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Molecular Attenuation Process in Live Vaccine Generation for Arenaviruses

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1. Introduction

The arenaviruses are part of a growing viral family denominated *Arenaviridae*. Currently, there are 22 recognized members (Salvato *et al.*, 2005), listed in Table 1. Based on the geographic origin and distribution of their hosts, the arenaviruses can be classified in two groups: the New World Arenaviruses (NWA) and the Old World Arenaviruses (OWA). The first group comprised native American arenaviruses, while the Old World group is conformed by African viruses and the ubiquitous Lymphocytic Choriomeningitis virus (LCMV). This geographic distribution is determined by the species host range, with LCMV as the only one arenavirus with a worldwide distribution, mainly because *Mus musculus* is its rodent reservoir. Specific members of *Neotominae* and *Sigmodontinae* from the *Cricetidae* rodent's family are the principal hosts for the New World Arenaviruses (Cajimat *et al.*, 2007). From these 22 recognized species, only 6 were consistently detected in humans and were related to a set of clinical symptoms that allowed to establish the description of a disease: Lymphocytic Choriomeningitis (LCM, caused by LCMV), Lassa Fever (LF caused by LASV), Argentine Hemorrhagic Fever (AHF, caused by JUNV), Bolivian Hemorrhagic Fever (BHF, caused by MACV), Venezuelan Hemorrhagic Fever (VHF, caused by GTOV), and Brazilian Hemorrhagic Fever (BrHF, caused by SABV). The physiopathology of the hemorrhagic fevers produced by NWA is very similar. AHF and BHF were described in the middle of the twentieth century. GTOV was isolated from humans during an epidemic outbreak (at first mistaken for hemorrhagic dengue) that happened in Venezuela (Salas *et al.*, 1991). Because GTOV and PIRV share the same (geographic) area, different studies were made with the aim of predicting a re-emergence of a Venezuelan hemorrhagic fever variant (Cajimat & Fulhorst, 2004). The Brazilian hemorrhagic fever was established from a human fatal case, isolating the SABV (Lisieux *et al.*, 1994). Recently, a serological screening for arenavirus among the population of Nova Xavantina, State of Mato Grosso in Brazil, show that 1,4% of the serum samples presented antibody titers against arenavirus (Machado *et al.*, 2010).

In 2003 a hemorrhagic fever case was reported in Bolivia, and after an exhaustive analysis a new virus, denominated Chapare, was described (Delgado *et al.*, 2008). On the other side, in the year 2000, another three isolated cases were reported, one of them, related to WWAV,

was a classical hemorrhagic fever (CDC, 2000), while the others, related to TCRV and FLEV, were mild feverish illness in laboratory workers (Charrel *et al.*, 2008).

Virus	Acronym	Evolutionary Lineage	Distribution	Reservoir	Human Disease
Flexal	FLEV	NWA-A	Brazil	*Oryzomys spp.*	LM
Pichindé	PICV	NWA-A	Colombia	*O. albigularis*	NR
Paraná	PARV	NWA-A	Paraguay	*O. buccinatus*	NR
Allpahuayo	ALLV	NWA-A	Perú	*Oecomys bicolor*	NR
Pirital	PIRV	NWA-A	Venezuela	*Sigmodon alstoni*	NR
Junín	JUNV	NWA-B	Argentina	*C. musculinus*	SD
Machupo	MACV	NWA-B	Bolivia	*C. callosus, C. laucha*	SD
Guanarito	GTOV	NWA-B	Venezuela	*Z. brevicauda*	SD
Sabia	SABV	NWA-B	Brazil	*Unknown*	LS
Chapare	-	NWA-B	Bolivia	*Unknown*	LS
Pinhal	-	NWA-B	Brazil	*Calomys tener*	NR
Tacaribe	TCRV	NWA-B	Trinidad	*Artibeus spp.*	LM
Cupixi	CPXV	NWA-B	Brazil	*O. capita*	NR
Amapari	AMAV	NWA-B	Brazil	*O. capita-N. guianae*	NR
Oiveros	OLVV	NWA-C	Argentina	*Bolomys obscurus*	NR
Pampa	-	NWA-C	Argentina	*Bolomys sp.*	NR
Latino	LATV	NWA-C	Bolivia	*Calomys callosus*	NR
Río Carcarañá	-	NWA	Argentina	*Bolomys obscurus*	NR
Catarina	-	NWA-RecA/B	USA, Texas	*Neotoma micropus*	NR
Skinner Tank	-	NWA-RecA/B	USA, Arizona	*Neotoma mexicana*	NR
North American	-	NWA-Rec A/B	USA	*Neotoma mexicana*	NR
Withewater Arroyo	WWAV	NWA-RecA/B	USA, Southwest	*N. albigula, N. mexicana*	LS
Tamiami	TAMV	NWA-RecA/B	USA, Florida	*Sigmodon hispidus*	NR
Bear Canyon	BCNV	NWA-RecA/B	USA, California	*Peromyscus sp.*	NR
Big Brushy Tank	-	NWA-Rec A/B	USA		
Tonto Creek	-	NWA-Rec A/B	USA		
LCM	LCMV	OWA	All world	*Mus musculus*	MD
Lassa	LASV	OWA	Nigeria, Ivory Coast, Guinea, Sierra Leone	*Mastomys sp.*	SD
Mopeia	MOPV	OWA	Mozambique	*Mastomys natalensis*	NR
Mobala	MOBV	OWA	Central African Republic	*Praomys sp.*	NR
Ippy	IPPYV	OWA	Central African Republic	*Arvicanthis sp.*	NR
Dandenong	-	OWA	Australia	*Unknown*	LM
Kodoko	-	OWA	Guinea	*Mus Nannomys minutoides*	NR
Morogoro	-	OWA	Tanzania	*Mastomys sp.*	NR
Lujo	-	OWA	South Africa	*Unknown*	LS

