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Allpahuayo Virus: A Newly Recognized Arenavirus (*Arenaviridae*) from Arboreal Rice Rats (*Oecomys Bicolor* and *Oecomys Paricola*) in Northeastern Peru

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Allpahuayo virus was initially isolated from arboreal rice rats (*Oecomys bicolor* and *Oecomys paricola*) collected during 1997 at the Allpahuayo Biological Station in northeastern Peru. Serological and genetic studies identified the virus as a new member of the Tacaribe complex of the genus *Arenavirus.* The small (S) segment of the Allpahuayo virus prototype strain CLHP-2098 (Accession No. AY012686) was sequenced, as well as that of sympatric isolate CLHP-2472 (Accession No. AY012687), from the same rodent species. The S segment was 3382 bases in length and phylogenetic analysis indicated that Allpahuayo is a sister virus to Pichinde in clade A. Two ambisense, nonoverlapping reading frames were identified, which result in two predicted gene products, a glycoprotein precursor (GPC) and a nucleocapsid protein (NP). A predicted stable single hairpin secondary structure was identified in the intergenic region between GPC and NP. Details of the genetic organization of Allpahuayo virus are discussed.

Key Words: Arenavirus; Allpahuayo virus; zoonoses; rice rats; Peru; S segment.

INTRODUCTION

To date, 15 different arenaviruses have been identified in the Americas (Tesh *et al.,* 1999). The sites of the original isolation and the presumed principal vertebrate hosts for each of the known New World arenaviruses are shown in Fig. 1. Most of these viruses have a rather restricted geographic distribution, and in some cases they are sympatric. In general, a single vertebrate species, usually a rodent, is believed to serve as the natural reservoir host for each virus (Buchmeier *et al.,* 1995; Bowen *et al.,* 1997; Childs and Peters, 1993).

Under the current taxonomic classification of the family Arenaviridae, there are two serogroups (complexes) within the genus *Arenavirus:* the Old World or lymphocytic choriomeningitis–Lassa (LCM–LAS) complex and the New World or Tacaribe complex (Buchmeier *et al.,* 1995). On the basis of their antigenic and phylogenetic relationships, the Tacaribe complex can be further subdivided into three lineages (Bowen *et al.,* 1997): lineage A, which includes Tamiami (TAM), Pichinde (PIC), Flexal (FLE), Parana (PAR), White Water Arroyo (WWA), and Pirital (PIR) viruses; lineage B containing Junin (JUN), Tacaribe (TAC), Sabia (SAB), Machupo (MAC), Guanarito (GUA), Amapari (AMA), and Cupixi viruses (Bowen, unpublished data); and lineage C comprising Latino (LAT) and Oliveros (OLI) viruses.

The members of the Arenaviridae family share various molecular properties. (1) They are lipid-enveloped, single-stranded RNA viruses, and (2) they have genomes consisting of two RNA molecules, large (L) and small (S), which are approximately 7 and 3.5 kb, respectively (Southern, 1996). The L segment encodes a viral RNA-dependent RNA polymerase and the Z protein, a potential structural/regulatory protein (Iapalucci *et al.,* 1989; Salvato *et al.,* 1988; Singh *et al.,* 1987). The S segment encodes the nucleocapsid protein (NP) and the precursor of the envelope glycoproteins (GPC) in an ambisense fashion (Bishop, 1986). The NP is translated from a complementary sense mRNA that is encoded in the 3′ half of the viral S RNA (Auperin *et al.,* 1984; Romanowski and Bishop, 1985; Clegg and Oram, 1985; Franze-Fernandez *et al.,* 1987). The GPC protein is translated from a viral sense mRNA encoded in the 5′ end of the viral S RNA (Auperin *et al.,* 1984, 1986; Romanowski *et al.,* 1985; Franze-Fernandez *et al.,* 1987). To date, all described arenaviruses have noncoding 3′ and 5′ regions as well as a noncoding intergenic region between the GPC and NP genes in the S segment.

The purpose of this report is to describe the antigenic and genetic characteristics of a new member of the Tacaribe complex, recently isolated from arboreal rice rats (*Oecomys bicolor* and *Oecomys paricola*) in the Amazon Region of northeastern Peru. The name of "All-

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FIG. 1. Map showing the original isolation sites of the 16 known Tacaribe complex viruses and their presumed reservoir hosts in the Americas.

pahuayo" has been proposed for this newly recognized virus after its place of origin.

RESULTS

Animal collections and virus isolations

A total of 952 small mammals (738 rodents and 214 marsupials) was trapped at the Allpahuayo Biological Station between July 1997 and November 1998. The genus and species of a few of these animals are still uncertain; definitive identification of the specimens and analysis of the collection data will be reported separately and constitute the doctoral thesis of one of the authors (C.L.H.).

