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SHORT COMMUNICATION

Oliveros Virus: A Novel Arenavirus from Argentina

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During the past few decades several newly recognized rodent-borne arenaviruses have been shown to be associated with severe hemorrhagic fever cases in South America. Changes in ecology and farming practices throughout the region have increased the concern over the potential public health threat posed by such emerging virus diseases. Oliveros (OLV) virus is a recently discovered arenavirus of the rodent *Bolomys obscurus* in Argentina. Genetic analysis of the small genomic RNA segment, which encodes the nucleocapsid protein and the envelope glycoproteins, shows that Oliveros is a novel, phylogenetically distinct member of the *Arenaviridae* family which differs in nucleotide sequence from the previously characterized members by approximately 35% or more. Despite this level of diversity, OLV virus possesses the same ambisense genome structure and many overall RNA and protein features in common with other arenaviruses. These data represent an important first step in the development of specific immunological and PCR diagnostic reagents to allow assessment of the prevalence and disease potential of this virus.

Arenaviruses are primarily rodent-borne viruses which include the etiologic agents of lymphocytic choriomeningitis and hemorrhagic fevers in humans. Arenaviruses have been divided into Old and New World groups on the basis of antigenic properties (*1*). Lymphocytic choriomeningitis (LCM), Lassa (LAS), Mopeia (MOP), Mobala, and Ippy viruses are members of the Old World group. The New World (Tacaribe complex) arenaviruses include Tacaribe (TCR), Junin (JUN), Machupo (MAC), Pichinde (PIC), Guanarito (GUA), Sabiá (SAB), Tamiami, Flexal, Amapari, Parana, and Latino viruses. Several Tacaribe complex arenaviruses are associated with severe hemorrhagic disease in humans. Argentine hemorrhagic fever (AHF), caused by JUN virus, emerged as serious occupational hazard of Argentine farm workers in the 1950s (*2*). First recognized in the 1960s, MAC virus, the etiologic agent of Bolivian hemorrhagic fever, has caused outbreaks of hemorrhagic fever with high mortality in rural areas of Bolivia (*2*). Since 1989, GUA and SAB viruses have also emerged as causes of severe hemorrhagic fever in South America (*3, 4*). GUA virus is the etiologic agent of Venezuelan hemorrhagic fever (*3*) and SAB virus has been associated with one human death in Brazil and two severe laboratory-acquired infections (*4, 5*).

Arenaviruses are enveloped viruses with a genome consisting of two single-stranded RNA segments, designated small (S) and large (L) (*6*), each of which contain two genes in an ambisense orientation (*7, 8, 9*). The S RNA encodes the nucleocapsid (N) protein at its 3′ end in the genome-complementary sense. The N protein is expressed through transcription of a subgenomic mRNA from the genomic S RNA template. The glycoprotein precursor (GPC) gene is found at the 5′ end of the S RNA in the genomic sense. The GPC is expressed via transcription of a subgenomic mRNA from the S antigenome, a replicative intermediate. Similarly, the L RNA encodes the virus polymerase (L protein) at the 3′ end and the Z protein (a putative regulatory protein) at the 5′ end. In both segments, the intergenic region separating the two genes has been predicted to form hairpin structures which may play a role in transcriptional termination (*10*).

From 1987 to 1990, a rodent survey for JUN virus was carried out in and near the AHF-endemic area of Argentina to assess the dynamics of JUN virus infection in its rodent reservoir (*11*). In 1990, a virus was isolated in Vero cells from the tissues of a *Bolomys obscurus* rodent trapped in the town of Oliveros, just north of the AHF-endemic area (*12*). To investigate the relationship of this virus [proposed name Oliveros (OLV) virus] to previously characterized arenaviruses, the nucleotide sequence of the entire virus S RNA genomic segment was determined and compared.

OLV virus RNA was extracted from infected Vero E6

The nucleotide sequence data reported in this paper have been submitted to the GenBank sequence database and assigned Accession No. U34248.