SD: Severe disease; MD: Mild disease; LS: Limited and severe disease; LM: Limited and mild disease; NR: not reported; OWA: Old World Arenavirus; NWA: New World Arenavirus; thereafter is indicated the lineage in which the New World members are classified: A, B, C and Rec A/B. The countries are listed based on viral isolation and not serology data. Table modified from Charrel & de Llambarie, 2003 and Charrel *et al.*, 2008.

Table 1. *Arenaviridae* family members list.

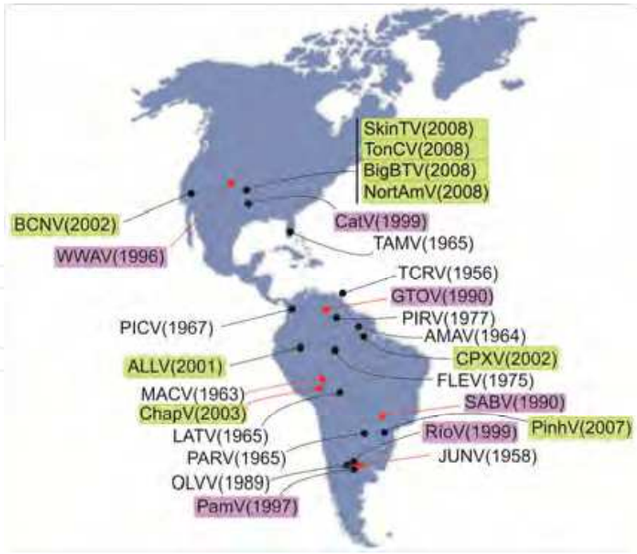


Fig. 1. Geographic distribution of New World Arenaviruses. The black points indicate those viruses not described as human pathogens, while the red points indicate the known pathogens that cause the different American hemorrhagic fevers. After each viral acronym, the year of isolation or description is added. The viruses identified after the year 2000 are shadowed in green, while those isolated in the past century's ´90s decade are in violet. The virus acronyms are indicated in Table 1, with the exception of the following: ChapV (Chapare), PamV (Pampa); RíoV (Río Carcarañá); PinhV (Pinhal); CatV (Catarina); NortAmV (North American); BigBTV (Big Brushy Tank); TontCV (Tonto Creek); and SkinTV (Skinner Tank), because they were not yet included as recognized arenavirus member.

In the course of the year 2008 the Dandenong virus was characterized, isolated from a transplanted patient, showing a high homology with LCMV (Palacios *et al.*, 2008). In that same year an outbreak of human nosocomial disease was reported in South Africa, with a high mortality rate (80%) and whose etiological agent was later characterized as an arenavirus. After 30 years, a new emerging member of the OWA group that caused a hemorrhagic fever (Briese *et al.*, 2009) was discovered. This virus was denominated Lujo virus, because the places where the first patients came from, the cities of Lusaka and Johannesburg. As can be seen in Figure 1, there is an evident increase in the information about arenavirus circulation in different places, with surprising findings in North America. Probably, their description is due to the active rodent capture program in different regions of USA and the search for arenaviral sequences by molecular techniques (with or without viral isolation). Most of these viruses still have to undergo taxonomic classification.

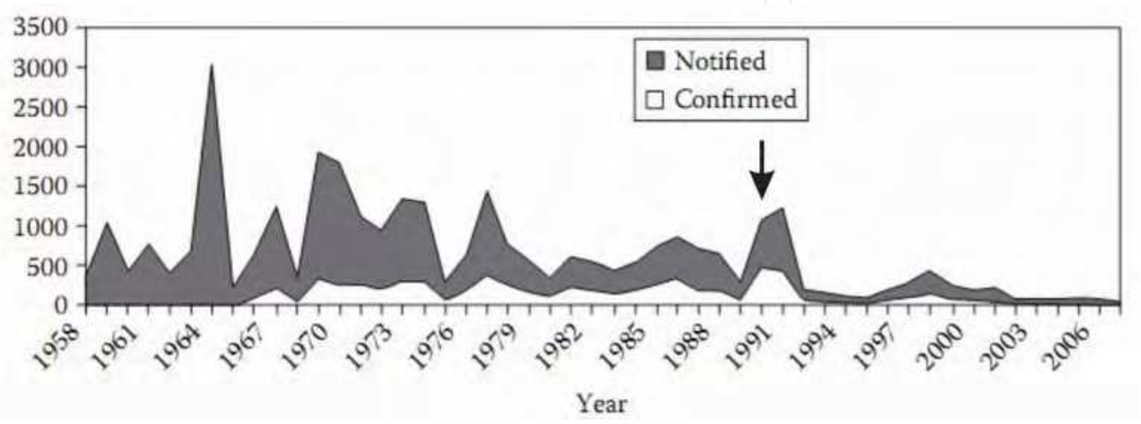
The LCM virus, detected in humans and rodents (Armostrong & Sweet, 1939; Lepine *et al.*, 1937; Rivers & Scott, 1935), is the causative agent of lymphocytic choriomeningitis and although it was associated to aseptic meningitis their infections in humans are unapparent in most cases. This virus has been a very important tool in the description of immunological mechanisms (Oldstone, 1987a, 1987b). The other arenaviruses, with the exception of LCMV, are found in restricted areas around the world. In fact, LCMV was also isolated in the AHF endemic region (Maiztegui *et al.*, 1972; Sabattini, 1977), where other arenavirus isolations were made, including OLVV and PamV which could be a variety of the same viral species. Another yet not completely characterized virus isolated in the AHF endemic area was