Of the 952 small mammals, 679 were tested for virus by culture in Vero cells (see Materials and Methods). Although several other viruses (Venezuelan equine encephalitis, group C, and a new phlebovirus) were isolated from the animals, arenaviruses were only obtained from the rice rats. Six isolations of a new arenavirus, designated Allpahuayo virus, were made from 41 arboreal rice rats (38 *O. bicolor,* 2 *O. paricola,* and 1 *Oecomys roberti*). Five of the six virus-positive rodents were identified as *O. bicolor;* the sixth was identified as *O. paricola.* Four of the six arenaviral isolates were obtained from rats captured in arboreal traps. The first isolate, CLHP-2098, from an *O. bicolor,* was designated as the prototype. The original isolate was made in Vero cells, but newborn mice subsequently inoculated intracerebrally with CLHP-2098 became sick on the seventh day after infection. Adult mice inoculated intraperitoneally with strain CLHP-2098 did not show signs of illness, but developed virus-specific antibodies as demonstrated by complement fixation (CF) and immunofluorescent antibody (IFA) tests.

CF tests with Allpahuayo virus

The reciprocal antibody and antigen titers are shown in Table 1 in a pairwise comparison of Allpahuayo with selected Tacaribe complex arenaviruses. These comparisons indicated that Allpahuayo was antigenically distinct from the other lineage A arenaviruses, but was most closely related to Pichinde and Parana viruses.

Transmission electron microscopy of ultrathin sections

At the surface of Vero cells, we observed groups of virions with ultrastructure typical for arenaviruses: round or slightly oval in shape, from 70 to 180 nm in diameter with dense internal granules measuring from 15 to 30 nm, and clearly defined envelope. The envelope of the virion has projections about 7 nm long with a periodicity of 10 nm (Fig. 2).

TABLE 1

Comparison of Complement Fixation Results for Allpahuayo Virus with Other Selected Tacaribe Complex Arenaviruses

	Antibodies						
Antigens	ALL	PIC	FLE	WWA	PAR	TAM	PIR
ALL	256/≥32[a]	64/≥32	64/16	8/8	128/32	<8/8	8/16
PIC	64/≥32	≥256/32					
FLE	32/≥32		≥256/32				
WWA	16/≥32			128/≥32			
PAR	64/≥32				≥512/32		
TAM	32/32					≥256/32	
PIR	8/16						256/16

[a] Reciprocal of highest antibody titer/reciprocal of highest antigen titer.

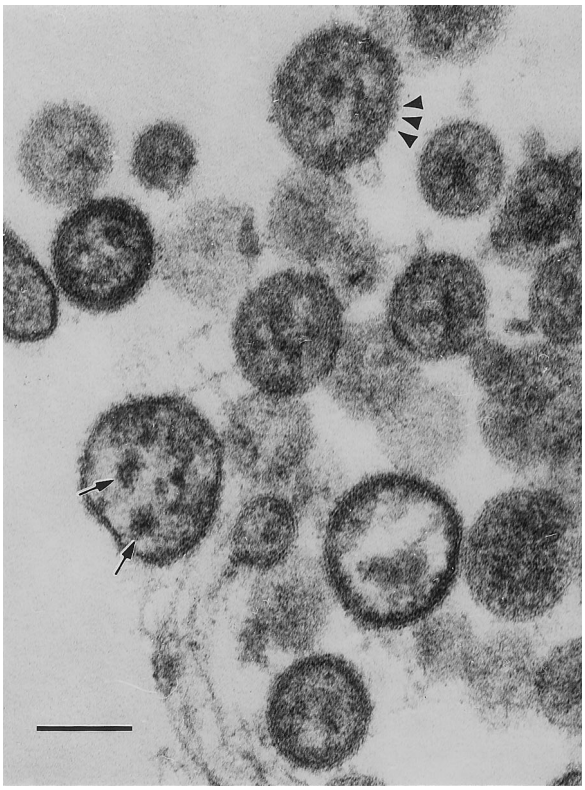

FIG. 2. A group of arenavirus virions at the surface of Vero cell. Virions vary in size, are mostly round or slightly oval, and have dense granules inside (arrows). The surface of the virions bears projections about 7 nm long with a periodicity of 10 nm (arrowheads). Bar = 100 nm.

Nucleotide sequence of Allpahuayo virus S RNA

The S segment sequence we determined for Allpahuayo virus strain CLHP-2098 was 3363 nucleotides in length, excluding the 3′ terminal 19 nucleotides where the cDNA primer presumably annealed (Fig. 3). The S segment of CLHP-2472 was also sequenced except for the 3′ and 5′ noncoding ends. The GPC gene was made up of 1548 nucleotides and the NP gene of 1699 nucleotides. The GPC open reading frame was situated between nucleotides 51 and 1598 in the sense direction. The NP reading frame was situated between nucleotide 3352 and 1654 in the antisense direction. The two open reading frames (ORFs) encoding N and GPC were arranged in opposite polarities (ambisense). The intergenic noncoding region of the S segment consists of 74 nucleotides.

