Virus Research 160 (2011) 150–158

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Viral diversity of Junín virus field strains

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ARTICLE INFO

Article history:
Received 23 March 2011
Received in revised form 2 June 2011
Accepted 3 June 2011
Available online 13 June 2011

Keywords:
Arenaviridae
Junin virus
Evolutive distance
Argentine Hemorrhagic Fever
RT-Nested-PCR

ABSTRACT

The Argentine Hemorrhagic Fever, an endemic disease present in a much of Argentina, is caused by the Junín virus (JUNV). Currently, there are sequences available from several strains of this virus, like those belonging to the vaccine lineage (XJ13, XJ#44 and Candid#1), as well as MC2 (rodent isolate) and IV4454 (human isolate). In this article, we report sequence information on two fragments of genomic segment S of viral isolates from the endemic area. A Nested-RT-PCR was used to amplify discrete genomic regions of 13 isolates of rodent and human origin. The bioinformatics studies revealed a great homogeneity of sequences among the JUNV isolates. The phylogenetic classification showed greater evolutionary distance between the old world arenaviruses (Lassa and LCM virus) than between the new world arenaviruses (JUNV and Machupo virus).

1. Introduction

Arenaviridae is a family composed of a growing number of enveloped viruses with a bipartite single-stranded RNA genome. Members of the *Arenaviridae* family were subdivided into two groups based on the geographical site of isolation, serological cross-reactivity and genetic data. The prototype of the family, lymphocytic choriomeningitis virus (LCMV), is a member of the Old World arenavirus group (OW), which also includes Ippy (IPPV), Lassa (LASV), Mobala (MOBV) and Mopeia (MOPV) viruses. The Junín virus (JUNV) is a member of the New World arenavirus group (NW), which also includes Allpahuayo (ALLV), Amapari (AMAV), Bear Canyon (BCNV), Flexal (FLXV), Guanarito (GTOV), Latino (LATV), Machupo (MACV), Oliveros (OLVV), Paraná (PARV), Pichindé (PICV), Pirital (PIRV), Sabiá (SABV), Tacaribe (TCRV), Tamiami (TAMV) and Whitewater Arroyo (WWAV) viruses (Salvato et al., 2005). In addition, there are several recently described species that have not yet been classified by the ICTV (http://www.ictvonline.org/virusTaxonomy.asp?version=2008).

Greater understanding of the taxonomy and evolution of the *Arenaviridae* has been achieved from nucleotide sequence data. It has been suggested that New World arenaviruses should be classified into four different lineages, named A, B, C and Rec (Charrel et al., 2002). Lineage A would contain ALLV, FLXV, PARV, PICV and PIRV; lineage B would contain AMAV, GTOV, JUNV, MACV, SABV and TCRV, lineage C would contain LATV and OLVV, and lineage Rec, with WWAV, TAMV and BCNV. Members of this last clade are thought to have an S RNA product of a natural recombination. Currently (ICTV website) the last three viruses are classified within Clade A.

The arenaviruses' natural history is characterized by the infection of a limited number of small rodent species which are considered reservoirs to maintain the viruses in nature (Ambrosio et al., 2006). The *Muridae* family of rodents serves this function in the natural virus cycle, with the *Murinae* subfamily as hosts for the OW arenaviruses, while the *Sigmodintinae* subfamily are the reservoirs for the NW arenaviruses (Gonzalez et al., 2007).

Junín virus (JUNV, virus code: 00.003.0.01.010.) is a South American arenavirus, the etiological agent of a severe endemo-epidemic disease called Argentine Hemorrhagic Fever (AHF). The clinical symptoms of AHF include hematological, neurological, cardiovascular, renal, and immunological alterations. The mortality rate for

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doi:10.1016/j.virusres.2011.06.004

Table 1
List of the isolates and strains of JUNV used in this study. A. Strains previously sequenced. B. Isolates from rodents in endemic area. C. Isolates from human cases in endemic area.

Source	Strain	Isolation year	GenBank ID[a]	Characteristics
A. Strains previously sequenced	XJ13	1958	NC_005081.1	Directly derived of XJ strain, isolated from a fatal case (Parodi et al., 1958).
	XJ#44	–	GQ121040.1	Vaccine genealogy (Parodi et al., 1958).
	Candid#1	–	FJ969442.1	Vaccine genealogy (Parodi et al., 1958).
	MC2	1967	D10072.2	Isolated from *Calomys* spp. (Berría et al., 1967).
	IV4454	1970	DQ272266.3	Fatal case (Medeot et al., 1992).
	Romero	1986	AY619641.1	Severe non fatal case (Kenyon et al., 1986).
B. Isolates from rodents in endemic area	AN_8640	1966	JF446289/JF446302	Isolated from *Calomys* spp.
	AN_5185	1966	JF446290/JF446303	Isolated from *Calomys* spp.
	AN_13365	1969	JF446291/JF446304	Isolated from *Calomys musculinus*
	AN_16501	1974	JF446292/JF446305	Isolated from *Calomys musculinus*
	AN_17058	1975	JF446293/JF446306	Isolated from *Calomys laucha*
	AN_17246	1975	JF446294/JF446307	Isolated from *Calomys musculinus*
	AN_17116	1975	JF446295/JF446308	Isolated from *Akodon* spp.
	AN_17064	1975	JF446296/JF446309	Isolated from *Akodon* spp.
C. Isolates from human cases in endemic area	H_Lye 63	1963	JF446284/JF446297	Fatal human case.
	H_FHA 5069	1971	JF446285/JF446298	Isolated from blood from a non fatal case.
	H_p1879	1971	JF446286/JF446299	Attenuated. Isolated from blood.
	H_FHA 5054	1971	JF446287/JF446300	Intermediate virulence.
	H_8027	1991	JF446288/JF446301	Non fatal case, virulent.

