to humans is presumed to occur through intake of aerosolized virus in the rodent excreta, direct contact with damaged skin or mucous membranes, or rodent bites (Childs et al., 1993; Douron et al., 1984; Glass et al., 1988; Lee and van der Groen, 1989). Generally, each strain of hantavirus has a unique rodent reservoir host species, and there is evidence for coevolution (Plyusnin and Morzunov, 2001). Hence, the distribution and ecology of the rodent host species defines the epidemiology of hantavirus diseases and distribution of the viral strains (Hjelle et al., 1995b; Khan and Khan, 2003).

Since the first recognition of HPS cases in the Southwestern US in 1993, and isolation of their causative agent Sin Nombre virus (SNV) from deer mice *Peromyscus maniculatus* (Nichol et al., 1993), more than 30 new hantaviruses, many of which cause HPS, have been identified from various rodent species throughout the Americas (Peters and Khan, 2002; Schmaljohn and Hjelle, 1997). Additional viruses which cause HPS in the US and Canada include Monongahela virus (MGLV) associated with *P. maniculatus* (Song et al., 1996), New York virus

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(NYV) harbored by *P. leucopus* (Hjelle et al., 1995c), Bayou virus (BAYV) harbored by *Oryzomys palustris*, (Khan et al., 1995; Morzunov et al., 1995) and Black Creek Canal virus (BCCV) harbored by *Sigmodon hispidus* (Ravkov et al., 1995). In Panama, Choclo virus harbored by *Oligoryzomys fulvescens* was identified as an etiologic agent of HPS (Vincent et al., 2000). Sigmodontinae-borne hantaviruses from the Americas that have not been associated with human disease include El Moro Canyon virus (ELMC) from *Reithrodontomys megalotis* (Rawlings et al., 1996), Rio Segundo virus (RIOSV) harbored by *R. mexicanus* (Hjelle et al., 1995a), Limestone Canyon virus (LSCV) harbored by *P. boylii* (Sanchez et al., 2001) and Calabazo virus harbored by *Zygodontomys brevicauda* (Vincent et al., 2000).

Studies of hantaviruses in Latin America have revealed an increasingly complex picture of their ecology. In contrast to North America, numerous rodent species have been identified to harbor unique strains of *Hantavirus* in Argentina (Calderon et al., 1999; Levis et al., 1998), Bolivia (Bharadwaj et al., 1997), Brazil (Figueiredo et al., 2003), Chile (Toro et al., 1998), Costa Rica (Hjelle et al., 1995a), Honduras (Milazzo et al., 2006), Panama (Vincent et al., 2000), Paraguay (Chu et al., 2006), Peru (Powers et al., 1999) and Venezuela (Fulhorst et al., 1997). These findings are not surprising given the diversity of sigmodontine rodents in Latin America, which followed a broad adaptive radiation after invading via the newly emerged Panama land bridge (Pardiñas et al., 2002). Moreover, this phylogenetic diversity is also reflected as ecological diversity, and a complex biogeographic history within continental South America (D'Elía, 2003). 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 as well as the geographic constraints of the landscape that have influenced rodent migration patterns and habitats (Engel et al., 1998).

The aforementioned research efforts in Central and South America reveal the phylogeographic complexity of *Hantavirus*, which reflect the rich biodiversity in these regions. Mexico, also, is one of the most biodiverse regions in the world, in part because it spans the intersection of North American with Central and South American faunae. The region contains a wide diversity of neotomine, sigmodontine and oryzomyine rodents (family Cricetidae) (Musser and Carleton, 2005; Ramírez-Pulido et al., 2005). At present, the only hantavirus that has been identified from Mexico is the ELMC virus from *R. megalotis*, a neotomine rodent (Hjelle et al., 1995a; Mantooth et al., 2001). To our knowledge, there have been no cases of HPS reported from Mexico. The objective of our study reported herein was to extend our knowledge of *Hantavirus* ecology and its genetic diversity within the sigmodontine rodents to Mexico; an important ecological intersection between North and South America. We collected several rodent species from a dry coastal thorn-scrub habitat in 2004 in the state of Colima, Mexico. Here we report identification of a unique genotype of *Hantavirus*, which we refer to herein as Playa de Oro virus (OROV), from *Oryzomys couesi*. Further, we report a second sequence with less than 1% difference to OROV, detected in *Sigmodon mascotensis*, possibly from spill-over infection, since these species are sympatric in the study area and have similar microhabitat preferences. Remarkably, *O. couesi* was reported to harbor Catacamas virus (CATV) in Honduras (Milazzo et al., 2006). The phylogenetic relationships between the CATV and ORO viruses showed substantial divergence at amino acid (8%) and nucleotide levels (24%). Intriguingly, these two viruses are harbored by two apparently different subspecies of *O. couesi.*