Phylogenetic analysis using complete GPC and NP gene sequences

The Allpahuayo virus GPC and NP amino acid sequences were aligned with other arenavirus GPC and NPs. In both comparisons, using all methods, the Old World viruses, Lassa and LCM, were grouped together (Fig. 4). New World viruses were grouped into three clades with Allpahuayo virus falling into Clade A together with Pichinde. The bootstrap value for this clade was 100%.

Phylogenetic analysis using partial S sequences

Phylogenetic analyses were also conducted using partial S segment sequences (a region of approximately 612–621 nucleotides in length encompassing partial GPC and NP regions; see Bowen *et al.,* 1996, 1997) of prototype strains of Old and New World arenaviruses. In nucleotide analyses using maximum parsimony, both with and without transversion weighting, Allpahuayo grouped with Flexal and Parana viruses. However, this grouping was supported by bootstrap values of only 61 and 57%, respectively. Using neighbor joining with nucleotide sequences resulted in the same grouping with a bootstrap value of 66%. Maximum likelihood grouped Allpahuayo with Pichinde together, as did maximum parsimony and neighbor joining analyses using amino acid sequences; bootstrap values were 72 and 88% for parsimony and neighbor joining, respectively. The neighbor-joining tree is shown in Fig. 4. By analysis of a conserved S segment region in the N protein, Allpahuayo prototype strain CLHP-2098 has a 74.6% amino acid sequence identity to Pichinde virus, 68.3% to Flexal, 66.3% to Parana, 64.4% to Pirital, 55.6% to White Water Arroyo, and 52.7% to Tamiami viruses.

Allpahuayo virus GPC and amino acid homology of arenavirus GPC gene product

The long GPC open reading frame is initiated by an AUG codon found at nucleotides 51–53 and terminated at a UAA termination codon at positions 1572–1574. The Allpahuayo virus GPC amino acid sequence was aligned with the GPC sequence of Tacaribe, Pichinde, Oliveros, lymphocytic choriomeningitis (LCM)–Armstrong, Lassa–Josiah, and Junin viruses (Fig. 5). As shown previously for other arenaviruses (Ghiringhelli *et al.,* 1991), the alignment showed conserved amino acid identity in two regions of the GPC translation product: (1) 57 amino acid residues at the N-terminus and (2) the C-terminal portion of GPC (data not shown). The Allpahuayo GPC sequence was 79% identical to Pichinde virus and 40–48% identical to Junin, Tacaribe, Sabia Oliveros, Lassa, and LCM viruses. The GPC gene of Allpahuayo virus encodes a predicted 512 amino acid protein. We identified nine putative glycosylation sites and a potential proteolytic cleavage site in a conserved region, which may cleave the GPC molecule into G1 and G2 proteins (Fig. 3). This possible cleavage site occurs at amino acid region RQLLGFF (Burns and Buchmeier, 1993).

Allpahuayo virus NP gene

The first AUG of the complementary S RNA sequence was observed at position 3328–3330 in the antisense