[a] For the isolates listed in parts B and C the GenBank IDs appear in the order: G and N fragment respectively (G/N).

AHF can be as high as 30%, but early treatment with immune plasma reduces fatal cases to less than 1%. The human population at risk is composed mainly of field workers, who are believed to become infected through cuts or skin abrasions or via airborne dust contaminated with urine, saliva or blood from infected rodents (Enría et al., 2008).

The two RNA segments of the Junín virus genome are named L and S and have approximate sizes of 7.2 and 3.5 kb, respectively. Each RNA segment directs the synthesis of two proteins; their open reading frames are arranged in opposite orientations (ambisense coding strategy) (Auperin et al., 1984) and are separated by a non-coding intergenic region that folds into a stable stem-loop structure (Ghiringhelli et al., 1991). The S RNA codes for the major structural proteins of the virion are the precursor of the envelope glycoproteins (GPC) and the viral nucleocapsid protein (N). Post-translational cleavage of GPC renders a stable signal peptide and the two viral glycoproteins (GP1 and GP2). Moreover, the L RNA segment codes for the viral RNA-dependent RNA polymerase is (L) and the small protein is Z.

A joint effort of the US and Argentine Governments led to the production of a live attenuated Junín virus vaccine, named Candid#1 (Parodi et al., 1958). After rigorous biological testing in rhesus monkeys, the vaccine was used in human volunteers, followed by an extensive clinical trial in the AHF endemic area (Ambrosio et al., 2006). This vaccine was derived from the 44th mouse brain passage (XJ#44) of the prototype strain of Junín virus (Enría and Barrera Oro, 2002). The sequence information of the L and S RNAs of Junín virus Candid#1 (avirulent strain) and XJ#44 (intermediate virulence) strains as well as the comparisons with the XJ13 wild-type strain (high virulence) and with other Junín virus strains, like Romero, IV4454 and MC2 strain, have previously been described. In that study, several different mutations were identified that may be involved in the virulence attenuation process (Goñi et al., 2010).

The usual method employed for AHF diagnosis is based on clinical examination of patients and several late response laboratory tests, like virus isolation in cell culture and laboratory animals, serological analyses and viral antigens detection. The only laboratory test currently available for early and rapid diagnosis of AHF is based on RT-PCR. It is sensitive enough to detect the low viremia found during the period in which immune plasma therapy can be used effectively. More recently, a number of molecular epidemiological methods and genomic variation studies have been developed for characterizing the molecular genetics of arenaviruses (Lozano et al., 1997; Drosten et al., 2003; Ledesma et al., 2009).

We used the previously reported virus sequences to identify conserved genomic regions in order to amplify a set of cDNA fragments that may prove useful in molecular screening of genomic variation of Junín virus in nature. In this study, we describe the design of an RT-Nested-PCR based method, employed for the amplification and sequencing of two different regions in the JUNV genome and the characterization of samples isolated in the endemic area (8 from rodents and 5 from humans). Phylogenetic analysis of the results identified clades that are more consistent between rodent isolates. Moreover, the evolutionary distance found for the NW arenaviruses (JUNV and MACV) is lower than the same parameter determined for two members of OW arenaviruses (LCMV and LASV). This fact may be related to the evolutionary origin of this viral family.

2. Materials and methods

2.1.1. Virus strains

In the sequence analysis, we included previously reported sequences (Table 1A, Goñi et al., 2010) and the sequences from the new virus isolates. The different strains employed in this study were isolated in the endemic area from human cases and captured rodents from the period of 1963–1975 and are listed in parts B and C of Table 1.

2.2. RNA isolation and cDNA synthesis

RNA was isolated from rodent tissues, virions and infected cells, using the QIAmp Viral RNA Mini Kit or RNeasy Mini Kit (Qiagen, Valencia, CA). For cDNA synthesis, two strategies were used: (i) with Arena primer or, (ii) with random hexamers (280 pmol/μl, Promega). In both cases, the cDNA synthesis was made with the SuperScript III *Reverse Transcriptase* (Invitrogen), following man-

ufacturer's instructions. The Arena primer comprises the highly conserved arena region, a 19-nucleotide fragment at the end of the genomic RNAs (Goñi et al., 2010).

2.3. Nested-PCR amplification

The accession numbers (GenBank ID) for the S genomic segments used are listed in Table 1A. The designed primers are listed in Table 2.

