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RAPID COMMUNICATION

Hantavirus Pulmonary Syndrome in Panama: Identification of Novel Hantaviruses and Their Likely Reservoirs

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Hantavirus pulmonary syndrome (HPS), a severe respiratory disease with high mortality caused by rodent-borne hantaviruses, has previously been identified in the United States and Canada as well as central and southern South America. In late 1999 and early 2000, an outbreak of acute illness compatible with HPS was reported in Los Santos, Panama, with the death of 3 of the 12 (25%) suspected cases. Hantavirus-specific antibodies were detected in patient sera, and virus RNA was detected by reverse transcriptase-polymerase chain reaction. Sequence analysis of virus genome N-, G1-, and G2-encoding fragments showed this to be a novel hantavirus, Choclo virus. Serologic and virus genetic analyses of rodents trapped in the area showed *Oligoryzomys fulvescens* to be the likely reservoir for the HPS-associated Choclo virus. In addition, *Zygodontomys brevicauda* rodents were shown to harbor another genetically unique hantavirus, Calabazo virus. © 2000 Academic Press

Introduction. The genome organization of hantaviruses is typical of other members of *Bunyaviridae,* consisting of three negative-stranded RNA segments, large (L), medium (M), and small (S), which encode the virus L polymerase, the G1 and G2 glycoproteins, and the nucleocapsid protein, respectively (*5, 7*). Hantaviruses in the Old World, such as Hantaan, Seoul, Puumala, and Dobrava viruses, are associated with hemorrhagic fever with renal syndrome (HFRS) and are prevalent in Asia and Europe (*1, 9*). The New World hantaviruses are associated with hantavirus pulmonary syndrome (HPS) (*13, 15, 17*). HPS is characterized by abrupt onset of fever, headache, myalgia, hypotension, and thrombocytopenia, followed by bilateral pulmonary infiltrates and rapid progression to respiratory failure. The mortality rate associated with HFRS ranges from less than 1 to 7% and from 40 to 50% for HPS. Each specific hantavirus is associated with a specific rodent that serves as host and vector, and the New World hantaviruses are hosted by various members of the subfamily Sigmodontinae.

In North America, most of the HPS cases have been reported from the western United States and Canada; sporadic occurrences have also been reported in other areas. Sin Nombre (SN) virus (*4, 13*) or viruses closely resembling the SN virus have been responsible for most of the cases. HPS has not been previously reported from south of the United States, Central America, the Caribbean Islands, and northern South America. In contrast, South American countries, such as Argentina, Chile, Paraguay, Peru, Brazil, and Bolivia, have previously had several HPS outbreaks (*14*). This is the first report to describe the presence of hantaviruses and HPS cases in Panama, Central America.

Between December 1999 and February 2000, cases of acute febrile respiratory illness exhibiting typical symptoms of HPS were identified in the town of Las Tablas in Los Santos Province, Panama. Serologic testing of samples obtained from the patients and trapped rodents indicated antibody reactivity with prototype Sin Nombre (SN) virus antigens. Further analyses of nucleotide sequences of S and M segment fragments amplified by polymerase chain reaction (PCR) indicate that the virus responsible for the outbreak in Panama has unique and novel genetic sequences. We have also tentatively identified *Oligoryzomys fulvescens* as the reservoir for this virus as evidenced by nearly 100% identity of viral nucleotide sequences obtained from both HPS patients and *O. fulvescens.*

Results. Detection of Hantavirus Infection in Patient Samples. Seven patients were found to be positive for the presence of hantavirus-specific antibodies, on initial

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TABLE 1

Primer Sequences Used to Amplify Nucleocapsid, G1, and G2 Diagnostic Fragments

Gene	Primer name	Sequence	Product size
Nucleocapsid	SS143C-N	TGGA(C/G)CCIGATGAIGTTAACAA	601 nt
(First round)	SS743R-N	TCIATCCA(G/A)TC(C/T)TTIACAAA	
Nucleocapsid	SS283C-N	CCAACAGGGITTGA(A/G)CC(T/A)GATGA	434 nt
(Second round)	PPT716R	AAICCIATIACICCCAT	
G1	SM1676C-N	GAITAICAIAA(G/A)AC(T/A)ATGGG	581 nt
(First round)	SM2256R-N	TT(A/G)AATGTI(G/C)CATCCATCCA	
G1	SM1691C-N	ATGGGITCIATGGTITGTGA	329 nt
(Second round)	SM2019R-N	TCIGCACTIGC(T/A)GCCCA	
G2[a]	SM2779C	TGTGAITATCAAGGIAAIAC	350 nt
(First round)	SM3128R	ACIG(A/T)IGCICCCATAICACAT	
G2[a]	SM2779C	TGTGAITATCAAGGIAAIAC	242 nt
(Second round)	SM3020R	CCCCAIGCICCITCAAT	