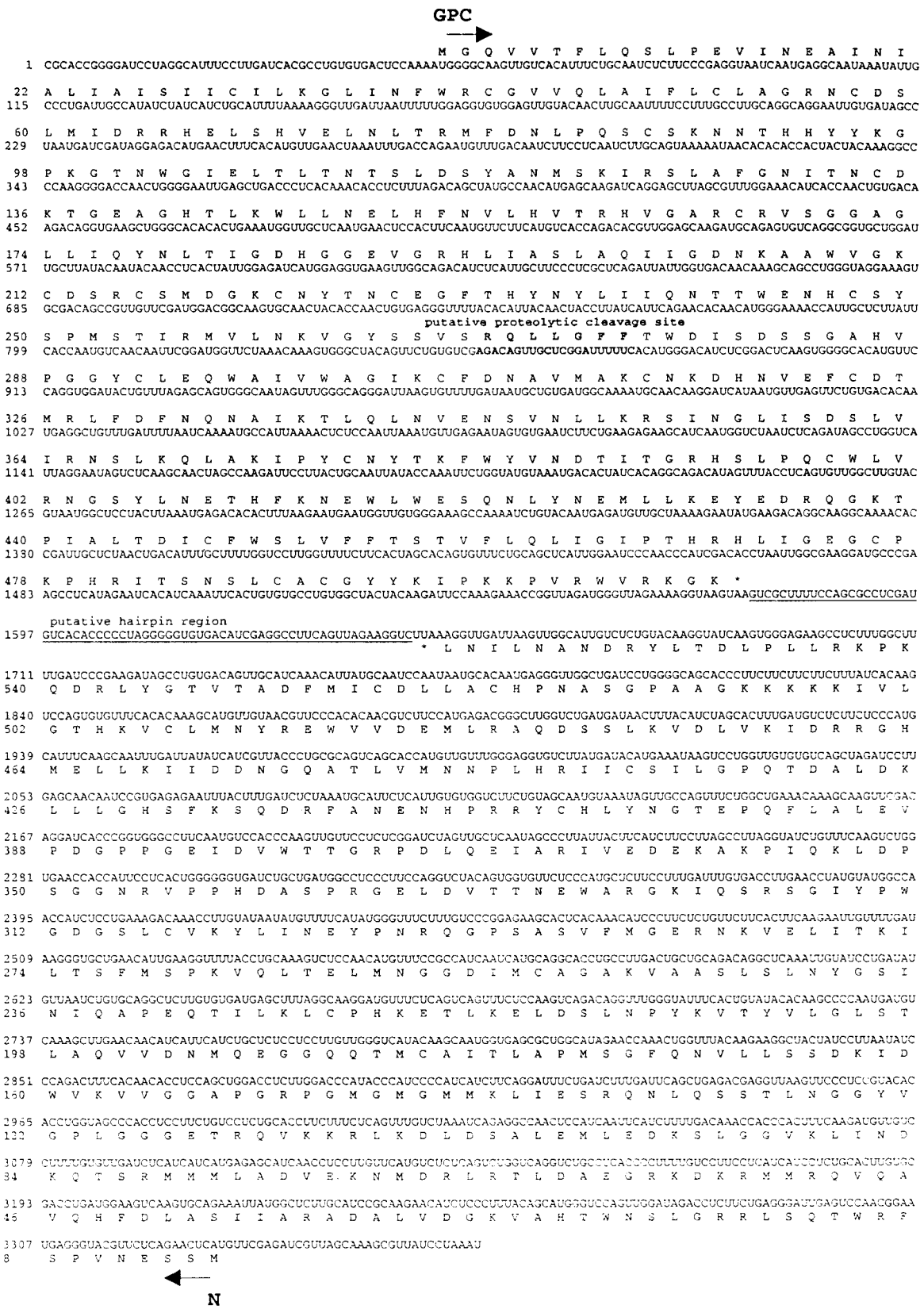

FIG. 3. Nucleotide sequence of Allpahuayo v S RNA. The 3391 nucleotide region of the viral S RNA is shown. The amino acid sequences of the envelope glycoprotein complex (GPC) and nucleocapsid protein (NP) translation products are shown in association with each ORF. The beginning and direction of each ORF are indicated by arrows. Translation termination codon and anticodon of GPC and N genes, respectively, are indicated by asterisks. Nucleotide numbering begins at the 5′ end of the S RNA. Amino acid residue numbering begins at the N-terminus of each protein.

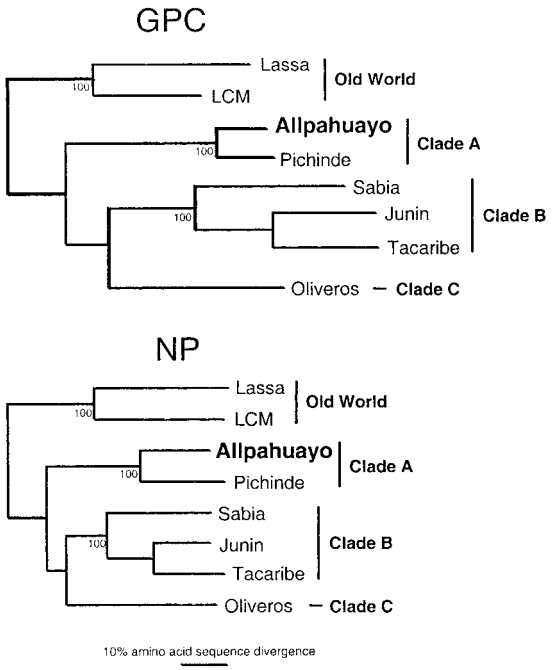

FIG. 4. Arenavirus phylogeny generated from deduced amino acid sequences of complete NP and GPC genes using the neighbor joining method. Scale bar indicates 10% amino acid sequence divergence. Viruses are divided into the Old World arenavirus group and the three lineages of the Tacaribe complex, labeled A, B, and C. Numbers indicate bootstrap values for groups defined by adjacent nodes.

direction (Fig. 3). This is the beginning of a long ORF that terminates at a UAA stop codon at nucleotides 1645–1647. The Allpahuayo virus NP showed a high degree of amino acid sequence conservation when compared to other arenaviruses (Fig. 5), sharing 76% sequence identity with Pichinde virus and between 51 and 58% identity with the other arenaviruses. The Allpahuayo NP gene encodes a 560 amino acid protein, including 83 basic or positively charged amino acid residues (lysine, arginine, and histidine).