denominated Río Carcarañá, and from the sequence data it could be a product of a recombination event between arenaviruses from lineages B and C. The arenavirus hemorrhagic fevers are characterized by a disease that develops in well-defined phases: prodromal, neurologic-hemorrhagic and convalescent (Enría *et al.*, 2004), with a short incubation period, high fever, headaches and a set of specific symptoms that depend on the arenavirus species related with the infection. The principal characteristics of the arenaviral hemorrhagic fevers are indicated in Table 2.

Agent (Virus)	Pathological Characteristics
Old World Lassa	Incubation: 3 to 21 days. Fever, headaches, myalgia, backaches, trembling and sickness. Generalized infection: hemorrhagic dissemination of the virus to several organs and systems via bloodstream, lymphatic system, respiratory and digestive tract. Black vomit, aqueous diarrhea (dehydration), decrease in quantity of lymphocyts and platelets, mild thrombocytopenia, abdominal, pleuritic and hepatic area pain. Extensive reticuloendothelial compromise: capillary injuries causing stomach, small intestine, kidneys, lungs and brain bleeding. Multifocal hepatocellular necrosis with Councilman-like bodies, hepatocites citoplasmatic degeneration and minimal inflammatory response. Adrenal focal necrosis and citoplasmatic inclusions. Respiratory system: interstitial pneumonia, cough, dyspnea, bronchitis, pneumonia and pleurisy. Cardiovascular system: pericarditis, tachycardia, bradycardia, hypertension, hypotension, thrombocytopenia, leukopenia and hiperuraemia, lymphadenopathy, elevated aminotransferases, decreased prothrombin levels, disorder of blood circulation and bleeding through the skin, lungs, gastrointestinal tract and other membranes mucosa. Nervous system: encephalitis, meningitis, uni- or bilateral hearing decrease, or convulsions.
New World Junín Machupo Guanarito Sabiá	Incubation: 6 to 14 days. First 4: decaying, fever, anorexia, nausea and vomiting, headaches and myalgia. Second stage: acute hemorrhagic syndrome (epistaxis and hematemesis, Melcom & Herskovits, 1981), or acute neurologic syndrome (Rugiero *et al.*, 1960). General: malaise, high fever, severe myalgia, anorexia, back pain, abdominal tenderness, conjunctivitis, retro-orbital pain, photophobia, and constipation. Acute phase of infection: lymphomonocytes periferic blood viral active replication (Ambrosio *et al.*, 1986). Oropharyngeal enanthem. Gums swollen, congested and bleeding (gingival border). Proteinuria high dehydration and hemoconcentration. In women, early menorrhagia. Multifocal hepatocellular necrosis with formation of Councilman-like bodies, nuclear pyknosis, cytoplasmic eosinophilia, cytolysis, inflammation and a mild cellular infiltration composed of mononuclear cells and neutrophils. Kidney damage: distal tubular cells and collecting ducts. Glomeruli or proximal tubules (Cossio *et al.*, 1975). In a few cases the presence of renal failure was described (Agrest *et al.*, 1969). Cardiovascular system: postural hypotension and relative bradycardia, arrhythmias are transient and benign. Different degrees of dehydration, uremia, proteinuria, hematuria and oliguria. Respiratory: dry cough, without sore throat. Pharyngeal enanthem broncho-pulmonary unchanged. Interstitial pneumonia or bronchial, pulmonary edema and hemorrhage.

Table 2. Arenaviral hemorrhagic fevers pathological characteristics. The principal pathological characteristics caused by Old World Arenaviruses (LASV, Lassa Fever), and New World Arenaviruses (JUNV, Argentinean Hemorrhagic Fever; MACV, Bolivian Hemorrhagic Fever; GTOV, Venezuelan Hemorrhagic Fever; SABV, Brazilian Hemorrhagic Fever) are listed.

As previously described, Argentine hemorrhagic fever (AHF) is a severe endemoepidemic disease characterized by vascular, renal, hematological, neurological, and immunological alterations with a mortality of 15 to 30% in untreated individuals. Since the disease was first recognized, annual outbreaks have occurred without interruption, principally in autumn and winter (Ambrosio *et al.*, 2006). In Figure 3 it is possible to see the number of notified and confirmed cases until 2008 (Enría *et al.*, 2008; Iserte *et al.*, 2010). In this figure, the black arrow indicates the start of vaccination of the population at risk at the endemic area, reflecting the decrease in the AHF annual case numbers. The vaccine efficacy for the 1992-2000 periods was estimated in a 98% (AHF National Control Program, 2007).



The arrow indicates the start of vaccination.