[a] Ref. (*8*).

screening of patient sera by enzyme-linked immunosorbent assay (ELISA) using SN virus antigens (*6*). Reverse transcriptase-PCR (RT-PCR) and subsequent nested PCR assays were performed on the seven antibody-positive, acute-phase sera from patients by using primers listed in Table 1. The primers specific for the nucleocapsid, G1, and G2 regions of the virus genome amplified 434-, 329-, and 242-bp bands, respectively, from the sera of six of the seven seropositive patients. The results indicated that the diagnostic primers previously used for the detection of Sigmodontinae-associated hantaviruses (*8*; Shoemaker and Nichol, unpublished results) successfully amplified the viral sequences from the serum samples of these HPS patients. No products were obtained from sera of one antibody-positive patient, most likely because of insufficient (30 μl) sample available.

Panamanian HPS-Associated Virus Is Genetically Distinct. The nucleotide and deduced amino acid sequences obtained from PCR products representing fragments of the nucleocapsid, G1, and G2 encoding regions of the Panama HPS-associated virus genome were compared with those from corresponding regions of previously characterized hantaviruses (Table 2). The 387-nucleotide nucleocapsid-encoding sequence fragment differed from the equivalent fragment of North American hantaviruses, such as SN, El Moro Canyon, and Black Creek Canal virus, by 19.9 to 24.3% and differed from South American hantaviruses, such as Laguna Negra, Oran, Castelo dos Sonhos, and Andes viruses, by 19.6 to 24.0%. Considerable differences were also evident on comparison of the deduced amino acid sequences (128 aa), with at least 3.9 to 6.3% differences in amino acid identity seen relative to South American hantaviruses, including Andes and Laguna Negra viruses and Castelo dos Sonhos. The nucleocapsid protein amino acid differences between the Panama outbreak virus and North American hantaviruses were in the range of 5.5 to 9.4%. The extent of difference observed in nucleotide and deduced amino acid sequences for this relatively conserved nucleocapsid region signifies that the virus detected in these Panamanian HPS cases represents a distinct hantavirus.

Similarly, considerable differences were seen on the basis of comparison of the 259-nucleotide fragment containing the region encoding part of the G1 protein. Differences in nucleotide sequences of the Panama HPS

TABLE 2

Comparison of Nucleotide and Deduced Amino Acid Differences between Choclo Virus and Representative North and South American Hantaviruses

Segment (protein) (*O. fulvescens* and patients)	% Nucleotide (amino acid) difference								
	SN	NY	BCC	ELMC	AND	LN	CAS	ARA	Calabazo
S (nucleocapsid)	23.3 (5.5)	24.0 (6.3)	24.0 (9.4)	19.9 (8.6)	20.9 (4.7)	19.6 (5.5)	24.0 (3.9)	21.4 (6.3)	22.0 (5.5)
M (G1)	26.3 (15.1)	28.6 (18.6)	25.9 (14.0)	30.5 (18.6)	25.5 (15.1)	29.7 (18.6)	30.9 (17.4)	27.4 (18.6)	25.1 (17.5)
M (G2)	26.3 (23.1)	27.3 (23.1)	30.3 (24.6)	31.3 (21.5)	22.2 (15.4)	23.7 (15.4)	22.7 (13.9)	27.7 (18.5)	22.7 (18.5)

Note. SN, Sin Nombre; NY, New York; BCC, Black Creek Canal; ELMC, El Moro Canyon; AND, Andes; LN, Laguna Negra; CAS, Castelo dos Sonhos; ARA, Araraquara.

virus compared with other known Sigmodontinae-associated hantaviruses ranged from 25.1 to 30.9%. The difference was also reflected at the amino acid level, for which approximately 14.0 to 18.6% differences were observed. The deduced viral G1 amino acid sequences from the patients were 35 and 50% different, respectively, from more distantly related Puumala and Hantaan viruses. A 64-amino-acid G2 coding region differed in the range of 21.5 to 24.6% from North American hantaviruses and in the range of 13.9 to 18.5% from the majority of South American hantaviruses. The divergence of G2 was as much as 42 and 48% from Puumala and Hantaan viruses, respectively. Thus, the differences in both nucleotide and amino acid sequences of the nucleocapsid, G1, and G2 proteins indicate that the hantaviruses responsible for the HPS outbreak in Panama are thus far unidentified viruses with novel genetic sequences. Comparison of amino acid sequences of the virus nucleocapsid, G1, and G2 from all the patients showed 99 to 100% identity to one another, indicating that a single virus was responsible for the HPS outbreak. Our attempts to isolate the viruses from the hantavirus antibody-positive human and rodent samples using Vero E6 cells were unsuccessful.