Intergenic region of Allpahuayo virus S RNA

A very stable hairpin loop secondary structure was predicted in the noncoding region downstream from the termination codons of N and GPC genes (from nucleotides 1575 to 1644 of v S RNA) (Figs. 3 and 6). This hairpin is stabilized by 18 pairs of nucleotides: 12 GC and 6 AU pairs.

5′ end of Allpahuayo virus S RNA

The noncoding upstream region of Allpahuayo virus GPC gene is made up of 50 nucleotides of the 5′ end of the S RNA. Although the 3′ end of the S RNA was not directly sequenced, it is likely to be similar to the conserved end of other arenaviruses since the oligonucleotide 19C, 5′ CGCACAGTGGATCCTAGGC 3′, formed a portion of the primer (M13-19C) used in cDNA synthesis (Ghiringhelli *et al.,* 1991).

DISCUSSION

We have identified Allpahuayo virus by serological and molecular studies as a newly recognized species in lineage A of the Tacaribe complex of New World arenaviruses. It appears to be most closely related to Pichinde virus. The genetic organization of Allpahuayo virus is similar to other arenaviruses (Auperin *et al.,* 1884, 1986; Romanowski *et al.,* 1985; Franze-Fernandez *et al.,* 1987; Ghiringhelli *et al.,* 1991). The typical proteolytic cleavage site proposed by Buchmeier *et al.* (1987) comprises a dibasic amino acid motif, typically two arginines (LCM, Lassa, Junin), but can comprise a different basic amino acid such as lysine (found in Pichinde virus). We observed a dibasic amino acid motif of two lysines as

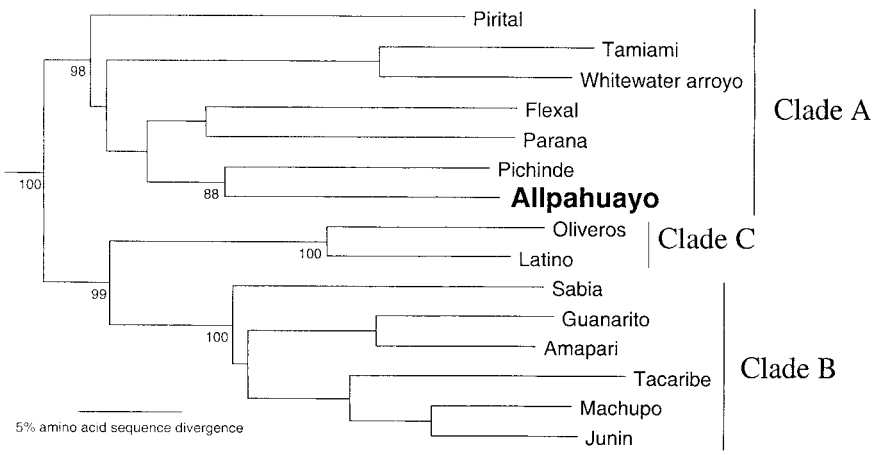

FIG. 5. Phylogenetic analysis of New World arenaviruses based on deduced, partial amino acid sequences of the nucleocapsid protein genes of Allpahuayo virus and other arenaviruses using neighbor-joining analysis. The tree was rooted using homologous Lassa, LCM, and Mopeia virus sequences as an outgroup. Numbers indicate bootstrap values for groups defined by adjacent nodes. The scale indicates 10% amino acid sequence divergence.