Fig. 3. AHF notified and confirmed cases (1958–2006).

The attempts to obtain a vaccine against AHF started in 1959. A collaborative effort conducted by the US and Argentine Governments led to the production of a live attenuated Junin virus vaccine. After rigorous biological testing in rhesus monkeys, the highly attenuated Junin virus variant, named Candid#1, was used in human volunteers, followed by an extensive clinical trial in the AHF endemic area. (Barrera Oro & Eddy, 1982, Maiztegui *et al.*, 1987). The vaccination consists in the administration of JUNV Candid#1 to generate the protective immune response. The diagram with the passage history in different systems until the attenuated strain was obtained can be observed in Figure 4. Records of the passage history of the XJ strain come from the Yale Arbovirus Research Unit, Connecticut, USA (J. Casals) and USAMRIID, Frederick, Maryland, USA (J. G. Barrera Oro). The phase I and II clinical studies were made between 1984 and 1988 in Argentine and USA. After these studies it was shown that Candid#1 is innocuous, because none of the inoculated volunteers presented alterations and the immunogenicity was demonstrated in 90% of the cases. Since 2005 the vaccine is being produced in the National Institute of Viral Human Diseases (INEVH) located in Pergamino city, Argentina.

For other arenaviruses, was not yet possible to obtain an effective vaccine to prevent the disease. This would be especially important for Lassa fever, which is endemic in West Africa with up to 500.000 reported cases (Ogbu *et al.*, 2007). In the effort to make an efficient vaccine to combat Lassa fever in Africa, several strategies were applied. One of them, is

based upon generation of a reassortant virus that contains a genomic segment from Mopeia virus (non pathogenic) and the other segment from Lassa virus (Lukashevich *et al.*, 2005). The obtained results are promising, but still have to demonstrate coverage of all natural variants of Lassa virus to ensure the protective capacity of a vaccine.

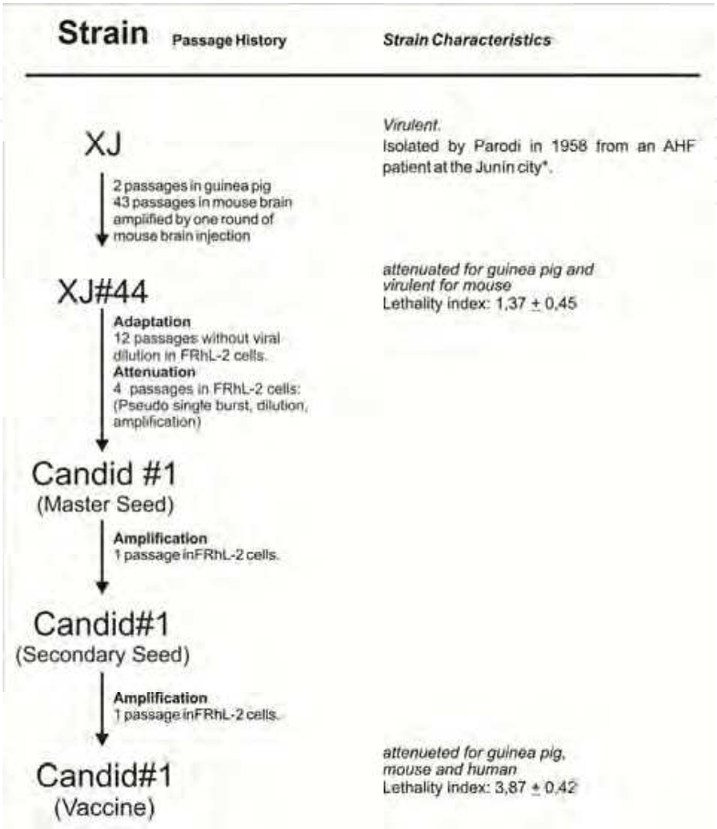


Fig. 4. Passage history of Junín virus, strain Candid#1. The genealogical relationships of the studied Junín virus strains are shown by arrows. The XJ strain was subjected to two passages in guinea-pigs (GP2) and 43 passages in mouse brain (MB43). Passage number 43 was amplified by one round of mouse brain injection (XJ#44). This brain homogenate was used to infect FRhL-2 cells. After 12 passages, one pseudo single burst growth was carried out, followed by cloning using two limiting dilution steps. After one amplification round, master and secondary seeds were obtained. The vaccine stock (Candid#1) was obtained by single amplification of the secondary seed. The lethality index was calculated as log10 p.f.u. that produce one LD50 (±1 SD) by intracerebral inoculation of mice (Parodi *et al.*, 1958).

2. Molecular features of arenaviruses

All arenaviruses shared morphological and biochemical properties. They are enveloped and their genome is composed of bipartite RNA (Martínez Segovia & Grazioli, 1969; Riviere *et al.*, 1985b). These RNAs are single-stranded and posses an approximate length of 7 kb (L RNA) and 3,5 kb (RNA S). The lipid envelope contains two viral glycoproteins, G1 and G2, inside of the virion there are three other arenavirus proteins, denominated N, Z and L. The majority of N protein is associated to the viral RNAs forming the nucleocapsids. The second,

a protein of 11 kDa, is denominated Z because it has a Zinc finger structural motif and could be the counterpart of the matrix proteins of other RNA viruses (Pérez *et al.*, 2003). Furthermore, all virions contain a minimal proportion of a RNA dependent RNA polymerase, denominated L protein.