2. Materials and methods

2.1. Rodent collections

The rodents used in the study were obtained from an inventory of small mammals performed in Colima, Mexico in January 2004. Rodents were captured using Sherman live traps (Sherman Trap Company, Tallahassee, FL), and were either sacrificed and processed in field camps as standard museum specimens, or were released at mark-recapture sites after collecting about 100 μl of blood from the animal. The following standard measurements were collected from each sacrificed animal: weight, total length, and tail, ear, and hind foot lengths. From the released rodents, only body weight was measured. The age, sex and reproductive condition of each individual were also recorded. Liver, lung, heart, kidney and muscle tissues from museum specimens, as well as blood specimens from released rodents, were collected and stored immediately in liquid nitrogen. Tissues were transported to the Museum of Texas Tech University where the samples were archived at −80 °C. All work was done under appropriate Animal Care and Use protocol approvals (Texas Tech University), collecting permit (SGPA/DGVS/O3250), export permit (Instituto de Biologia, UNAM #7) and import permits (Centers for Disease Control).

2.2. Antibody detection

Blood from rodent specimens was centrifuged at 7000 rpm in a refrigerated centrifuge and the supernatant was diluted 32-fold in PBS buffer. To detect antibody, SNV-infected Vero E6 cells were grown on a 10 spot glass slide and fixed with pure acetone, followed by irradiation with ^{60}Co for 4 h in a container packed with dry ice. Forty microliters of diluted plasma from each rodent was added to each well of the antigen slide and incubated for 30 min at 37 °C in a moist chamber. Slides were washed twice with PBS for 5 min and rinsed with distilled water. Slides were air-dried inside a biological safety cabinet, 25 μl of fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc.) was added, incubated for 30 min, and washed as described earlier. Slides were mounted with mounting media (90% glycerol in PBS buffer) and observed under an Axioscope 200 fluorescent microscope (Zeiss).

2.3. RT-PCR amplification

Total RNA was extracted from blood and amplified by a nested RT-PCR as described previously (Chu et al., 2003). Extracted RNA was diluted with 10 μl of RNase-free distilled

water, and subjected to RT with random hexamers using a SuperScript RT kit (Invitrogen) in a total reaction volume of 21 μl. Ten microliters of the RT-reaction was used for the first amplification by PCR with small (S) segment generic outer primers using a PCR kit (Invitrogen). Two microliters of the amplified PCR product was taken for a second round of PCR amplification with S segment generic inner primers as described previously (Chu et al., 2003). PCR products were analyzed by 1.2% agarose gel electrophoresis in TRIS-acetate-EDTA (TAE) buffer. The remaining RT-reaction from the RNA-positive specimens was divided for further amplification of its M or S segments. The second amplification targeted 1537 nt of the M segment (not including primer sequences) by nested PCR. The M outer forward primer (5′-TGCWGGIGYICCWATGTAYAG-3′) and M outer reverse primer (5′-CCHAGIAGCCAYTCWCCWGA-3′) were used for primary PCR. The M inner forward primer (5′-GG1GCWAGTTGTGARGCHTA-3′) and M inner reverse primer (5′-TGRAAWGARTCYYTWGTDGCCAT-3′) were used for the second round of PCR. For further amplification of the S segment, we amplified a 1038 nt piece (not including primer sequences) by hemi-nested PCR. We used the S outer forward primer (5′-GTAGTAGACTCCTTGAGAAGC-3′) and S outer reverse primer (5′-TGGTCCAGTTGAATYCCCAT-3′) for primary PCR. We used the S inner forward primer (same as above mentioned outer forward primer) and S inner reverse primer (5′-CCTACAGACTTTGATGCCAT-3′) for the second round of PCR. To amplify the remaining 3′ terminus region of the S segment, we employed a hemi-nested PCR approach in which we used the S outer forward primer (5′-GTTTGGGTGTTTGCATGTGC-3′) and S outer reverse primer (5′-GTAGTAGACTCCTTGAGAAGC-3′) for primary PCR, and the S inner forward primer (5′-CCCACCGACAGCATTATATG-3′) and S inner reverse primer (same as above mentioned outer reverse primer) for the second round of PCR. The initial PCR was performed for 35 cycles at 94 °C for 30 s; at 50 °C, for 60 s; and at 72 °C for 3 min. The nested PCR was performed for 35 cycles at 94 °C for 30 s; at 55 °C, for 60 s; and at 72 °C for 3 min. Amplified PCR products were separated in 1.2% agarose gels in TAE buffer and visualized following ethidium bromide staining.

2.4. Cloning and sequencing of amplified RT-PCR products

The PCR amplified cDNA from each sample was extracted from an agarose gel, purified (Bio101), and ligated into the pCR 2.1 Topo vector plasmid (Invitrogen). M13 forward and reverse primers, and internal sequencing reverse (5′-TGTTGTTTGCCAAGGGTA-3′) primers for M segment PCR product were used for sequencing. We sequenced at least three clones for each PCR product in both directions using the BigDye 3.0 terminator sequencing system (ABI) as described by the manufacturer.