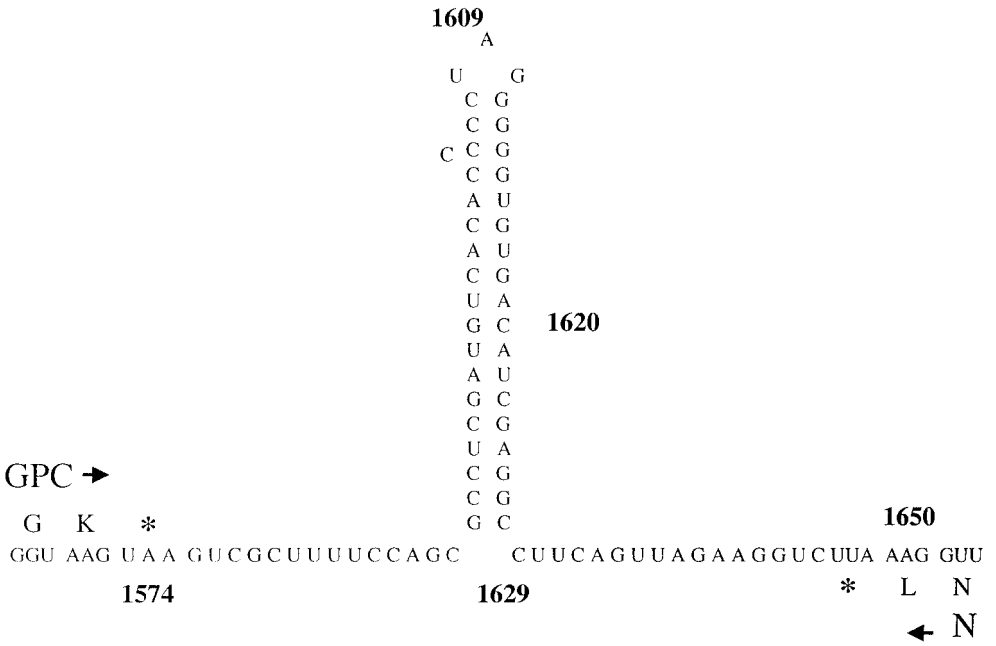

FIG. 6. Intergenic region of Allpahuayo S RNA. Nucleotides 1598 to 1657 with the ends of the GPC and N genes (sense and antisense strand, respectively) and the noncoding intergenic noncoding regions are shown. Deduced translation products are indicated as well as the direction of translation. Asterisks indicate the stop codon and anticodon for the GPC and N genes, respectively.

comprising the putative proteolytic site of Allpahuayo virus.

The intergenic noncoding region of Allpahuayo virus was shown to form a predicted hairpin structure that is stabilized by 12 GC and 6 AU nucleotide pairs. Single hairpin structures are found in the majority of arenaviruses so far described, e.g., LCM, Lassa, and Pichinde viruses (Romanowski and Bishop, 1985; Auperin *et al.,* 1984; 1986; Franze-Fernandez *et al.,* 1987). Double hairpin loops have been described in Junin, Tacaribe, and Mopeia viruses (Wilson and Clegg, 1991; Ghiringhelli *et al.,* 1991). The finding of a single hairpin loop in Allpahuayo virus is not surprising considering its similarity with Pichinde.

Allpahuayo isolates CLHP-2098 and CLHP-2472 had 14.4% nucleotide sequence divergence when comparing a conserved region of the NP gene. These isolates were obtained from the same forest during the same year. Larger genetic variations have been observed among other arenaviruses, where considerable nucleotide divergence was found in virus isolates separated by less than 35 km (Fulhorst *et al.,* 1999; Weaver *et al.,* 2000; Garcia *et al.,* 2000; Bowen *et al.,* 2000). Differences of up to 20.8% nucleotide divergence were found among Pirital virus isolates separated by less than 35 km (Fulhorst *et al.,* 1999). Nineteen percent nucleotide sequence divergence has been detected among Guanarito isolates in Venezuela separated by only ca. 300 km (Weaver *et al.,* 2000), and considerable divergence was seen between strains isolated at the same site. The extent of sequence divergence observed in our study is quite large given that Allpahuayo isolates were within the same forest. Studies need to be conducted to better understand the evolution of genetic diversity among arenavirus populations in their rodent hosts.

It is not known if Allpahuayo poses a public health threat. To date there have been no human cases of arenavirus infection reported in Peru. Allpahuayo is a member of clade A of the Tacaribe complex, while most of the New World arenaviruses causing hemorrhagic fever in humans are included in clade B (Bowen *et al.,* 1997). However, Pichinde and Flexal viruses (other members of clade A) have produced a nonhemorrhagic febrile illness in accidentally infected laboratory workers, and Whitewater Arroyo virus has recently been associated with three fatal cases of hemorrhagic fever in California (CDC, 2000). In any case, there are no people presently living in the Allpahuayo Forest reserve where this virus was isolated, so the risk to humans at this time would appear to be low. The range of Allpahuayo virus is unknown; however, one of its presumed natural hosts, *O. bicolor,* is widely distributed in the Amazon Basin (Eisenberg and Redford, 1999).

MATERIALS AND METHODS

Study site

Small mammal collections were made at the Allpahuayo Biological Station, located 3°54′S, 73°25′W at 120–140 met elevation, about 25 km southwest of the city of Iquitos, Loreto Department, Peru. The Biological Station encompasses approximately 2750 hectares, primar-

ily of primary forest, with patches of secondary forest and a few scattered agricultural plots; it is owned and managed by the Instituto de Investigaciones de la Amazonia Peruana (IIAP). The climate of the region is tropical with an average temperature of 25.9°C and the mean annual precipitation of 2949 mm. One of the most striking characteristics of this and other rainforests of the western Amazon is their tremendous biodiversity (Voss and Emmons, 1996; Eisenberg and Redford, 1999). For example, within the area of the Allpahuayo Biological Station a total of 1729 plant species have been recorded (Vasquez-Martinez, 1997). The specimens examined in this study were obtained as part of an offshoot of a biodiversity survey of the small mammal fauna that was conducted between July 1997 and November 1998.

Animal trapping

A number of different sampling methods were used in order to obtain as complete inventory as possible of the small mammal population. Both grids and line transect formations were implemented. An array of traps was also used, including Tomahawk (Tomahawk Trap Co., Tomahawk, WI), Sherman (Sherman Trap Co., Tallahassee, FL), Victor snap, and pitfall traps. Sampling was done on the ground and in trees. Grids and transects were assessed for 10 consecutive nights at each site; traps were baited with yucca, dried fish, or a peanut butter mixture.