For the different PCR, the *Amplitaq DNA Polymerase* (5 U/μl, PerkinElmer, EE.UU.) was used, following manufacturer's instructions. In all cases, the final reaction volume was 50 μl, of which 7% was the cDNA template. For the second round, the volume of the DNA product was 2%. The amplification profiles optimized for the Nested-PCR amplification were: (i) first round: 94 °C × 2 min, and 40 cycles 94 °C × 1 min, 55 °C × 40 s, 72 °C × 1 min, and final extension of 10 min; (ii) second round, similar conditions of the previous profile but using 50 °C for the annealing temperature in this case (Table 2).

The N and G fragments were amplified by RT-Nested-PCR for all samples listed in Table 1B and C. In addition, the JUNV Candid#1 and JUNV XJ#44 strains were used as control.

Proper sample manipulation protocol was strictly followed to prevent cross-contamination, with all preparations made in appropriately divided work spaces (Carlos III, Health Institute, Madrid).

2.4. Sequences and bioinformatics tools

Both strands of the amplified products were further sequenced with ABI Prism BigDye Terminator Cycle sequencer v3.1 ready reaction (Applied Biosystems) and analyzed with the ABI PRISM 377 DNA Analyzer (Applied Biosystems). The identity of these sequences was assessed with GenBank database by means of the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/).

For the subsequent bioinformatics analysis, the following tools were used:

2.4.1. Motif search

The protein sequences were scanned in order to find putative domains and motifs. The tools used were ScanProsite (ExPASy Proteomics Server, ScanProsite, http://www.expasy.ch, Gasteiger et al., 2003) for identifying common motifs, and NETNGLYC 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/; Gupta et al., in preparation) to search for N-glycosylation sites.

2.4.2. Phylogenetic analyses

The nucleotide sequences from the N and G fragments of the different arenaviruses whose complete genome is at the GenBank were aligned independently using Clustal X. Each alignment was bootstrapped (1000 replicas) and a neighbor joining analysis was performed using the MEGA4 and *p*-distance method (Tamura et al., 2007).

2.4.3. Evolutionary distance

This parameter was determined through quantification of the number of nucleotide (or aminoacid) substitutions that occur between the two compared sequences. Different models can be applied, selecting the *p*-distance in the MEGA4 program (Tamura et al., 2007). It was thereby possible to calculate the standard deviation by bootstrap analysis.

3. Results and discussion

3.1. RT-Nested-PCR design

Previously known JUNV segment S sequences were aligned by ClustalX (CLUSTer ALignment, MEGA4, Tamura et al., 2007). In this analysis, other arenaviruses were not included, but the Tacaribe virus was used in the laboratory optimization assays.

Two different regions were selected for the analysis: one comprises the nucleotide positions 303–941 (in the glycoprotein precursor coding sequence), called G fragment, and the other is located between nucleotides 1632 and 2095 (in the nucleoprotein coding sequence), called N fragment (Fig. 1A). These regions were selected because they include some mutations detected in previous studies, and their ends revealed a high conservation degree between strains. The method was optimized using JUNV Candid#1 strain and TCRV as samples (Fig. 1B).

Genome amplification of 13 field JUNV strains (Table 1B and C) rendered the expected G and N amplification products because of the use of RT-Nested-PCRs, a highly sensitive approach for RNA samples with a low quantity of genetic material (Ré et al., 2007). The amplified fragments were purified and sequenced using the second round primers (Table 2).

3.2. Bioinformatics analysis

3.2.1. Amino acid alignments

From the alignment of the different *in silico* translated proteins of fragments G and N, only the positions in which it was possible to identify variations between strains are shown (Table 3). For fragment G, the analyzed region spanned positions 107–262 of the glycoprotein precursor (JUNV XJ13 reference) or, more specifically, between residues 49 and 193 of glycoprotein 1 (G1), as well as the first 11 amino acids from glycoprotein 2 (G2), after the consensus sites for glycoprotein processing observed in other arenaviruses (York et al., 2004). For the N fragment there are 118 residues analyzed, between residues 435 and 552 of the nucleoprotein, covering the carboxyl terminal of the last third of the protein.

Previously, we defined two types of mutations among JUNV strains (Goñi et al., 2010). The type 1 mutations are the positions where the nucleotide or amino acid sequence of one of the previously sequenced field strains of Junín virus (XJ13, Romero, IV4454 or MC2) was different from all other previously sequenced field strains, while the type 2 mutations reflect the positions with mutations among the vaccine strains (XJ13, XJ#44 and Candid#1) (Goñi et al., 2010). Here we analyzed some of the positions with type 2 mutations (potentially related to the virulence attenuation process) within the G fragment. However, for the positions included in this analysis, the variation observed for the attenuated JUNV strains was also present in some of the new field samples, taken from both rodents and human cases. As a consequence, the relative importance of this variation in the attenuation process should be re-evaluated.

To continue with the characterization of these viral sequences we analyzed predicted hydrophobicity and secondary structure profile. The profiles are all generally similar. For the sequenced strains, small regions can be observed with differences in the calculated hydrophobicity values, but no radical changes are apparent.