Each RNA segment directs the synthesis of two proteins; their open reading frames are arranged in opposite orientations (ambisense coding strategy) and are separated by a non-coding intergenic region that folds in a stable secondary structure (Auperin *et al.*, 1984). Furthermore, the ends of both genomic RNAs are complementary. The first 19 nucleotides at the 3' end base-pair with the complementary sequence at the 5' end forming a panhandle structure, which is conserved among the arenaviruses. The S RNA codes for the major structural proteins of the virion: the precursor of the envelope glycoproteins (GPC) and the viral nucleocapsid protein (N). Posttranslational cleavage of GPC renders a signal peptide (SP) and the two viral glycoproteins (G1 and G2). The L RNA segment codes for the viral RNA dependent RNA polymerase (L) and the small protein (Z). N and L proteins are translated from anti-genome-sense mRNAs, complementary to the 3′ portion of the viral S or L RNA, respectively. The GPC and Z proteins are translated from viral or genome-sense mRNAs corresponding to the 5′ region of the viral S or L RNA, respectively.

The secondary and tertiary structures present in the viral genomic RNAs play an essential regulatory role during the transcription, translation and assembly of new viral particles (Álvarez *et al.*, 2005). The intergenic region in both genomic fragments is arranged into a stable hairpin loop structure, which is crucial in the regulation between transcription and replication of the viral genome (Tortorici *et al.*, 2001b), while the panhandle structure at the ends of the genomic RNAs, could act as a promoter initiating replication, as occurs in Flavivirus (Álvarez *et al.*, 2005; Pérez & de la Torre, 2003; Salvato & Shimomaye, 1989).

In Figure 5, the morphological characteristics of the arenavirus particles are shown. They are pleomorphic with a diameter of 50 to 300 nm (Dalton *et al.*, 1968; Gschwender *et al.*, 1975; Murphy *et al.*, 1968, 1970; Murphy & Whitfield, 1975; Ofodile *et al.*, 1973; Speir *et al.*, 1970).

Once the virus enters the cell the ribonucleoproteins are released into the cytoplasm, and, transcription and replication are mediated by the L protein within the cytoplasm. The glycoproteins and Z are co-translated and processed in the ER and Golgi, while the N, and L proteins are translated on free ribosomes. Virus assembly initiates at the Golgi or plasma membrane. The N and L mRNAs are transcribed from the RNA S or L 3´ end, respectively. On the contrary, the GPC and Z mRNAs are transcribed from the 3′ end of the antigenomic S or L RNA. These processes and mechanisms are detailed in Figure 6.

The genomic S RNA is transcribed to only two antigenomic forms: the 1,8 kb N mRNA and the 3,4 kb full length antigenomic S RNA. This antigenomic S RNA serves as the replicative form of the virus and also as the template for GPC mRNA transcription. When translation is inhibited, transcription of Junín virus S RNA yields only the N mRNA. Apparently, the non-coding intergenic region form a secondary very stable hairpin loop acting as a transcription terminator (Franze Fernández *et al.*, 1987; Ghiringhelli *et al.*, 1991; Tortorici *et al.*, 2001a). This implies that the synthesis of a full length antigenomic copy of S RNA requires an antiterminator. This function is supplied by the N protein (Tortorici *et al.*, 2001a).