Rodents were handled and processed according to recommended safety procedures (Mills *et al.,* 1995). Animals were euthanized with Metafane; then blood, spleen, and lung samples were collected aseptically. These specimens were placed in separate plastic cryovials for storage at −70°C. The samples were subsequently transported to the University of Texas Medical Branch on dry ice for virus isolation attempts. Specimens, including museum skins and skulls, were deposited at the Museum of Texas Tech University, Lubbock, TX and the Museo de Historia Natural de la Universidad Mayor de San Marcos, Lima, Peru. A duplicate set of tissue samples was deposited at the Museum of Texas Tech University, Lubbock, TX.

The studies reported herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996.

Virus isolation

A portion of the frozen spleen from each animal was thawed and homogenized in 2.0 ml of phosphate-buffered saline pH 7.2 (PBS), containing 30% (v/v) heat-inactivated (56°C for 30 min) fetal bovine serum, using sterile Ten Broeck tissue grinders. A measured 0.3-ml volume of the centrifuged (5000 *g* for 10 min) homogenate of each sample was inoculated into a 12.5-cm^2 flask of Vero cells, which was maintained at 37°C. Cultures were examined every 2 days for evidence of viral cytopathic effect (CPE) and the maintenance medium was changed every fifth or sixth day. After 14 days, if no CPE was observed, some of the cells were scraped from the flask and placed on 12-well glass microscope slides. After drying and acetone fixation, the cells were examined by indirect fluorescent antibody (IFA) test for the presence of arenaviral antigen, using a Tacaribe complex, polyvalent, hyperimmune mouse ascitic fluid, and a commercial, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) (Fulhorst *et al.,* 1997).

Antigen and immune reagents

The following viruses were used to prepare the antigens and immune reagents: Tacaribe (strain TRVL 11573), Pichinde (strain AN 3739), Parana (strain MARU 12056), Tamiami (strain CDC W10777), Flexal (strain BeAn 293022), Pirital (strain VAV-488), Whitewater Arroyo (strain Av 9310135), and Allpahuayo (strain CLHP-2098). Antigens for use in complement fixation tests were prepared from infected newborn mouse brain by the sucrose–acetone extraction method (Beaty *et al.,* 1989). Immunizing antigens were 10% crude brain suspensions of infected mice in PBS.

Specific hyperimmune mouse ascitic fluids (MAF) were prepared against the arenavirus prototype strains listed above. The immunization schedule consisted of four weekly intraperitoneal injections of mouse brain antigen mixed with Freund's adjuvant, as described previously (Fulhorst *et al.,* 1997). Following the fourth injection, sarcoma 180 cells were also injected intraperitoneally to induce ascites formation. The Tacaribe complex, polyvalent immune ascitic fluid was prepared by pooling individual MAFs made to Tacaribe, Pichinde, and Pirital viruses.

CF tests

CF tests were performed using a microtechnique (Beaty *et al.,* 1989), with two full units of guinea pig complement. Titers were recorded as the highest dilution giving 3+ or 4+ fixation of complement.

Transmission electron microscopy of ultrathin sections

Immediately after removing the medium, infected monolayers of Vero cells were fixed in a mixture of 1.25% formaldehyde and 2.5% gluteraldehyde, containing 0.03% trinitrophenol and 0.03% $CaCl_2$ in 0.05 M cacodylate buffer pH 7.3, and washed in 0.1 M cacodylate buffer. Cells were scraped off the plastic, pelleted in buffer, postfixed in 1% OsO_4 in cacodylate buffer, *en block* stained with 1% uranyl acetate in 0.1 M maleate buffer pH 5.2, dehydrated in ethanol, and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections

were cut on Reichert Ultracut S ultramicrotome, stained with 2% aqueous uranyl acetate and 0.4% lead citrate, and examined in a Philips 201 electron microscope at 60 kV.

RNA extraction

RNA was extracted from infected Vero cells 9–10 days after inoculation with virus. Most of the culture medium was removed from the culture flask and 1.0 ml of Trizol LS (BRL Laboratories, Bethesda, MD) was added directly to the cell monolayer. RNA was extracted by adding 200 μl of chloroform and 40 μg of tRNA to ca. 1 ml of culture medium. After incubation for 15 min on ice, the mixture was centrifuged at 14,000 rpm, 4°C for 15 min. The aqueous layer was removed and mixed with an equal volume of isopropanol. RNA was precipitated by storing the samples at −20°C for 1 h and then pelleted by centrifugation at 14,000 rpm, 4°C for 15 min. RNA pellets were washed with 70% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water.