3.2.2. Phylogenetic analysis

For the phylogenetic analysis, the S segment information for 76 arenaviruses was collected (Table 4). Reported sequences were filtered, taking into account that only natural variants were selected, without a high number of culture passages (for example, for JUNV Candid#1 and XJ#44 strains were discarded).

Table 2
Primers used for the nested-PCR and amplification product characteristics.

Primers	Sequence (5′–3′)	Fragment name	Fragment length (pb)	Annealing temperature (°C) First round	Annealing temperature (°C) Second round
303 S	CTgTgTCCTTCTCAATggTggg	–	638	55	–
941 AS	CAAAACACTTCATTTTggCTgC				
374 V	AgCCATCTTTACATTAAggg	G	529	–	50
903 C	TCTAgACAATAgCCTCCAgg				
1632 S	CACTgCTTACAgTgCATAggC	–	463	55	–
2095 AS	gAAAACCACATgATgAgAAAgg				
1653 V	CTTCgggAggAACAgCAAgC	N	392	–	50
2048 C	gACATAgAAgATgCAATgCCAgg				

With all these sequences and those obtained for the different isolates in this study, a phylogenetic analysis was calculated (Fig. 2). Only the homologous N and G regions were considered. The tree obtained for the N fragment presents the same distribution of branches as the complete S segment analysis, while the tree of the G fragment shows a different topology regarding some clades from the new world (data not shown). In Fig. 2 the trees obtained for the JUNV strains are highlighted. The bootstrap test shows that there are many branches with very low support values. Therefore, the true tree topology is difficult to obtain. However, if we observe the nodes with a bootstrap value higher than 70, we find that only one group of three samples (AN_17116, AN_16501, and AN_17064) is maintained. The other subgroups separated by high bootstrap values are not similar between the trees calculated for the G and N fragments.