2.5. Sequence comparisons and phylogenetic analyses

Sequence alignments and comparisons were performed to get sequence divergence values based on neighbor joining method with the AlignX programs from the Vector NTI software package, version 10.0 (Invitrogen, Inc.). Modeltest (Posada and Crandall, 1998) was used to select the optimal model of substitution for each data set, and model parameters were further optimized in (Swofford D.L., 1999) PAUP* (version 10). Phylogeny reconstruction was carried out with both maximum likelihood and Bayesian methods. With maximum likelihood methods, a heuristic search strategy with general time reversible (GTR) model of nucleotide substitution (six parameters), an invariant rate parameter (PINVAR) and gamma distribution was used, with starting trees obtained by random stepwise addition of taxa (10 replicates) and branch swapping, as implemented in PAUP*. Bayesian phylogeny reconstruction was performed with MrBayes (Huelsenbeck and Rannala, 1997) for 1 million generations, sampling every 100 generations, with the final tree being constructed from a consensus tree was estimated by using a burnin of 2,500 trees. The S- and M segments of Prospect Hill virus (PHV) were used as outgroup sequences for phylogeny reconstruction.

3. Results

3.1. Rodent collections, serology and viral RNA detection

A total of 600 small mammals, belonging to 1 species of marsupial (*Tlacuatzin*), 2 species of pocket mouse (*Liomys*), 7 species of New World mice and rats (family Cricetidae), mostly within the subfamily Sigmodontinae, tribes Oryzomyini and Sigmodontini, were trapped at Playa de Oro (19°08′N, 104°31′W), Municipality of Manzanillo, in Colima, Mexico. Three species belonging the New World mice and rats had antibodies to SNV antigens as determined by IFA (Table 1). *O. couesi* was the predominant species in the collection. Sera from 23 of the 358 *O. couesi* rodents (6.4%) had antibody-positive reactions against SN virus antigen with reciprocal antibody titers ranging from 64 to 4096. The second most abundant species in the collection was *S. mascotensis*, from which 6 of the 87 animals (6.9%) showed reciprocal antibody titers that ranged from 128 to 4096. The third most abundant species was *Baiomys musculus*, from which we detected 1 animal from the 77 collected (1.3%) with an antibody titer of 256.

Hantaviruses have three single-strand, negative-sense RNA segments. The large (L) genome segment encodes the viral RNA-dependent RNA polymerase, the medium (M) genome segment encodes the glycoprotein (GPC), which is post-translationally cleaved into Gn and Gc, and the small (S) genome segment encodes the nucleocapsid (N) protein. These segments are approximately 6500, 3500 and 2000 nt, respectively. For screening purposes, we targeted the S segment RNA since this is the most abundant viral mRNA. We used a nested RT-PCR strategy with S segment specific generic primer pairs. We detected hantaviral RNA from blood samples of 13 rodents; 12 *O. couesi* and 1 *S. mascotensis* (Table 1).

We consistently detected higher prevalence of antibody and viral RNA in males than females (Table 1). Twelve of the 312 (3.8%) males were RNA-positive, while only 1 of the 286 (0.3%)

Table 1
Presence of hantaviral antibody and RNA among small mammals collected in the state of Colima, Mexico

Species	Antibody-positive				RNA-positive			
	Male	Female	Unknown	Total	Male	Female	Unknown	Total
Order Didelphimorphia, family Didelphidae								
Tlacuatzin canescens	0/6	0/4	0	0/10	0/6	0/4	0	0/10
Order Rodentia, family Heteromyidae								
Liomys pictus	0/6	0/12	0	0/18	0/6	0/12	0	0/18
Liomys spectabilis	0/5	0/1	0	0/6	0/5	0/1	0	0/6
Family Cricetidae, subfamily Neotominae, tribe Baiomyini								
Baiomys musculus	1/33 (3.0%)	0/42	0/2	1/77 (1.3%)	0/33	0/42	0/2	0/77
Tribe Reithrodontomyini								
Osgoodomys banderanus	0/4	0/5	0	0/9	0/4	0/5	0	0/9
Peromyscus perfulvus	0/12	0/4	0	0/16	0/12	0/4	0	0/16
Reithrodontomys fulvescens	0/9	0/9	0	0/18	0/9	0/9	0	0/18
Subfamily Sigmodontinae, tribe Oryzomyini								
Oryzomys couesi	21/190 (11.1%)	2/168 (1.2%)	0	23/358 (6.4%)	11/190 (5.8%)	1/168 (0.6%)	0	12/358 (3.4%)
Tribe Sigmodontini								
Sigmodon mascotensis	5/47 (10.6%)	1/40 (2.5%)	0	6/87 (6.9%)	1/47 (2.1%)	0/40	0	1/87 (1.1%)
Subfamily Tylomyinae								
Nyctomys sumichrasti	0	0/1	0	0/1	0	0/1	0	0/1
Total	27/312 (8.7%)	3/286 (1.0%)	0/2	30/600 (5.0%)	12/312 (3.8%)	1/286 (0.3%)	0/2	13/600 (2.2%)

females was RNA-positive. With respect to the presence of antibody by IFA, 27 of the 312 male rodents (8.7%) had antibody as compared to only 3 of the 286 females (1.0%).