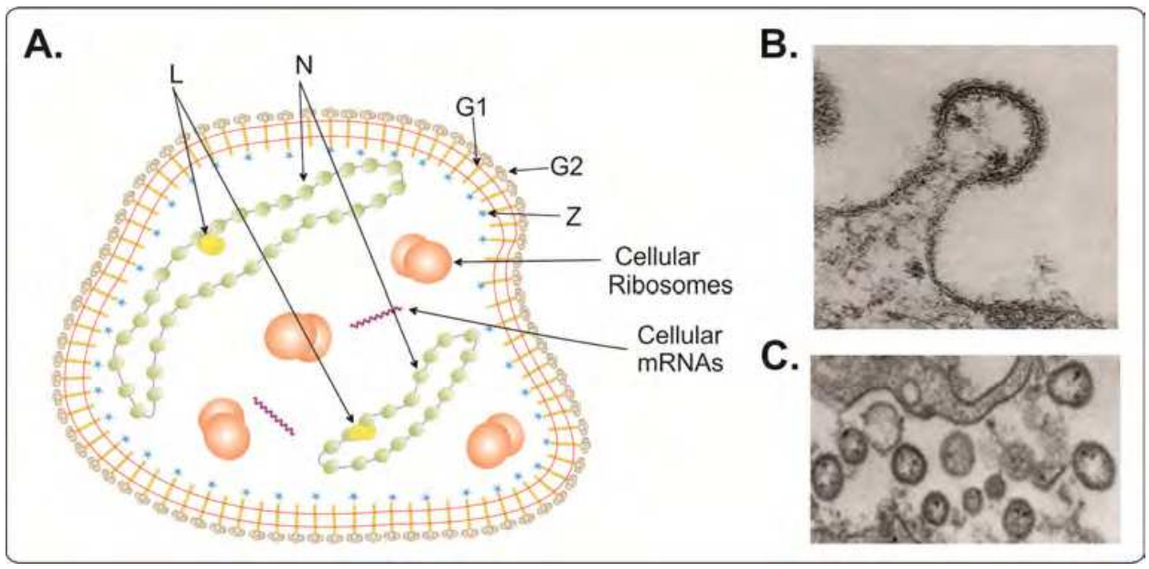


Fig. 5. Arenavirus viral particle.A. Virion structure diagram. The arenavirus particles have a lipid bilayer envelope (red lines), with envelope glycoprotein's (G1 and G2), and internal components that include two circular nucleocapsids with bead aspect, associated to the viral polymerase (L), cellular messengers and ribosomes. The nucleocapsids contain RNAs S and L and proteins (N, in several copies and L in few copies). The Z protein is found associated to the bilayer by the inner face. B. Electronic micrograph of a section showing a virion budding from an infected Vero cell. The viral envelope is more dense and different to the host cell membrane. The ribosomes are presents in the budding particle (173.000 X, Murphy *et al.*, 1968). C. Electronic micrograph of Junín virus particles in the extracellular space on the periphery of infected Vero cells (95.000 X). The particles are constituted by a heavy membrane envelope, containing several dense granules of 20 to 25 nm (Murphy *et al.*, 1970).

The glycoprotein precursor is processed into 3 peptides: the peripheral protein G1, the trans-membrane G2 and the signal peptide (Buchmeier & Oldstone, 1979; York *et al.*, 2004). Due to their characteristics, G1 is the protein that interacts with the cellular receptor. The Old World and C clade of New World arenaviruses share the same cellular receptor, the α-distroglycan (Cao *et al.*, 1998; Spiropoulou *et al.*, 2002). Later, it was found that for clade B of the New World arenaviruses, the receptor is a protein: the transferrin 1 receptor (TfR1, Radoshitzky *et al.*, 2007).

The arenavirus nucleoprotein has a weight of 63kDa and constitutes the principal component of the nucleocapsid, being the most abundant protein in the virion (near to 70%). This protein presents a dual function: structural and non-structural. On one side, it is involved in essential genome replication steps promoting the synthesis of the full length antigenomic segments, and on the other side it is associated to the viral genome to form the nucleocapsid. For that, the determinations of N-RNA interactions are very interesting study targets. Among the N described motifs, there is one RING finger domain whose folding requires zinc (Tortorici *et al.*, 2001b). There is one conserved region between amino acids 497 and 530 ($C_{497}X_2H_{500}X_{23}C_{525}X_4C_{530}$, Parisi *et al.*, 1996) and their function was experimentally studied (Tortorici *et al.*, 2001b). These results confirm the identity of the domain and also

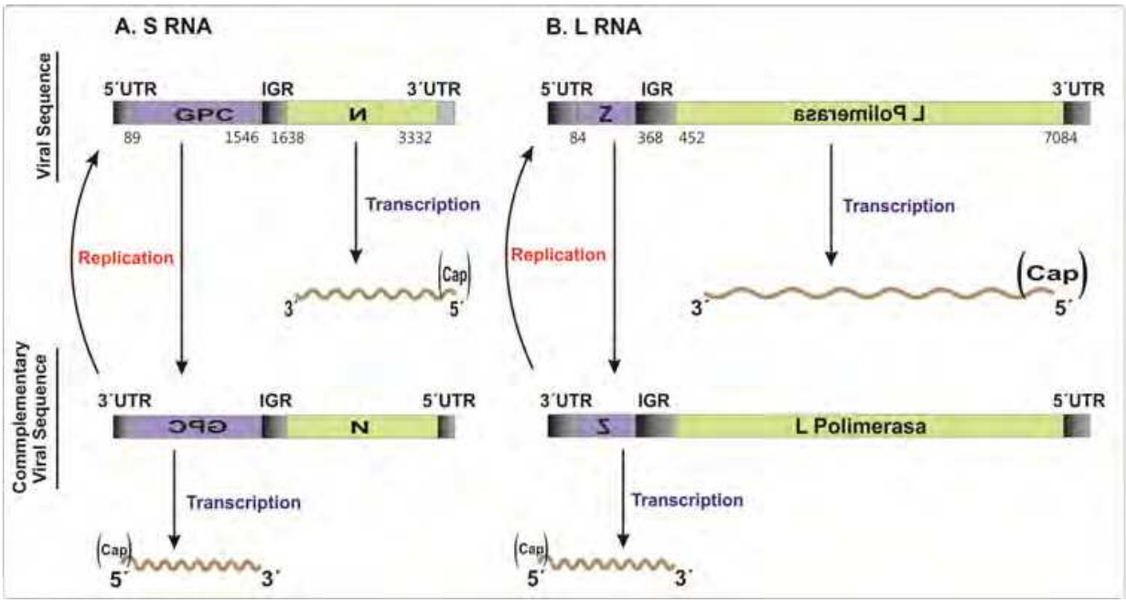


Fig. 6. Arenavirus replication/transcription strategy. The S and L genomic segments are schematized as horizontal rectangles. The ORFs that present a genomic or viral polarity (GPC and Z) are violet, while the antigenomic or viral complementary genes (N and L) are in green. The intergenic region (IGR) and untranslated terminal regions (UTR) are shown in gray. A. S RNA replication/transcription diagram. B. L RNA replication/transcription diagram.

showed the possibility of the presence of other motifs that enhance this activity. Another important functional aspect of the N protein is its capacity to avoid elimination by the adaptative immune response of the host. It was demonstrated that in LCMV infections, N inhibits the response by β-interpheron production interference (Martínez-Sobrido *et al.*, 2006).