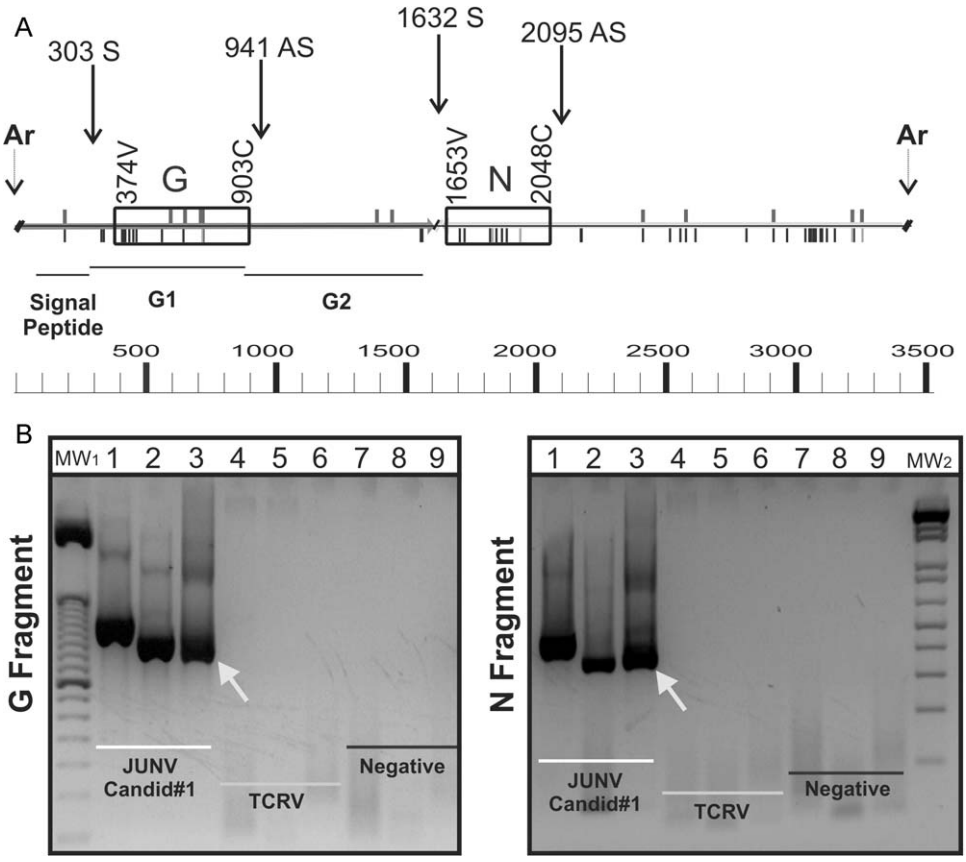

Fig. 1. Design and optimization of RT-Nested-PCR. (A) The horizontal bars represent the RNA S genomic segment, while the filled arrows show the open reading frames that code for the glycoprotein precursor (GPC) in the 5′ half, and for the nucleoprotein in the 3′ half (ambisense coding, Auperin et al., 1984). The different components for GPC protein are indicated: signal peptide, G1 and G2 (York et al., 2004). With open boxes, the selected regions for amplification are indicated, denominated G and N. The vertical bars above the genomic segment represent the variation positions found between vaccine strains, while in the lower part the differences identified when comparing the field strains are shown (Goñi et al., 2010). At the end of the open box, the primers used for the second round in the Nested-PCR are indicated, while those used in the first round are indicated with black arrows. The Arena primer (Ar) shows complementarity in both genomic segment ends (gray arrows). (B) Agarose electrophoresis visualization of G fragment (right) obtained for JUNV Candid#1 strain, TCRV and negative reactions, and N fragment (left). For both fragments, lanes 1–3 show the products obtained using cDNA template synthesized with random hexamers from RNA purified of cell cultures infected with JUNV Candid#1 strain. Lanes 4–6 correspond to TCRV template, and lanes 7–9 are negative controls. In lanes 1, 4 and 7, first round primers were assayed (303S/941 AS for G fragment and 1632S/2095 AS for N), while for lanes 2, 5 and 8, second round primers were used directly (374V/903C for G and 1653V/2048C for N fragments). In lanes 3, 6 and 9, the nested-PCR was performed. MW1: 1 kb Bio-Rad, MW2: 50 pb Bio-Rad.

Table 3
Positions with residue differences between G and N fragments encoded proteins Letters in bold indicate the residues that are different from the residue present in XJ13 strain. The amino acids are shown in the one letter code. The type 2 mutations are shadowed in gray.

Residue position	XJ13	XJ#44	Candid#1	MC2	IV4454	Romero	AN_8640	AN_5185	AN_13365	AN_16501	AN_17058	AN_17246	AN_17116	AN_17064	H_Lye63	H_FHA5069	H_p1879	H_FHA5054	H_8027
G Fragment																			
107	S	S	S	S	**T**	S	S	S	S	S	S	S	S	S	S	**T**	**T**	**T**	S
109	Q	Q	Q	**K**	**M**	**M**	Q	Q	Q	**M**	Q	Q	**M**	**M**	Q	**M**	**M**	**M**	Q
111	S	S	S	S	**T**	S	S	S	S	S	**T**	S	S	S	S	**T**	**T**	**T**	S
116	A	A	A	A	A	**E**	A	A	A	A	A	A	A	A	A	A	A	A	A
121	Q	Q	Q	**E**	Q	Q	Q	Q	**E**	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
125	I	I	I	I	**V**	I	I	I	I	I	**V**	I	I	I	I	**V**	**V**	**V**	I
133	S	S	S	S	S	S	S	S	S	**N**	S	S	**N**	**N**	S	S	S	S	S
143	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	**R**	**R**	**R**	W
151	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	**V**	**V**	**V**	A
157	H	H	H	**Y**	**Y**	H	H	H	H	H	H	H	H	H	H	H	H	H	H
168	T	**A**	**A**	T	T	T	**A**	**A**	T	T	**A**	**A**	T	T	**A**	T	T	T	**A**
184	V	V	V	**I**	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
186	E	E	**G**	E	E	E	**G**	**G**	E	E	**G**	**G**	E	E	**G**	E	E	E	**G**
206	S	S	**P**	S	S	S	**P**	**P**	S	S	**P**	**P**	S	S	**P**	S	S	S	**P**
208	P	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**	**L**
209	N	N	N	**D**	**S**	N	N	N	N	N	N	N	N	N	N	**S**	**S**	**S**	N
244	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	**H**	Q
245	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	**F**	L
N Fragment																			
476	K	K	K	**R**	**R**	**R**	K	**R**	**R**	**R**	**R**	K	**R**	**R**	**R**	**R**	**R**	**R**	**R**
493	Y	Y	Y	**F**	**F**	Y	Y	Y	Y	Y	Y	Y	Y	**F**	Y	**F**	Y	Y	**F**
495	H	H	H	H	H	H	H	H	**N**	H	H	H	H	H	H	H	H	H	H
499	Y	Y	Y	**H**	**H**	Y	Y	**H**	Y	Y	**H**	Y	Y	Y	**H**	**H**	Y	**H**	**H**
506	N	N	N	**S**	**S**	N	N	**S**	N	N	**S**	N	N	N	**S**	**S**	N	**S**	**S**
511	E	E	E	**D**	**D**	**D**	E	**D**	**D**	**D**	**D**	E	**D**	**D**	**D**	**D**	**D**	**D**	**D**
514	T	T	T	**A**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
546	P	P	P	**L**	P	**L**	P	P	**L**	**L**	P	P	**L**	**L**	P	P	**L**	P	P
552	P	P	P	P	P	**S**	P	P	**S**	P	P	P	P	P	P	P	P	P	P

Table 4
Arenavirus list employed for the sequence analysis. The table indicates the virus name (with the acronym when correspond), the clade to which it belongs, the quantity of sequences employed and the GenBank IDs.