3.2. Amplification, genetic characterization and comparison of S and M segments from ORO with other American hantaviruses

The majority of the RNA-positive specimens came from rodents collected in the capture and release sites, from which we had a limited amount of blood for amplification of viral genetic material. We amplified, cloned and sequenced a 1038 nt fragment of the S segment and a 1537 nt fragment of the M segment, excluding primer pair sequences, from RNA-positive blood specimens of *O. couesi* and *S. mascotensis*. Only one RNA-positive rodent (*O. couesi*) had lung tissue collected, which was used to obtain a complete S segment sequence.

The 1953 nt S segment sequence amplified from *O. couesi* represents the full length S segment (referred to as ORO_6521). The complete S segment nucleotide sequence of 1953 nt was derived from 2 PCR amplicons obtained from the lung tissue of *O. couesi*, which had an 112 nt overlap. The open reading frame (ORF) of the N protein gene spans 1287 nucleotides (428 amino acids) from position 43 to 1329 in the complementary RNA (cRNA) sequence. As noted for some of the other hantaviruses such as PUU, PH, SN, BAY, BCC and TUL viruses (Ravkov et al., 1995), an overlapping coding sequence of 192 nucleotides which encodes a 63 amino acid ORF from position 122 to 313 in the cRNA was identified. We could identify 42 nucleotides of 5′ non-coding region (NCR) and 624 nucleotides of 3′ NCR.

A comparison of the nucleotide sequences of the 1038 nucleotide length S segment fragments among the three *O. couesi* (referred to as OROV6521, OROVB265 and OROVA353) showed a 1% nucleotide difference (data not shown). Thirteen point mutations were identified which were silent. Pairwise comparison of the nucleotide sequence data from the OROV6521 S segment with that of other representative hantaviruses in the New World revealed that it was most similar to BAY, CAT and BCC viruses (Table 2). Comparisons of the complete OROV S segment nucleotide sequence to BAY, CAT and BCC viruses showed 76% similarity. Amino acid comparisons revealed 93, 92 and 90% similarity of OROV6521 to BAY, CAT and BCC viruses, respectively (Table 2). Nucleotide sequence similarities of 3′ non-coding region (NCR) of OROV to BAY, CAT and BCC viruses were 67, 64 and 62%, respectively. In contrast the 3′ non-coding similarity of CATV to BAYV and BCCV were 80 and 68% (Table 3).

The 1537 nucleotide M segment from the 2 different rodent species of *O. couesi* and *S. mascotensis* showed 1% nucleotide and amino acid difference with 26 point mutations and 3 amino acid changes. The ORF contained the proposed glycoprotein precursor cleavage site, WAASA (Lober et al., 2001). All 7 point mutations in the Gn region were silent, but 5 out of 19 mutation sites in the Gc region were degenerative. Pairwise comparison of the partial OROV M segment sequence data with that of other representative hantaviruses revealed a nucleotide sequence similarity of 78% to BAY, CAT and BCC viruses. Comparisons with the deduced amino acid sequence derived from the M segments of OROV6521, OROV2699 and OROVA318 with BAY, CAT and BCC viruses showed 91, 92 and 90% similarity, respectively (Table 4).

Table 2
Nucleotide and amino acid similarity of newly identified hantavirus, ORO, to other American hantaviruses based on the S segment

	ORO	CAT	BAY	BCC	MUL	AND	LN	EMC	RS	SN	NY
ORO	–	76	76	76	72	66	66	64	64	61	61
CAT	92	–	82	78	78	72	73	68	70	66	67
BAY	93	95	–	76	75	68	68	65	64	62	62
BCC	90	92	92	–	74	66	66	64	63	63	62
MUL	89	92	93	90	–	66	66	64	62	62	62
AND	88	88	89	86	85	–	72	67	66	62	62
LN	88	88	87	86	85	90	–	65	66	61	62
EMC	84	85	84	83	83	83	82	–	75	60	61
RS	83	84	84	82	81	81	81	91	–	58	58
SN	85	86	86	83	82	86	85	84	83	–	75
NY	86	87	88	86	84	87	87	84	83	93	–

Above: nucleotide sequence similarity. Below: amino acid similarity.