Serologic and Genetic Search for Virus Rodent Reservoir. Specific hantaviruses are usually associated with a distinct rodent host in which the virus establishes a chronic, asymptomatic infection. Identification of the rodent reservoir is important in understanding human hantavirus diseases associated with specific rodent hosts as well as the virus biology in its natural host, predicting the probabilities of disease outbreaks, and establishing measures of prevention. Rodents were trapped from the outbreak area, and blood samples were screened for the presence of hantavirus-specific antibodies. Of 120 rodents collected, 6 (5%) had IgG antibodies reactive with SN virus antigens. The positive rodents included 4 of 50 cane mice, *Zygodontomys brevicauda,* and 2 of 15 pygmy rice rats, *O. fulvescens.* Tissues from the antibody-positive animals were further screened by RT-PCR and nested PCR, using the primers successfully used to detect the hantavirus in the human samples. Specific virus N, G1, and G2 region PCR products of appropriate sizes were obtained for all of the antibody-positive rodents.

The deduced virus amino acid sequences obtained from one patient and *O. fulvescens* matched 100% of nucleocapsid and G1 proteins and 98.5% of G2 protein. This high degree of identity strongly implicates *O. fulvescens* as the vector for the virus causing the recent hantavirus outbreak in Panama. On the other hand, nucleotide differences of 22.0, 25.1, and 22.7%, for N, G1, and G2, respectively, were seen when virus gene sequences obtained from patients and *O. fulvescens* were compared with those obtained from *Z. brevicauda* (Table 2). The nucleotide and amino acid sequences obtained from *Z. brevicauda* did not match the viral sequences obtained from the patients, suggesting that the hantavirus harbored in *Z. brevicauda* was not the cause of the current outbreak.

Comparison of Viral Sequences from O. fulvescens and Z. brevicauda. Interesting differences were noted between the virus gene sequences obtained from the two rodent species (Table 2). At the nucleotide level, an approximately 22% difference was noted between the N sequences of the hantavirus harbored in *O. fulvescens* (Choclo virus) and that found in *Z. brevicauda* (Calabazo virus); the corresponding difference in a 128-amino-acid region was 5.5%. Significant differences were also observed in their M segments. For example, the variation in the amino acids of G1 and G2 amino acids were 17.5 and 18.5%, respectively. The extensive variation confirms that the viruses harbored in *Z. brevicauda* and *O. fulvescens* are different in their nucleotide and amino acid sequences and do not match any of the hantavirus sequences identified so far.

Phylogenetic analysis of the nucleotide sequences of the hantavirus genome fragments containing the G1 and G2 regions also supports the distinct nature of the viruses harbored in *Z. brevicauda* and *O. fulvescens* and suggests their placement between the viruses associated with the North American peromycine rodents and the main group of viruses associated with the South American sigmodontine rodents (Fig. 1).

Discussion. Since the identification of SN virus as the cause of HPS (*13*), numerous cases of this acute illness have been confirmed in various regions of the United States and Canada and central and southern South America. This is the first report of confirmed HPS cases and the identification of the rodent reservoir in the broad central region of the Americas. Interestingly, not just one but two newly discovered hantaviruses were identified during this outbreak study: Choclo virus associated with the HPS cases and *O. fulvescens* and Calabazo virus associated with *Z. brevicauda.*

The North American prototype hantavirus (SN virus) and the Panamanian patient sequences differed by 5.5% in the amino acids of the nucleoprotein. The variation was much higher in the amino acids of G1 and G2 proteins (15.1 and 23.1%, respectively). Similarly, modest variation in the nucleocapsid and more extensive variation in G1 and G2 amino acid sequences were noted when the Panamanian viruses were compared with North and South American hantaviruses. It is reasonable to attribute the lesser variation in the nucleocapsid protein to the lesser selection pressure it must undergo compared to the viral surface glycoproteins.