Clades	Virus	Quantity	Viral strain (GenBank ID)
Clade A	Total	11	
	PARV	2	12056 (NC_010756.1); 10256 (AF512829.1)
	FLEV	2	BeAn293022 (NC_010757.1); Pinheiro (AF485257.1)
	ALLV	3	CLHP-2472 (NC_010253.1); Moncayo (AY012686.1); CLHP-2098 (AY081210.1)
	PIRV	2	VAV-488 Charrel (AF277659.1); VAV-488 Archer (NC_005894.1)
	PICV	2	Auperin (K02734.1); Munchique CoAn4763 P2 (EF529744.1)
Clade B	Total	22	
	JUNV	4	IV4454 (DQ272266.3); MC2 (D10072.2); Rumero (AY619641.1); XJ13 (NC_005081.1)
	MACV	10	Carvallo (NC_005078.1); MARU 216606 (AY624206.1); Chicava (AY624355.1); 9301012 (AY924205.1); Mallele (AY619645); MARU 222688 (AY924207); 200002427 (AY924204.1); 9430084 (AY924203.1); MARU 249121 (AY924208.1); 9530537 (AY571959.1)
	AMAV	2	BeAn 70563 (AF512834.1); BeAn 70563 (NC_010247)
	Chapare	1	(NC_010562.1)
	TACV	1	Iapalucci (NC_004293.1)
	CPXV	1	BeAn 119303 (NC_010254.1)
	SABV	1	SPH114202 (NC_006317.1)
	GTOV	2	CVH-960101 (AY497548.1); INH-95551 (NC_005077.1)
Clade C	Total	2	
	OLIV	1	(NC_010248.1)
	LATV	1	MARU 10924 (NC_010758.1)
Clade Rec A/B	Total	13	
	North American Arenavirus	3	AV D1240007 (EU123329.1); AV 96010024 (EU123331.1); AV 96010151 (EU123330.1)
	BCNV	3	AV A0070039 (NC_010256.1); AV 98470029 (AY924392.1); A0060209 (AF512833.1)
	WWAV	2	AV 9310135 (NC_010700); 9310141 (AF485264.1)
	TAMV	2	CDC W-10777 Archer (NC_010701.1); W 10777 Charrel (AF512828.1)
	Skinner Tank	1	V AV D1000090 (EU123329.1)
	Catarina	2	AV A0400212 (DQ865245.1); AV A0400135 (DQ865244.1)
Clade LCMV	Total	11	
	Dandenong	1	(EU136038.1)
	LCMV	10	Traub (DQ868487.1); Cepa Y (DQ118959.1); Armstrong 53b (AY847350.1); CH-5692 (DQ868483.1); Pasteur (DQ868485.1); Marseille#12 (DQ286931.1); M1 MAI-Pas#6 (AB261991.1); GR01 (FJ895883.1); SN05 (FJ895884.1); CABN (FJ895882.1)
Clade Africa	Total	17	
	LASV	10	Pinneo (AY628207.1); LP (AF181853.1); Acar 3080 Hajjaj (AY628208.1); 803213 (AF181854.1); Weller (AY628206.1); Nigeria GA391 (X52400.1); Z148 (AY628205.1); Macenta (AY628201.1); Josiah (AY628203.1); NL (AY179173.1)
	MOPV	3	AN20410 (NC_006575.1); AN 21366 800150 (M33879.1); Mozambique (DQ328874.1)
	MOBV	1	ACAR 3080 MRC5 P2 (NC_007903.1)
	IPPV	1	Dak An B 188 d (DQ32887.1)
	Morogoro	1	3017/2004 (/NC_013057.1)
Unclassified	Lujo	1	(NC_012776.1)
	Total	76	

Interestingly, if we include only those samples isolated from rodents (*Calomys* spp. and *Akodon* spp. captured in the endemic area), a similar distribution can be identified for both fragments. If the five samples isolated from human cases are observed, when analyzing the G fragment sequences, similarities stand out between H_Lye63 and H_8027 on one hand and H_FHA5069, H_p1879 and H_FHA5054 on the other. However, when the N fragment profile is observed, the grouping is not present. The tree topology could reflect the continuous natural variation of the viral species. That is, the strains isolated in different years are related to their potential progenitor isolated in previous years.

3.2.3. Evolutionary distance

The previous analyses focused on the differences among the strains of Junín virus selected for this study. In order to observe these variations from a different point of view, an evolutionary distance analysis was performed. The sequences used for the analysis are some of those listed in Table 4. The same 48 sequences used for the distance analysis were subjected to a nucleotide similarity comparison. The results are summarized in Table 5. It is surprising that the similarity among LCMV strains is almost as great as between LCMV and LASV. Another noteworthy fact is that the similarity values of the N fragments, when comparing New World viruses to Old World viruses, are almost identical,