Table 3
Nucleotide sequence similarity of the 3′ non-coding region and coding region from the S segment of ORO to other American hantaviruses

	ORO	CAT	BAY	BCC	MUL	EMC	RS	SN CC107	SN NMR11	NY	MNG
ORO	–	64	67	62	60	40	36	41	48	41	50
CAT	80	–	80	68	68	45	47	50	51	50	51
BAY	80	83	–	65	68	42	37	41	50	42	54
BCC	79	82	81	–	61	39	34	39	50	42	51
MUL	79	81	80	81	–	38	33	41	47	42	47
EMC	79	76	76	75	76	–	62	31	42	31	42
RS	76	74	75	73	72	79	–	25	45	25	47
SN CC107	76	76	76	75	76	76	75	–	86	67	67
SN NMR11	76	76	77	75	75	76	75	90	–	66	68
NY	78	77	77	76	76	77	74	82	83	–	71
MNG	75	77	77	76	75	77	74	82	83	85	–

Above: nucleotide sequence similarity of 3′ non-coding region (NCR). Below: nucleotide sequence similarity of coding region (CR).

3.3. Phylogenetic comparisons of S and M segments from ORO with American hantaviruses

The phylogenetic relationships of OROV and other American hantaviruses were analyzed by maximum likelihood and Bayesian analysis with bootstrap confidence limits. On both M and S segment phylograms, ORO, BAY, CAT, BCC viruses formed a nested where OROV was basal with 100 posterior probabilities values. In the S segment based phylogram (Fig. 1A), BAY and CAT viruses showed a sister relationship in a terminal node of subclade IIIA. In the M segment based phylogram, a similar picture was observed with OROV as basal and BAYV and CATV showing a sister relationship in the terminal node of subclade IIB (Fig. 1B).

4. Discussion

Since the first report of HPS in the US in 1993, 485 HPS cases have been reported in North and Central America, and approximately 1425 cases have been reported in South America

Table 4
Nucleotide and amino acid similarity of newly identified hantavirus, ORO, to other American hantaviruses based on the M segment

	ORO 6521	ORO 2669	ORO A318	CAT	BAY	BCC	SN	NY	BR	EMC	AND	LN
ORO 6521	–	99	99	78	78	78	75	75	75	73	75	76
ORO 2669	99	–	99	78	78	78	75	75	75	73	75	76
ORO A318	99	99	–	78	78	78	75	75	75	73	75	76
CAT	92	92	92	–	79	78	74	75	76	74	73	76
BAY	91	91	91	95	–	79	74	75	74	74	75	75
BCC	90	90	90	91	92	–	76	75	74	74	74	76
SN	88	88	88	87	87	86	–	79	80	74	74	74
NY	86	86	86	87	86	85	97	–	80	73	75	74
BR	86	86	87	86	86	84	95	94	–	75	76	75
EMC	83	83	83	83	83	83	85	84	84	–	72	73
AND	83	83	83	84	85	83	87	86	86	82	–	76
LN	84	84	85	86	85	85	85	85	84	82	89	–

Above: nucleotide sequence similarity. Below: amino acid similarity.

[http://www.paho.org/English/AD/DPC/CD/hantavirus-1993-2004.htm]. Despite the widespread occurrence of HPS cases north and south of Mexico, there have been no reported cases of HPS in Mexico. However, Vado-Solis et al., reported 4 people with antibodies to hantaviral antigens with unknown histories among 616 people from the general population in the state of Yucatan, but did not identify antibody to hantavirus antigen among 92 collected rodents (Vado-Solis et al., 2003). Furthermore, several rodent species such as *R. megalotis*, *R. sumichrasti*, *P. maniculatus*, *P. melanotis* and *P. hylocetes*