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cell culture medium by the method of Chomczynsky and Sacchi (*13*), treated with 10 m*M* methyl mercury hydroxide, and then reverse transcribed using primer 19C (5′-CGCACAGTGGATCCTAGGC-3′). Oligonucleotide primer 2420C (5′-GTTTTGATAGCATTTTTGTTGTAATC-3′) was designed from conserved regions of arenavirus GPC genes and used in conjunction with primer 19C to amplify a portion of the OLV S RNA GPC gene and the 5′ noncoding region (NCR) by the polymerase chain reaction (PCR). PCR products were then cloned into the TA cloning vector pCRII (Invitrogen Corp.), and individual clones were sequenced using the dye termination cycle sequencing technique (Applied Biosystems, Inc.). Clones containing OLV virus S RNA sequence were identified by alignment and comparison of the insert sequence with previously reported arenavirus S RNA sequences. To obtain clones from the 3′ end of the S RNA, cDNA synthesis was carried out with primer 19C, using the method of Gubler and Hoffman (*14*). The resulting double-stranded cDNA was blunt-ended with Klenow and ligated to *Sma*I-digested pBluescript II (KS+) (Stratagene Cloning Systems, Inc.). Individual clones were then sequenced. The remaining S RNA sequence, with the exception of terminal sequences, was determined by direct sequencing of PCR products. All sequences originally obtained from clones were verified by direct sequencing of PCR products.

To obtain the sequence at the 5′ terminus of the S RNA, a poly(A) tail was added onto the OLV virus S cDNA, using terminal deoxynucleotidyl transferase (*15*), and the tailed template was PCR amplified, using the OLV virus S RNA complementary primer (5′-ATGCCACACAGATGAGGGCAAT-3′) and a primer that annealed to poly(A) tails (5′-CGGGATCCCGTTTTTTTTTT-3′). PCR products were then cloned into the pCRII vector and individual clones sequenced. The 3′ terminal sequence of the OLV virus S RNA was obtained by end-ligation with T4 RNA ligase (*16*) and performing cDNA synthesis, using an OLV virus S RNA complementary primer (5′-CTCTTAATAGCATTCCTATTGTAATC-3′). The template was then amplified by nested PCR, using two PCR primer pairs (outside pair, 5′-ACTGTGTGAAGTTTAGAGTGATAC-3′ and 5′-CCCAAGGGATCTCAGTATAGCT-3′; inside pair, 5′-ATGCCACACAGATGAGGGCAAT-3′ and 5′-TTGCGAGACTTGACTGAAGTCA-3′). PCR products were then cloned into the pCRII vector and sequenced.

Sequence compilation, analysis, and alignment were performed using the Wisconsin Sequence Analysis Package, version 8.0 (Genetics Computer Group, Inc.). The OLV virus S RNA genomic sequence was deposited into the GenBank sequence database (Accession No. U34248).

The OLV S RNA was determined to be 3535 nt long. The 5′ NCR is 116 nt in length, somewhat longer than the 5′ NCRs of other arenavirus S RNAs. The 5′ terminus of the S RNA apparently contains a nontemplated G residue as described previously for other arenaviruses, though some sequence heterogeneity was observed (*17, 18, 19*). Of 12 clones obtained by poly(A) tailing of the cDNA, 8 contained an additional G, 3 contained the extra G plus three to seven additional, nonconserved bases, and 1 clone was missing the additional G but contained four additional, nonconserved bases. The sequences containing additional heterogeneous bases could represent mRNAs, since the virus RNA source for these studies was infected cell culture medium that had been prepared by freezing and thawing the entire cell culture flask prior to harvest (this was done to increase virus titer by liberating virus from infected cells).

The GPC gene is 1557 nt long and encodes a 518-amino-acid gene product. The GPC contains 13 potential asparagine-linked glycosylation sites (4 in the putative G1 protein and 9 in the putative G2 protein), of which 5 (located at residues 90 to 92, 180 to 182, 389 to 391, 414 to 416, and 419 to 421) are conserved amongst all arenaviruses. In addition, the GPC contains 12 cysteine residues that are conserved amongst all arenavirus GPCs (9 in the putative G1 protein and 3 in the putative G2 protein). The putative G1–G2 cleavage site of the OLV virus GPC contains the dibasic R–R motif (at positions 280 to 281) described in other arenaviruses (*20*).