The major ORF in the virus genome corresponds to the RNA dependent RNA polymerase denominated L protein. In the virion, this protein is associated to the viral nucleocapsid forming the ribonucloeprotein complex, and posseses the sequence motifs characteristically conserved between the RNA dependent RNA polymerases of the negative-stranded RNA viruses. The polymerase activity requires oligomerization through the formation of a L-L complex that is essential for the enzymatic function (Sánchez & de la Torre, 2005). Because of its size this protein is the target for different mutations that affect its capacity to different extents.

Finally, the smaller protein of this viral family is denominated Z protein. The role of Z in the virus life cycle is not completely elucidated, and homologues of Z are not found in other ambisense or negative-stranded RNA viruses. Z is a structural component of the virion (Salvato *et al.*, 1992), and by means of *in vivo* and *in vitro* experiments, the interaction of Z with several cellular factors has been reported, including the promyelocytic leukemia protein and the eukaryotic translation initiation factor 4E (Borden *et al.*, 1998a, b). Because of this latter interaction, it was proposed that Z inhibits Cap-mediated translation (Campbel *et al.*, 2000; Kentsis *et al.*, 2001). Other researchers suggested that Z could be a transcriptional regulator of the viral cycle (Garcín *et al.*, 1993) or even an inhibitor of viral replication (López *et al.*, 2001). Furthermore, Pérez and coworkers (Pérez *et al.*, 2003) proposed, for LCMV and

LASV, that Z is the functional counterpart of the matrix proteins found in other negative-stranded enveloped RNA viruses. Z protein have characteristic late domains (LDs), also found in matrix proteins from negative-stranded RNA viruses and in Gag protein from retroviruses. LDs, have an essential role in the viral budding process (Freed, 2002). Three types of motifs have been defined within viral LDs: P[TS]AP, PPxY, and YxxL (Pornillos *et al.*, 2002), where ''x'' is any amino acid. Later, Martín Serrano and coworkers (Martín Serrano *et al.*, 2004), redefined the last as: YPxL/LxxLF. LDs are highly conserved and have been shown to mediate interaction with host cell proteins, in particular with members of the vacuolar protein-sorting pathway (Bieniasz, 2006; Urata *et al.*, 2006). For instance, the PTAP motif from Ebola virus VP40 matrix protein and from HIV Gag protein interacts with Tsg101, a member of the vacuolar protein-sorting pathway.

3. Sequence analysis of the Junín virus vaccine related strains

To characterize the mutations associated with the attenuated phenotype in Junín virus, we obtained the complete nucleotide genomic sequence from the vaccine genealogy related strains and another field strains of Junin virus (Goñi *et al.*, 2006, 2010).

Sequence data was analyzed using a series of bioinformatics tools. When we compared the complete genomic sequence of Candid#1, XJ#44 and XJ13 strain, we found a set of differences that could be associated with the attenuated phenotype (Figure 7). Alignment of the coding sequences of the S RNA genes of Junín virus vaccine related strains showed twelve nucleotide changes implied in amino acid substitutions. As depicted in Figure 7.A.1, one of these changes is found in the signal peptide (I_{35}→V→V), four of them are at the middle portion of G1 (T_{168}→A→A; E_{186}→E→G; S_{206}→S→P and P_{208}→L→L), two are at the carboxyl terminus of G2 (F_{427}→F→I and T_{446}→T→S), and five more are at the amino half of N (V_{47}→V→E; K_{59}→R→R; I_{158}→V→V; E_{268}→D→D and T_{322}→I→I). An alignment of the coding sequences of the L genes of Junín virus strains showed only nine nucleotide changes between XJ13, XJ#44 and Candid#1 strains implicated in amino acid substitutions (Figure 7.B.1). Seven of these changes may be related with the attenuation process (H_{76}→Y→Y; V_{415}→V→A; D_{462}→N→N; L_{936}→L→P; R_{1156}→K→K; S_{1698}→S→F and I_{1883}→I→V) and two changes (R_{881}→G→R; S_{921}→G→S) could be considered reversions. All these changes are presented as XJ13 residue → XJ#44 residue → Candid#1 residue. On the contrary, no changes were found in the amino acid sequence as well as at the nucleotide level of the Z protein, among these three Junín virus strains.

3.1 Mutation analysis

Furthermore we compared the nucleotide sequences obtained from vaccine related strains with other reported Junín virus strains. There are only two other Junín virus strains whose genome was fully sequenced, Romero and MC2 strains. Romero strain, classified as a high virulence strain, was isolated from an AHF patient and was passed twice in fetal rhesus lung cells and once in Vero cells (McKee *et al.*, 1985; Yun *et al.*, 2008). This strain is named Rumero in Genebank, but this was derived from a typographical mistake. On the other hand, the MC2 strain was isolated from a rodent from the endemic area of AHF, and was classified as intermediate virulence strain (Berría *et al.*, 1967; Candurra *et al.*, 1989; Weissenbacher *et al.*, 1987). Moreover the complete S RNA and Z gene sequences were

obtained for the Junín virus strain Cba-IV4454. This strain, classified as an intermediate virulence strain, was isolated from an AHF patient. Differences between deduced amino acid sequences from the six analyzed Junín virus strains are shown as vertical bars in Figure 7 and were classified into two types:

Type 1: Positions where the nucleotide or aminoacid sequence of one of the field strains of Junin virus (XJ13, Romero, IV4454 or MC2) was different from all other field strains, shown as vertical gray bars in Figure 7 located below each RNA scheme.