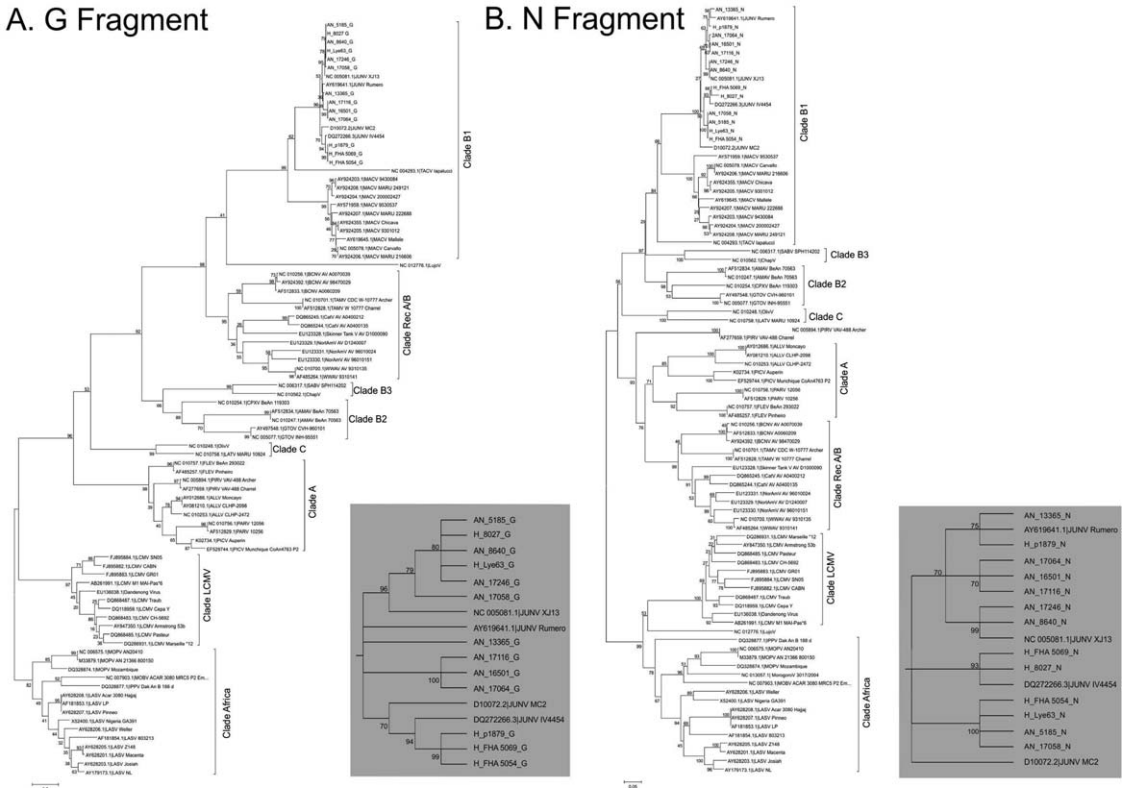

Fig. 2. Phylogenetic trees for N and G fragments from different arenavirus. Each analysis was made using the MEGA4 program as described previously. All clades are indicated with the corresponding names. The gray shadowed areas correspond to the JUNV samples. In this view, the branches with a statistically support value of bootstrap below 70 were condensed in order to emphasize the reliable portions of the tree pattern. (A) Phylogenetic analysis results from sequences corresponding to the G fragment. (B) Phylogenetic analysis results from sequences corresponding to the N fragment.

Table 5
Similarity % based on G and N fragment sequences. To estimate the evolutionary divergence over sequence pairs between groups, the average number of nucleotide differences per site over all sequence pairs between groups and among groups was calculated. Standard error estimates were obtained by a bootstrap procedure (500 replicates). JUNV, Junín virus; MACV, Machupo virus; LASV, Lassa virus; LCMV, Lymphocytic Choriomeningitis virus.

Comparison	Similarity of sequences, % ±SD	
	G fragment	N fragment
Within JUNV	94.5 ± 0.6	95.4 ± 0.7
Within MACV	91.2 ± 0.8	92.5 ± 0.9
Within LASV	82.4 ± 1.1	82.4 ± 1.3
Within LCMV	76.9 ± 1.2	81.0 ± 1.4
Between LASV and LCMV	76.0 ± 1.6	81.4 ± 1.8
Between JUNV and MACV	68.3 ± 1.9	80.1 ± 2.0
Between MACV and LCMV	52.6 ± 2.0	68.3 ± 2.0
Between JUNV and LCMV	52.0 ± 1.9	68.3 ± 2.0
Between MACV and LASV	48.1 ± 2.1	68.1 ± 2.2
Between JUNV and LASV	48.4 ± 2.0	67.2 ± 2.2

while they are lower and different when comparing the G fragments.

4. Conclusions

The increasing sequence information from complete arenavirus genomes, especially of the JUNV species, contributed to the identification of putative markers of virulence attenuation (Goñi et al., 2010). With the objective of analyzing the mutation distribution in nature, a molecular technique of RT-Nested-PCR was designed and afterwards used with rodent and human samples from the endemic area. As reported in a previous study with another group of strains (García et al., 2000), the first sequence analysis suggested a high degree of homology of the selected genomic regions.