The OLV virus N gene (1677 nt) encodes a 558-amino-acid protein. The N protein is composed of over 12.5% basic amino acids; 34 of these residues are conserved as either K or R residues in all arenavirus N proteins. The abundance of positively charged residues in the OLV virus N protein correlates well with its role as an RNA binding protein. The 3′ NCR is 88 nt long. The 19 nt at the 3′ end of the OLV virus S RNA were highly conserved with those of other arenaviruses. Of 11 clones obtained by ligation of the RNA 3′ and 5′ ends, 9 contained the predicted sequence (i.e., the complement to primer 19C) and 2 clones contained single substitutions in this region. A single clone contained an intact 5′ terminus and an extra nontemplated G; other clones, however, contained two to four base truncations of the 5′ terminus. Prior to ligation, the OLV S RNA termini were not treated with tobacco acid pyrophosphatase (TAP) to convert the tri- or diphosphate group present on the 5′ ends of arenavirus genomes (*21*) to a monophosphate group that can then be ligated to the 3′ end using T4 RNA ligase. The clones containing truncated 5′ ends probably represent virus genomes that had been digested by exonucleases, thus exposing a monophosphate group that could participate in the ligation reaction. Meyer and Southern (*18*) reported that the termini of LCM virus genomes and antigenomes could only be ligated if first treated with TAP.

The intergenic region is 97 nt in length. Predicted secondary structure of this region displayed in the N gene coding (genome-complementary) sense consists of two hairpin structures (Fig. 1). When the intergenic region is

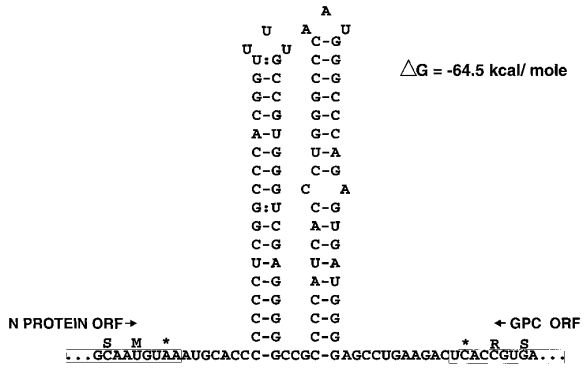

FIG. 1. Secondary structure of the intergenic region of the OLV virus S RNA displayed in N coding (genome-complementary) sense as predicted by the MFOLD program of the Wisconsin Sequence Analysis Package, version 8.0. The 3′ ends of the N and GPC genes are indicated by boxed text.

folded in the GPC coding (genomic) sense, a third hairpin structure with a 3-bp stem is predicted to form between the GPC stop codon and the 18-bp structure. This small stem–loop structure is not predicted to form, however, when the intergenic region is folded in the N gene coding sense due to the energetic instability introduced by replacement of a noncanonical U–G bp with an A–C mismatch. Like OLV virus, the intergenic regions of the S RNAs of MOP, JUN, and TCR viruses are also predicted to form two hairpin structures (*22, 23, 24*). Mapping of the 3′ ends of the mRNAs of TCR virus strongly suggests that these stem–loop structures play a role in transcriptional termination (*10*).

Pairwise analyses of the OLV virus N and GPC gene sequences and their deduced amino acid sequences with those of other arenaviruses indicate that OLV virus is unique (Table 1), sharing no greater than 64.6% sequence identity with any other arenavirus. This finding is confirmed by preliminary serological data in which OLV virus was found to be antigenically distinct from seven other arenaviruses (JUN, MAC, SAB, GUA, TCR, LAS, LCM) by neutralization assay (*12*).

Arenavirus phylogeny was inferred by maximum parsimony (MP) analysis of available N gene sequences. The sequences were edited to match the shortest length sequence available in the GenBank database, which in this case was the partial length N gene sequence (730 nt) of GUA virus. MP analysis (Fig. 2) revealed that the arenaviruses can be separated into two clades that correspond to the Old World and New World (Tacaribe complex) groups. Within the Tacaribe complex clade, OLV virus occupies a position between PIC virus, the most ancestral member of the complex, and GUA virus, which is ancestral to TCR, MAC, and JUN viruses. The position of OLV virus was supported by 71% or more of bootstrap replicates. Under conditions thought to be typical of most phylogenetic analyses, bootstrap proportions of $\geqslant$70% usually correspond to a probability of $\geqslant$95% that the corresponding clade is real (*32*). The topology of the region of the tree occupied by TCR, MAC, and JUN viruses, though supported by only 65% of bootstrap replicates in the partial N gene analysis, received 96% bootstrap support when full-length N gene sequences were analyzed (data not shown). The topology of the tree within the Old World clade was supported by 100% of all bootstrap replicates. Arenavirus phylogenies inferred using full-length N gene (minus GUA) and GPC gene (minus GUA and MAC) were congruent with the phylogeny inferred using the partial-length N gene sequence (data not shown) and concordant with previously published phylogenies estimated using fewer arenavirus strains (*29, 30, 33*).