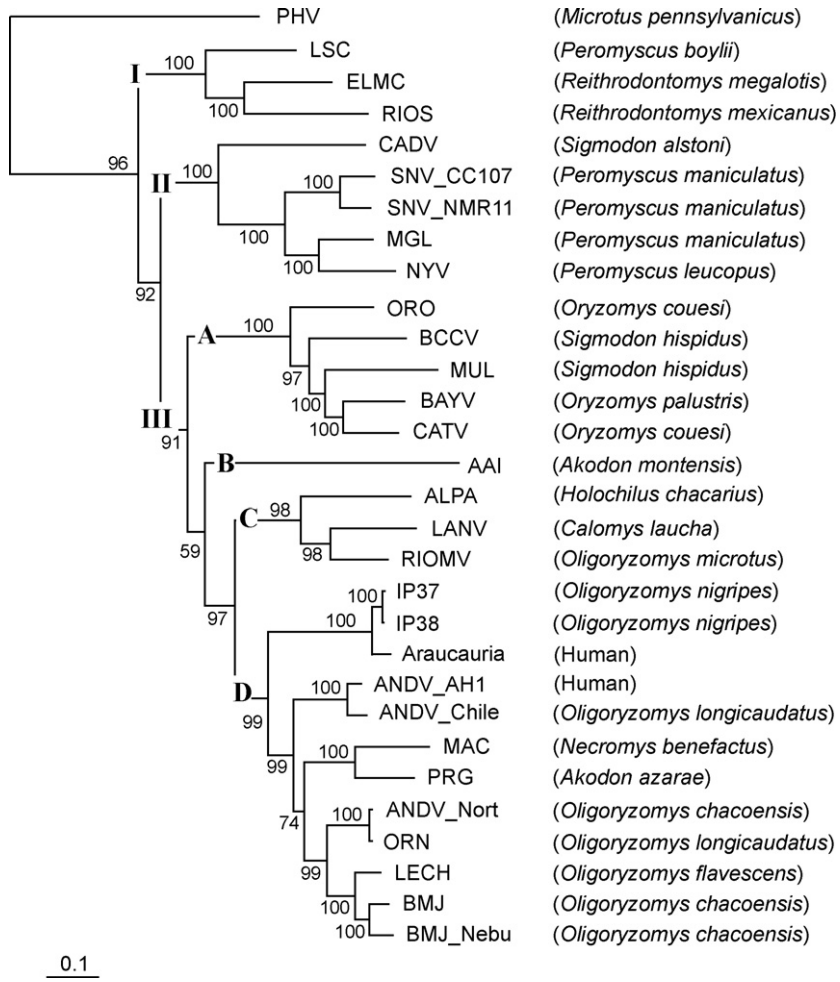

Fig. 1. Phylogenetic tree based on maximum likelihood analysis of the S and M segments of American hantaviruses. The cladogram was based on (A) the complete S segment and (B) a partial (1537 nt) fragment of the M segment of North and South American hantaviruses. PHV was used as an outgroup for all phylogenetic analyses. The scale bar shows the mean number of substitutions per site. The numerical values at the nodes indicate the percentage of 1000 bootstrap replicates that supported each interior branch. Abbreviations and GenBank accession numbers (GenBank): AAI (Ape Aime) virus from Paraguay [GenBank AY515599 and AY515604 (M segment) and DQ345764 (S segment)]; ALP (Alto Paraguay) virus from Paraguay [GenBank accession numbers AY515597 and AY515602 (M segment) and DQ345762 (S segment)]; ANDV_AH1 (Andes virus, strain AH-1) from Argentina [GenBank AF324901 (M segment) and AF324902 (S segment)]; ANDV_CHL (Andes virus, strain Chile 9717869) [GenBank AF291703 (M segment) and AF291702 (S segment)]; Araraquara virus from Brazil [GenBank AF307327 (M segment) and AF307325 (S segment)]; Araucaria virus from Brazil [GenBank AY740633 (S segment)]; BAY (Bayou) virus from the USA [GenBank L36930 (M segment) and L36929 (S segment)]; BCC (Black Creek Canal) virus from the USA [GenBank L39950 (M segment) and L39949 (S segment)]; BMJ (Bermejo) virus from Argentina [GenBank AF028025 (M segment) and AF482713 (S segment)]; BMJV-ÑEB (Bermejo virus, strain Neembucu) from Paraguay [GenBank AY515598 and AY515603 (M segment) and DQ345763 (S segment)]; BRV-Indiana (Blue River virus, strain Indiana) from the USA [GenBank AF030551 (M segment)]; BRV-Oklahoma (Blue River virus, strain Oklahoma) from the USA [GenBank AF030552 (M segment)]; CAS (Castelo dos Sonhos) virus from Brazil [GenBank AF307326 (M segment)]; CAT (Catacamas) virus from Honduras [GenBank DQ177347 (M segment) and DQ256126 (S segment)]; CAD (Cano Delgadito) virus from Venezuela [GenBank DQ284451 (M segment) and DQ285566 (S segment)]; ELMC (El Moro Canyon) virus from the USA [GenBank U26828 (M segment) and U11427 (S segment)]; IPV 37 (Itapua virus, strain 37) from Paraguay [GenBank AY515600 andAY515605 (M segment) and DQ345765 (S segment)]; IPV 38 (Itapua virus, strain 38) from Paraguay [GenBank AY515601 andAY515606 (M segment) and DQ345766 (S segment)]; LEC (Lechiguanas) virus from Argentina [GenBank AF028022 (M segment) and AF482714 (S segment)]; MUL (Muleshoe) virus from the USA [GenBank U54575 (S segment)]; LAN (Laguna Negra) virus from Paraguay [GenBank AF005728 (M segment) and AF005727 (S segment)]; MCL (Maciel) virus from Argentina [GenBank AF028027 (M segment) and AF482716 (S segment)]; MGL (Monongahela) virus from the USA [GenBank U32651 (M segment) and U32591 (S segment)]; NY (New York) virus from the USA [GenBank U36802 (M segment) and U47135 (S segment)]; ORO (Playa de Oro) virus from the Mexico [GenBank EF534080-82 (M segment) and EF534077-79 (S segment)]; PH (Prospect Hill) virus from the USA [GenBank M34011 (M segment) and U47136 (S segment)]; PRG (Pergamino) virus from Argentina [GenBank AF028028 (M segment) and AF482717 (S segment)]; RIOS (Rio Segundo) virus from Costa Rica [GenBank U18100 (S segment)]; SNV_CC107 (SNV, strain CC107) from the USA [GenBank L33474 (M segment) and L33683 (S segment)]; SNV_NMR11 (SNV, strain NMR11) from the USA [GenBank L37903 (M segment) and L37904 (S segment)].