The protein sequences obtained by *in silico* translation of the G and N fragment were analyzed with different tools. With the purpose of determining the different mutations, the information from the amino acid alignment was collected in Table 3. It was possible to note the formation of groupings of different samples when analyzing the G fragment. As noted above, the analysis of this fragment covered the sequence involved in the cleavage between G1 and G2 proteins. The possible recognition site is formed by the sequence QLPRRSLK$_{251}$↓AFF (Beyer et al., 2003), or between the L$_{250}$ and K$_{251}$ (Lenz et al., 2001), and cutting is done by the protease SKI-1/S1P. The sequenced samples reported here showed a high conservation degree in this zone. Only for isolate H_FHA5054, changes were observed at positions 244 and 245 (Q→ H and L→ F respectively), although it is possible that these modifications may not affect the recognition site. Another important site in this fragment was determined by NETGLYC 1.0, comprising the target sequence N$_{166}$R$_{167}$T$_{168}$K$_{169}$ involved in the N-glycosylation of this protein. By observing the distribution of this target in the different isolates it can be seen that only 58% of the strains have the target sequence, while the other 42% have an alanine residue (A$_{168}$). The bioinformatics studies did not show important changes in the protein properties (data not shown). On the other hand, a recent study resolved the importance of two positions strongly related to the N-glycosylation for JUNV and MACV (Bowden et al., 2009). It was found that both residues and their environment are highly conserved. In any case, it was recently shown for LCMV, that each N-linked glycan in the arenavirus glycoprotein is involved in GPC expression, fusion with the host receptor and infectivity (Bonhomme et al., 2011).

Greater conservation was detected when observing the N fragment's amino acid sequence. This characteristic could be directly related to the important role of this protein in the viral cycle (Hastie

et al., 2011; Levingston Macleod et al., 2010). The residue changes appear outside the exonuclease active site and Zn^{2+} coordination regions among all samples (data not shown).

Here, two different species from each group (Old World and New World) were analyzed so that the phylogenetic characteristics of each viral species could be described. When the results for the two species of NW were compared, MACV was slightly different from JUNV for both G and N fragments. Still, the different characterizations made for these strains showed the monophyletic nature of the viral species and their close relationship with JUNV (Cajimat et al., 2009). On the other hand, LCMV and LASV species have shown a high genetic diversity with respect to the previously analyzed virus. For the four identified lineages in LCMV, their diversity likely reflects the long and complex phylo-geographic history of the house mouse host (Albariño et al., 2010). For LASV, a distribution of strains in four genetic lineages was found, showing a correlation between genetic and geographic distances (Bowen et al., 2000) contrary to that observed for LCMV (Albariño et al., 2010). Considering the genetic variability and the availability of sequences, the establishment of a genetic tool for viral classification may well be very close. With the metagenomic approach, we can access more data with the aim of solidifying this new strategy in virus taxonomy.

In this study it was possible to observe the high homogeneity of nucleotidic sequences for JUNV field samples. Taking into account the previous efforts made to define a unique genetic criterion for classification (Charrel et al., 2008), we propose a technique for JUNV that combines amplification and sequencing of fragments located within the S segment and that allows a rapid isolate characterization. The N fragment has a lower variability than the G fragment, but both present complementary information useful in case of a recombination event or host jumping event (Cajimat et al., 2007). We detected a higher intra-species evolutionary distance for the two OW viruses analyzed (LASV and LCMV), when compared to the value obtained for two viruses of NW (JUNV and MACV). This could be due to: (i) an evolutionary history more ancient for the viruses with a higher distance (OW), or (ii) an inaccurate taxonomic classification, where more than one virus could be denominated as only one viral species, or (iii) independent evolutions for strains present in relatively isolated areas. These last two hypotheses probably apply to LASV, where different viral species could be defined according to genetic and geographic parameters.

On one hand, for members of the B1 subclade (TCRV, JUNV and MACV), differences were reported between G1 proteins (Cajimat et al., 2009; Oldenburg et al., 2007). On the other hand, the TfR1 protein was identified as the receptor for the hemorrhagic arenaviruses (GTOV, JUNV, MACV and SABV, Radoshitzky et al., 2007), while the non-pathogenic viruses of clade B use their hosts' orthologue (TCRV and AMAV) (Abraham et al., 2009). This feature was also observed for the zoonotic transmission of viruses that cause the American hemorrhagic fevers (Radoshitzky et al., 2008). Some of the changes observed in the G fragment (63% of G1) could be related to the host jump process, of a strain or isolate from rodents to humans, or to the receptor affinity variation in human isolates, leading to changes in the virulence (attenuation or increase) trough modification of the cellular tropism.

Additional studies that employ a reverse genetics system for JUNV are necessary to investigate the role of different mutations in the virulence attenuation process or host jumping events, with the principal objective of generating better surveillance programs, prevention strategies (vaccines) and therapies. To this date, there are reports of such two systems for this arenavirus (Albariño et al., 2011; Emonet et al., 2011). Our results contribute to the generation of the sequence data of field isolates that should prove highly useful in the selection of residues potentially involved in different viral mechanisms of survival and are a possible target for mutagenesis.

Acknowledgments

This research was supported by National University of Quilmes (UNQ), Carlos III National Institute of Health (ISCIII) and by PICT 38138 from ANPCYT and PIP5813 from CONICET (Argentina). SEG and BIS have a CONICET scholarship. MEL is a research investigator of CONICET. AT is a research investigator. MSC is a Titular professor in the National University of Córdoba (UNC). SEG, JAI and MEL are Professors in the UNQ.

We thank the collaboration of the ISCIII staff who collaborated in this study.

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