Clearly, the phylogenetic relationships of the New World arenaviruses are complex, with no correlation between virus phylogeny and the geographic distribution of arenaviruses, e.g., the two Argentine viruses, OLV and

TABLE 1

Percentage Sequence Identity Between OLV Virus and Other Arenaviruses

	Arenavirus[a]							
	LCM	LAS	MOP	PIC	GUA	TCR	MAC	JUN
N gene (nt)	55.7–57.6	56.3–56.5	57.6	61.7	61.4[b] (730 nt)	61.3	63.2	62.9
N protein (aa)	51.0–51.2	50.8–52.2	50.7	57.7	64.6[b] (243 aa)	60.4	62.0	59.7
GPC gene (nt)	54.9–55.4	55.6–55.8	56.5	56.9	ND[c]	56.8	ND[c]	58.4
GPC (aa)	44.7–45.2	47.4–47.9	46.9	48.3	ND[c]	48.9	ND[c]	47.0

[a] LCM virus strains Armstrong 53b (GenBank Accession No. M20869) (*25*) and WE (M22138) (*26*), LAS virus strains Nigerian GA391 (X52400, M36544) (*27*) and Josiah (J04324) (*28*), MOP virus (M33879) (*22*), JUN virus (D10072) (*23*), MAC virus (X62616) (*29*), TCR virus (M20304, M65834) (*24*), GUA virus (L42001) (*30*), PIC virus (M16734) (*7*).

[b] Partial sequence used to calculate identity; length given in parentheses.

[c] Not determined due to unavailability of sequence data.