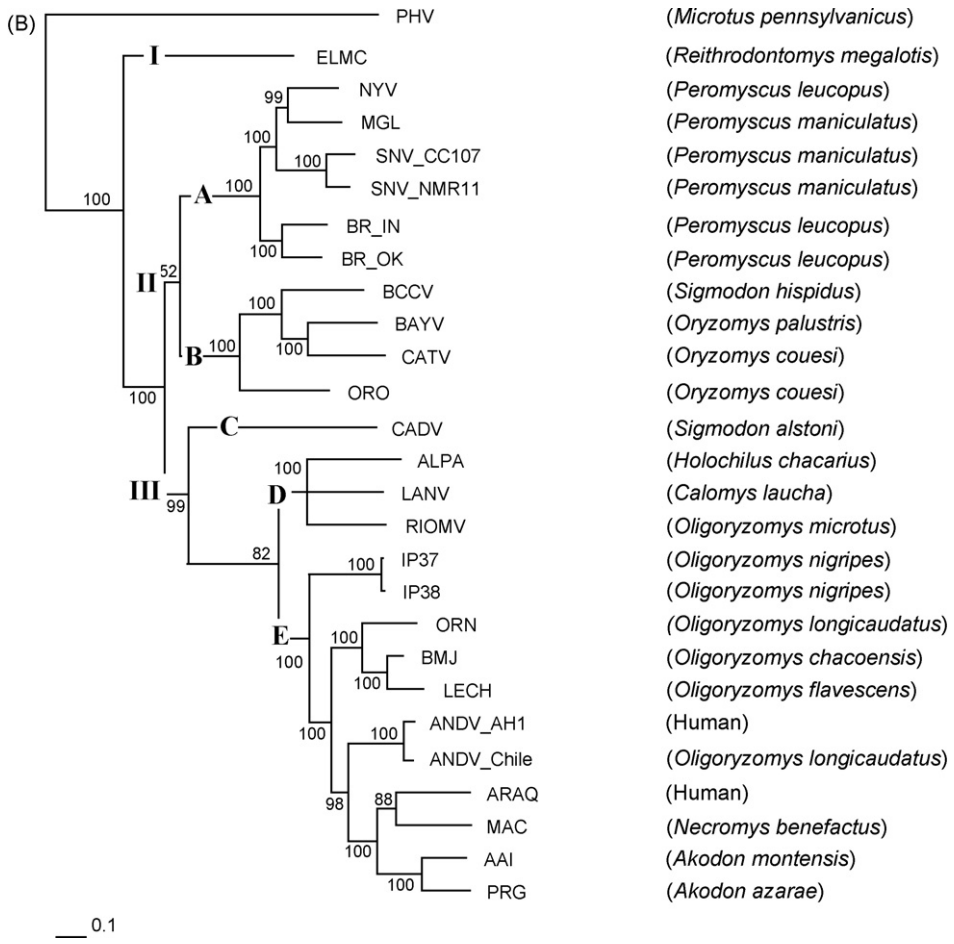

Fig. 1. (*Continued*).

collected in the states of Oaxaca and Zacatecas have been shown to have hantaviral antibody or RNA (Hjelle et al., 1995a; Mantooth et al., 2001; Suzan et al., 2001). Given the rich biodiversity of Mexico (Ceballos et al., 2005) and the complex phylogenetic picture of hantaviruses emerging in Latin America, we hypothesized that additional viruses likely were present in other related rodent species. Additional phylogenetic information on the hantaviruses in Mexico, a key country in the radiation of the sigmodontine rodents, will help to reveal the evolution of these viruses in the Americas. Of the 120+ sigmodontine species currently known from Mexico (Ramírez-Pulido et al., 2005), approximately 80 are from 5 genera (*Peromyscus*, *Reithrodontomys*, *Oligoryzomys*, *Oryzomys*, *Sigmodon*) that include species which are known reservoirs for hantavirus.