Several sigmodontine rodents, and particularly members of the genus *Oligoryzomys,* have been shown to be hantavirus reservoirs, including Bermejo, Lechiguanas,

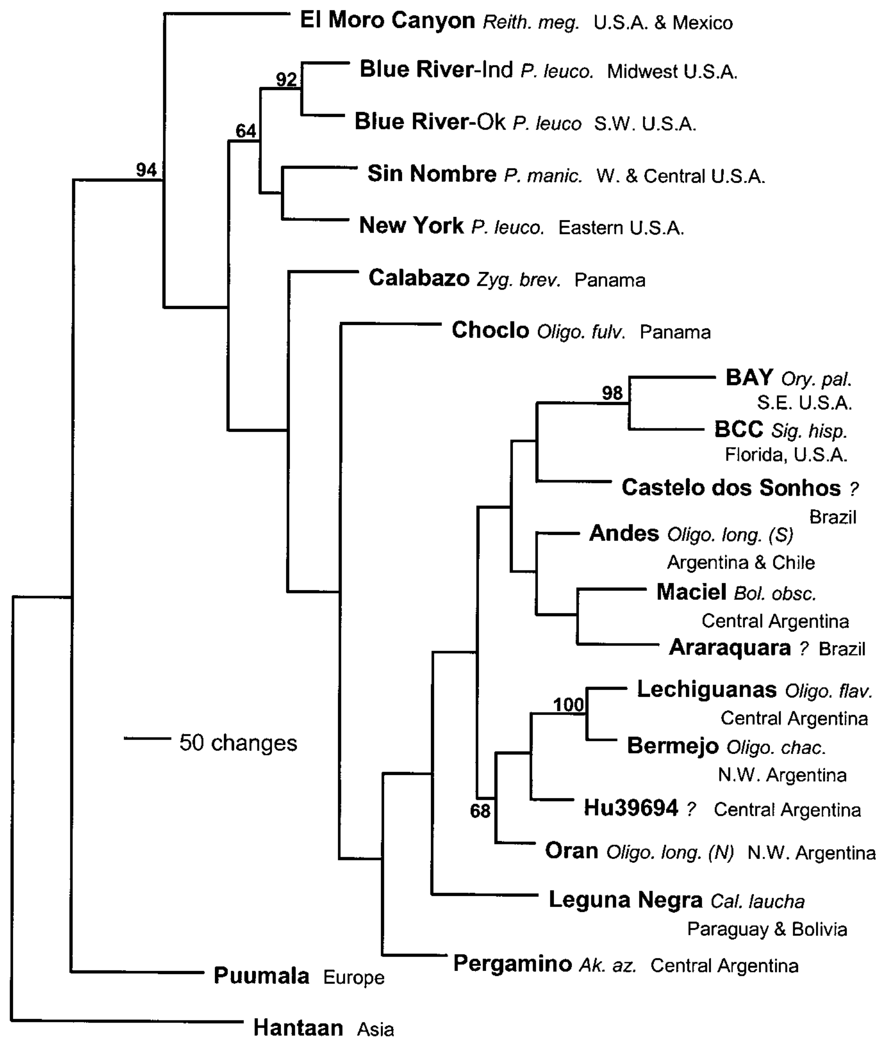

FIG. 1. Phylogenetic analysis of hantavirus M segment nucleotide sequences. Phylogenetic analysis of the M segment sequence fragments (259-nt G1 encoding and 198-nt G2 encoding regions) was carried out by the maximum-parsimony method using PAUP*4.0 and a 3:1 weighting of transversions over transitions. A single most-parsimonious tree was obtained. Horizontal distances represent nucleotide step differences (see scale bar), while vertical branches are for visual clarity only. Bootstrap analysis was carried out on 500 pseudo-replicates of the data set, and values greater than 50% are shown on the tree. Each label includes the virus name, primary rodent reservoir species, and known geographic distribution of the virus. BAY, Bayou; BCC, Black Creek Canal; *Reith. meg., Reithrodontomys megalotis; P. leuco., Peromyscus leucopus; P. manic., Peromyscus maniculatus; Zyg. brev., Zygodontomys brevicauda; Oligo. fulv., Oligoryzomys fulvescens; Ory. pal., Oryzomys palustris; Sig. hisp., Sigmodon hispidus; Oligo. long. (S), Oligoryzomys longicaudatus,* southern form; *Bol. obsc., Bolomys obscurus; Oligo. flav., O. flavescens; Oligo. chac., Oligoryzomys chacoensis, Oligo. long. (N), O. longicaudatus,* northern form; *Cal. laucha, Calomys laucha; Ak. Az., Akadon azarae;* ?, unknown host. GenBank accession numbers for sequences are as follows: El Moro Canyon, U26828; Blue River IND and OK, AF030551 and AF030552; Sin Nombre, L25783; New York, U36801; BAY, L36930; BCC, L39950; Castelo dos Sonhos, (Ref. *19*); Andes, U51040, U51041, AF042119-AF042121; Maciel, AF028027; Araraquara, (Ref. 19); Lechiguanas, AF028022; Bermejo, AF028025; Hu39694, AF028023; Oran, AF028024; Leguna Negra, AF005728; Pergamino, AF028028; Puumala, X61034; Hantaan, M14627.