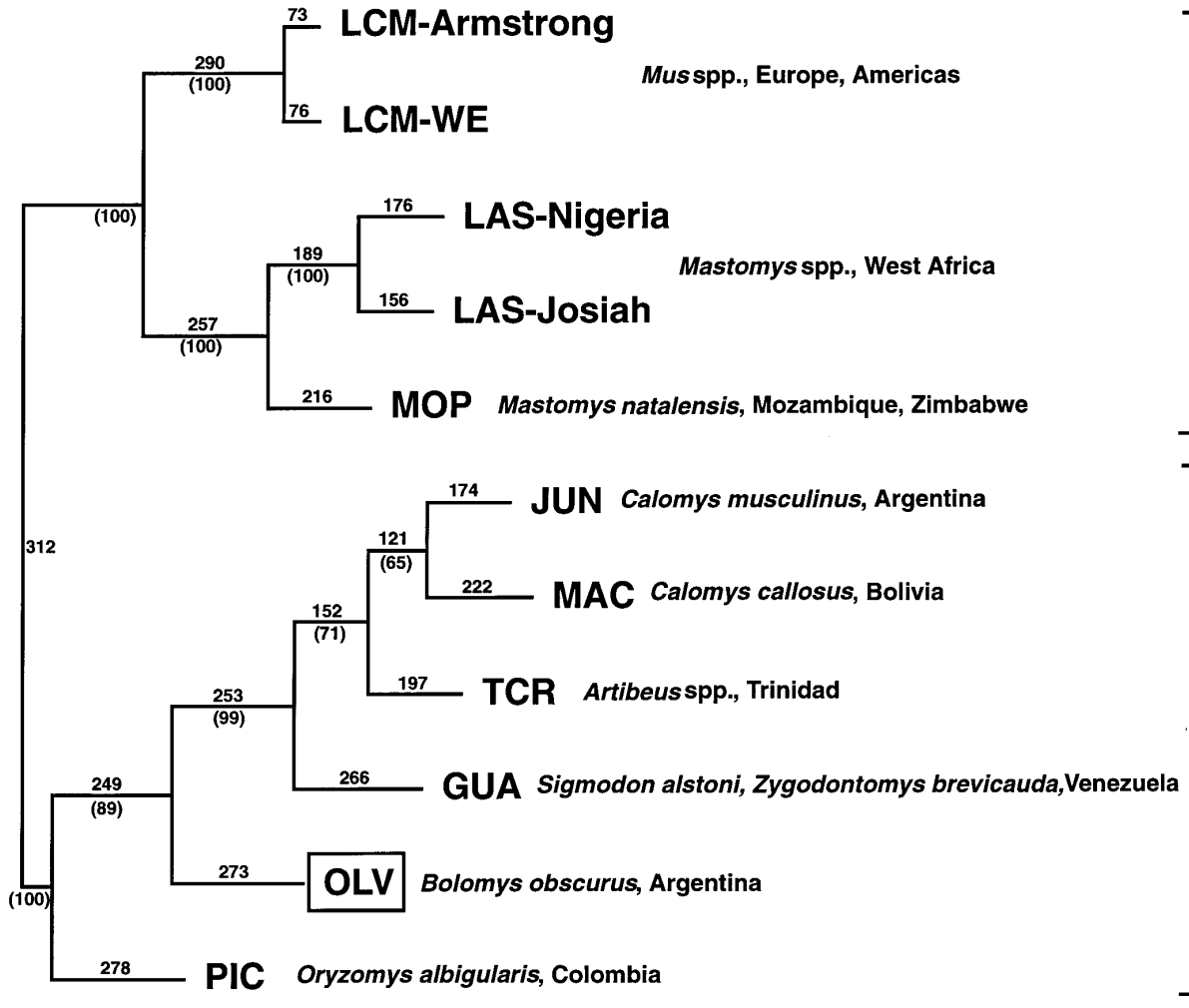

FIG. 2. Arenavirus phylogeny inferred from a 730-nt region of the N gene. Analyses were performed with Phylogenetic Analysis Using Parsimony (PAUP), version 3.1.1 (*31*), using the branch-and-bound search option and a 4:1 weighting of transversions over transitions. Bootstrap confidence intervals were calculated by carrying out 1000 heuristic search replicates. Numbers above horizontal branches indicate estimated branch lengths and include a fourfold weighting of transversions. The percentage of bootstrap support for each interior branch is indicated by numbers in parentheses below each interior branch. Vertical branches are for visual clarity only. The position of OLV virus is indicated by boxed text. The confirmed or suspected host and geographic distribution (*2*) of each virus are listed. The Old World and Tacaribe complex (New World) groups are indicated by brackets.

JUN, are only distantly related. There is some indication that virus phylogeny may mirror host phylogeny, since both JUN and MAC viruses are associated with rodents of the genus *Calomys;* however, this picture is clouded by the fact that the hosts of TCR virus are believed to be *Artibeus* bats rather than rodents. At this point, the pathogenic nature of New World arenaviruses cannot be accurately predicted by phylogenetic relationships. The known pathogenic members of the Tacaribe complex (JUN, MAC, GUA) are found in the more distal region of the Tacaribe complex lineage, but so is TCR virus which has been associated only with a single, non-fatal laboratory-acquired infection (2). PIC is not pathogenic for humans, whereas the status of OLV virus as a human pathogen remains to be determined. Trends in arenavirus evolution may become clearer once the complete phylogeny of the Arenaviridae is established.

In Argentina, cases of hemorrhagic fever occur annually that are diagnosed clinically as AHF but cannot be attributed to JUN virus; the etiologic agent(s) of this disease is unknown. If OLV virus is indeed a cause of arenavirus hemorrhagic fever, a significant number of people could be at risk of exposure to OLV virus because the described range of *B. obscurus* covers Argentina (including Buenos Aires province), Uruguay, and Paraguay (*34*). Changes in ecology and farming practices throughout the region have increased the concern over the potential public health threat posed by such emerging virus diseases. The availability of data for the S RNA of OLV virus permits the design of PCR primers, probes,

and recombinant antigens for investigating the status of OLV virus as a human pathogen through epidemiologic and ecologic studies.

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