RT-PCR

To amplify the S RNA of prototype Allpahuayo virus CLHP-2098 and an additional sympatric isolate CLHP-2472 from another *O. bicolor,* the RNA was first denatured by incubation with 10 mM methyl mercury hydroxide for 10 min at room temperature. Excess methyl mercury was bound by adding 2.0 μl 700 mM 2-mercaptoethanol and 4 u of Rnasin (Promega, Madison, WI) and by incubating for 15 min at room temperature. Reverse transcription was carried out in a 30 μl volume containing 1 μM primer M13-19C (5′-TGTAAAACGACGGCCAGTGCGCACAGTGGATCCTAGGC-3′). AMV reverse transcriptase buffer (Life Sciences Inc., Hercules, CA), 1 mM dNTPs, 20 u of Rnasin (Promega), and 30 u of AMV reverse transcriptase XL (Life Sciences Inc.). After incubating for 2 h at 42°C, 30 μl of RNA hydrolysis solution (0.4 N NaOH, 40 mM EDTA) was added and the reaction was incubated for 30 min at 65°C. The cDNA was recovered by ethanol precipitation with ammonium acetate and resuspended in 10 μl nuclease-free water.

S segment cDNAs were amplified using the Expand high-fidelity PCR system (Boehringer Mannheim) in a 100 μl reaction containing Expand buffer with 2 mM $MgCl_2$, 600 nM of primer M13-19C, 2% DMSO, 1 μl cDNA suspension, and 3.5 u of Expand enzyme. PCR amplification was carried using a profile that consisted of an initial 2 min at 94°C, followed by 30 cycles of 94°C for 15 s, 45°C for 30 s, and 72°C for 2 min with the extension time increased in 20-s increments from cycles 11 through 30, followed by a 7-min extension at 72°C.

Sequence determination

To determine the nucleotide sequence of the S segment, PCR products were 3′ end-tailed with dATP in a 50 μl reaction containing 1× PCR buffer (Boehringer Mannheim), 800 nM dATP, 3 mM $MgCl_2$, and 5 u Taq polymerase (Boehringer Mannheim). The reaction was incubated for 1 h at 75°C and then loaded onto an 0.8% agarose gel in Tris–acetate–EDTA buffer with 0.02% methylene blue dye, excised from the gel, purified using QIAquick PCR gel extraction kit (Quiagen, Valencia, CA), and ligated to the pCRII plasmid vector (Invitrogen, Carlsbad, CA). Plasmid DNA was sequenced using the Big Dye terminator cycle sequencing kit (Applied Biosystems, Forest City, CA) using an ABI 377 XL automated sequencer. Sequence was read from both strands from a minimum three independent clones of the prototype virus. Determination of the predicted secondary structure of the intergenic noncoding region was made via the M-Fold program (Zuker *et al.,* 1999). The 5′ noncoding region sequence was determined by using the 5′ RACE system for rapid amplification of cDNA ends, Version 2.0 (Life Technologies, Rockville, MD). First-strand cDNA was synthesized from a viral RNA template by using a designed primer (5′-ATAAGCAATCCAGCACCGC-3′). The cDNA was then tailed with a homopolymer and amplified via PCR using an anchor primer which annealed to the homopolymer and a designed nested primer (5′-GTTGGTGATGTTTCCAAACG-3′).

Phylogenetic analysis

Sequences were initially aligned with corresponding NP gene sequence of 613–631 nucleotides within the S sequences available for arenaviruses from the GenBank sequence database [Accession Nos: U43685 (AMA), U43687 (FLE), U43686 (GUA), D10072 (JUN), Jo4324 (LAS), U43688 (LAT), M20869 (LCM), X62616 (MAC), U34248 (OLV), U43689 (PAR), K02734 (PIC), U62561 (PIR), U41071 (SAB), M20204 (TAC), U43690 (TAM), and AV9310135 (WWA)]. The entire S segment sequence of Allapahuayo virus was also aligned with available S segment sequences of other Arenaviruses. Sequences were aligned using the PILEUP (Devereux *et al.,* 1984) software implemented in the Wisconsin Package version 8.0. For alignment of different arenavirus sequences, deduced amino acid sequences were first aligned using the default parameters, and nucleotide sequences were aligned manually to preserve proper codon alignments. Phylogenetic analyses were conducted using the PAUP 4.0 (Swofford, 1998) using the maximum parsimony, neighbor joining, and maximum likelihood program. For distance analyses, the Kimura two-parameter and F84 formulas were used (Swofford, 1998). Maximum likelihood analysis included empirically determined nucleotide frequencies and a proportion of invariant nucleotide sites estimated at 0.16 based on the assumption that amino acids conserved across the partial N sequences of all arenaviruses are not free to vary, and on the number of nondegenerate sites within these codons. A

gamma value of 0.29 and transition:transversion ratio of 5.0 were estimated from Guanarito virus sequences described previously (Weaver *et al.,* 2000) Bootstrap analyses (Felsenstein, 1985) were conducted with 1000 resamplings of the original data set.

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