Andes, and Oran (*10–12*) viruses in South America. In most cases, a defined rodent host serves as the reservoir for each virus (*2, 3, 16*) and the predominant species of rodents captured in an outbreak locality have been demonstrated to be the virus reservoirs. Of the rodents captured during the Panama investigation, 50 were identified as *Z. brevicauda cherriei* and only 15 were identified as *O. fulvescens.* Thus, it is a little unusual that the virus responsible for the HPS outbreak was not harbored by the most abundant rodent, *Z. brevicauda,* but by the less abundant *O. fulvescens.* Obviously, rodent habitat and relative trap location can strongly influence the trap success for different species. It is interesting to note that one antibody- and PCR-positive *O. fulvescens* was captured at the household of a confirmed HPS patient.

Our studies also provide evidence for the presence of another novel hantavirus in the predominantly captured rodent, *Z. brevicauda.* The nucleotide and the deduced amino acid sequences of nucleocapsid, G1, and G2 fragments obtained from the tissues of *Z. brevicauda* are

consistently different from those obtained from the patients and *O. fulvescens.* Matching sequences were not obtained from any of the viruses detected from human patients or from other already characterized hantaviruses, showing that the *Z. brevicauda*-borne virus was yet another newly discovered hantavirus. The absence of an association of Calabazo virus with HPS cases may reflect the lower pathogenicity of this virus to humans. However, it may just reflect less contact of the infected rodents with humans. Since *Z. brevicauda* was the predominant species captured in that area, it raises the possibility that this rodent-borne virus may also emerge as a potential human pathogen under favorable environmental conditions that may favor higher numbers of infected members of this species or in some other way increasing its contact with humans.

Epidemiologic studies indicate several interesting features about this outbreak. The case fatality during the outbreak in Panama was low (25%) compared with previous outbreaks in Chile (54%), southwestern Argentina (58%), and the United States (42%) (*10*). *Oligoryzomys*-borne hantaviruses, such as Lechiguanas from central Argentina, Oran from northwestern Argentina, and Andes from southwestern Argentina, have been shown to cause high mortality (up to 58%). On the other hand, Laguna Negra virus isolated from western Paraguay and Bolivia (*8*) and the present virus from Panama appear to be linked to lower case-fatality rates. In addition, antibody-positive humans were as high as 31% in some towns within the outbreak area (CDC, unpublished data). It remains to be seen whether the Calabazo virus harbored in *Z. brevicauda* is responsible for the unusually high prevalence of hantavirus antibody in humans observed in some of the outbreak areas, resulting in a mild or unapparent infection. A detailed study of the sequences of viruses obtained from both the rodent species, as well as studies regarding host tropism and their replication in their natural hosts, should provide better insight into these novel hantaviruses.

Materials and Methods. Extraction of RNA from human and rodent specimens. Serum samples from patients and blood samples of rodents captured in the outbreak area were initially screened for hantavirus-specific antibodies by ELISA (*6*). Positive samples in the ELISA were further characterized to confirm the etiologic agent by RT-PCR and sequence analysis. RNA was isolated from the sera of patients and tissues of rodents by following methods described previously (*8*). In addition, a blood clot obtained from one antibody-positive patient was used for RNA isolation and subsequent RT-PCR and sequencing analysis.

RT-PCR, Sequencing, and Analysis. RNA obtained from the samples was subjected to reverse transcription and first round polymerase chain reaction using the Access RT-PCR kit (Promega Corp., Madison, WI). The sequences of some of the primers used and the cycle conditions were previously published (*8*) and the sequences of the new primers are presented in Table 1. The products obtained from the second PCR were electrophoresed on a 1.5% agarose gel using a Tris–acetate/EDTA buffer system to identify the products of appropriate sizes and to purify them. The DNA fragments were eluted from agarose using the Qiaex II gel extraction kit (Qiagen Inc., Valencia, CA) and sequenced by following the dye termination cycle sequencing method (Applied Biosystems Inc., Foster City, CA). Sequences were analyzed by Sequencher version 3.1.1 (Gene Codes Corp., Ann Arbor, MI) and comparisons of the human and rodent virus sequences as well as those of other hantaviruses were performed by the SEQLAB version of the Wisconsin Sequence Analysis Package (Genetics Computer Group, Inc., Madison, WI). Phylogenetic analysis was done using the PAUP* version 4.0b4a Macintosh computer software program (*18*). Maximum-parsimony analysis of virus RNA nucleotide differences was performed using a 3:1 weighting of transversions to transitions.

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