In this study we collected and analyzed marsupials and rodents from Colima, Mexico in area where *O. couesi* and *S. mascotensis* predominated. *S. mascotensis* exhibited the highest prevalence of hantaviral antibody, followed by *O. couesi* and *B. musculus* with RNA prevalence highest in *O. couesi*. The presence of the same virus in these two hosts is most likely due to spill-over among these two micro-sympatric rodent species, which experience a high likelihood of cross-infection because of the increased probability of encounters in overlapping home ranges. Although the reservoir host will develop a strong neutralizing antibody response against hantavirus, viral RNA can be detected from the tissues, saliva, urine and feces of antibody-positive rodents (Hjelle and Yates, 2001). In contrast, spill-over infections are usually characterized by an acute infection followed by subsequent clearance of virus and hence, lack of RNA (Plyusnin and Morzunov, 2001; Klingstrom et al., 2002). It is impossible to show strain specificity by IFA or ELISA since all New World strains show some level of antibody cross-reactivity. The only way to confirm the identity of the infecting virus is through direct sequencing of the viral genome or possibly by a cross-neutralization test. To identify the circulating hantaviral genotype among the rodents collected in this region, amplified PCR products from the tissues of two different rodent species were sequenced and compared. We have found that same genotype virus is circulating among these two rodent species. Our data suggest that *O. couesi* is the primary host of this newly identified virus because of the high prevalence of antibody and hantaviral RNA.

The deduced 428 amino acid sequence from the entire S segment of the OROV showed a 7, 8 and 10% difference from BAY, CAT and BCC viruses, respectively. Similarly the deduced 512 amino acids from partial OROV M segment showed a 9, 8 and 10% difference compared to BAY, CAT and BCC viruses, respectively. The similarity of OROV's 3′ NCR region to BAY, CAT, BCC and MUL viruses was 67, 64, 62 and 60%, respec-

tively. Interestingly CATV's 3′ NCR region sequence similarity to BAY, BCC, MUL viruses showed 80, 68 and 68%, respectively. Usually less related viruses show much higher nucleotide sequence difference in NCR than coding region (CR). For example, the *Peromyscus*-derived hantaviruses, SNV_CC107 and SNV_NMR11, show only 4% difference between their CR and NCR regions. However, these regions in SNV show a 15% difference when compared with NY and MGL viruses. Similarly, CATV virus showed a close relationship to BAYV. The nucleotide sequence of CATV showed a difference off 3% to BAYV, and a 16% difference to OROV.

According to the most recent guidelines, a virus is considered to be a new species if; (1) it has been found in unique ecological niche, (2) exhibits more than a 7% difference in amino acid identity in a comparison of the complete glycoprotein precursor and nucleocapsid protein sequences, (3) shows at least a 4-fold difference in two-way cross-neutralization tests and (4) does not form reassortants with other species in nature (Fauquet et al., 2005). While we have not isolated this newly identified ORO virus in Mexico due to limited museum tissue specimens, we believe that ORO virus is a unique genotype within *Hantavirus* because of following reasons. Firstly, the sequences exhibited more than 7% differences as compared to other Oryzomyne rodent derived BAY, CAT, MUL and BCC viruses in a same clade in both M and S segment based phylograms. Secondly, even we though we are unable to perform a two-way cross-neutralization test with the other viruses, we can deduced that OROV would show more than 4-fold difference to other viruses based on studies with New World (NYV) and Old World (Saaremaa virus) neutralization tests (Gavrilovskaya et al., 1999; Sjolander et al., 2002). For these viruses, amino acid sequence differences of the M segment among New World (NYV, SNV) and Old World (Dobrava virus, Belgrade virus and Saarema viruses) are only 3–4%, however, these groups show significant differences on cross-neutralization reaction.

Intriguingly, CAT and ORO viruses were identified from same rodent species, *O. couesi*, yet CAT virus formed a sister group with BAY virus from *O. palustris* instead of ORO virus in our phylogenetic analysis based on S and M segments. Similar relationships have been reported previously for SNV and MGL viruses which are harbored in different genetic lineage of *P. maniculatus* (Dragoo et al., 2006), BCC and MUL viruses harbored by *S. hispidus* (Rawlings et al., 1996), and Hantaan and Saarema viruses harbored by different genetic lineages of *Apodemus agrarius* (Nemirov et al., 2002). Phylogenetic analysis based on S and M segments have shown that CATV forms a sister clade with BAYV, and that OROV formed a subclade separate from BAYV and BCCV. Phylogenetic analysis showed that this newly identified hantavirus strain, OROV, formed a separate subclade with BAYV, CATV and BCCV. Future analyses such as cross-neutralization will be performed once the virus has been isolated to ascertain the relationship of OROV to other viruses in *Hantavirus*. The findings from this study and previous studies in Mexico suggest that a thorough evaluation of the extent and distribution of hantaviruses in Sigmodontinae reservoir hosts and the general human population will be beneficial toward assessment of the public health risks associated with hantaviruses. Although we know of no HPS case to date in Mexico, the potential risk of human infection from OROV or other undiscovered viruses cannot be ignored.

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