Type 2: Positions with mutations among the vaccine strains, shown as vertical red bars in Figure 7 located over each RNA scheme.

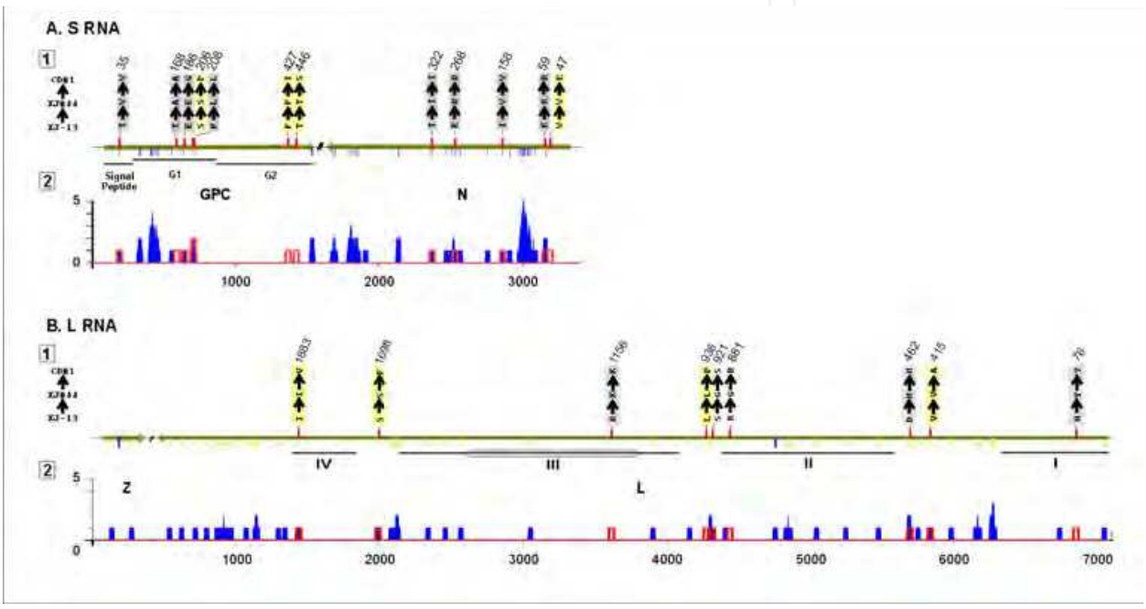


Fig. 7. Schematic of the changes detected in Junín virus proteins. Comparisons were done among all fully sequenced genomes from different Junín virus strains. A nucleotide rule is depicted below the diagram to facilitate the location of each position. **A.** S RNA, **1:** the open reading frames corresponding to the N and GPC genes are shown as open rectangles with arrowheads indicating the direction of translation. Non-coding sequences are shown as horizontal thin lines. The three cleavage products of GPC protein are shown by horizontal lines below the diagram. Amino acid changes detected between vaccine genealogy strains (XJ13, XJ#44, and Candid#1; type-2 mutation) are represented as vertical red lines over the genes. Above them, the detected changes and the position are indicated. Amino acid changes between field strains of Junín virus (XJ13, Romero, IV4454 and MC2; type-1 mutation) are indicated as vertical lines below the genes; **2:** plot of relative mutation frequency for type-1 mutations (blue areas) and for type-2 mutations (red line). **B.** L RNA, **1:** the open reading frames corresponding to the Z and L genes and the position of the changes in the amino acid sequence are shown as in (A.1.). The four conserved regions of the RNA polymerase of arenaviruses as described by Vieth *et al.* (Vieth *et al.*, 2004) are shown by horizontal lines below the diagram of the L RNA. Inside region III, the polymerase domain is signalized with an open box; **2:** plot of relative mutation frequency for type-1 mutations (blue areas) and for type-2 mutations (red line).

To identify those type 2 mutations that could be more confidently related with the attenuation process, we compare the homologous positions at the genomes obtained from field strains of Junín virus (XJ13, Romero, IV4454 and MC2). At positions GPC_{35}, N_{158}, N_{268} and N_{322} the same variations present between vaccine related strains was found among field strains. At positions L_{881} and L_{921}, there is a variation from XJ#44 to Candid#1. However, Candid#1 derived sequence is identical to the field strains (including XJ13) derived sequences. Thus, it is probable that these positions represent naturally generated mutations, non-related with the attenuation process. Furthermore, sequence variations among field strain are subject to natural selection pressure, whereas sequence variations among vaccine genealogy strains were subjected to an arbitrary selection pressure. We calculated a mutation frequency index defined as number of mutations per amino acid, and the graph was constructed with a program designed in our group by J.A. Iserte (unpublished results), using an overlapped windows-based strategy (11 residues) counting the number of mutations over each window and plotting the value at the middle of the window. This analysis was made along the Junín virus genome for both types of mutations (Figure 7.A.2 and 7.B.2). The regions of the S or L RNA with a high mutation frequency index for type 1 mutations are shown as closed blue areas while regions with a high mutation frequency index for type 2 mutations are shown as open, red outlined, areas. Mutations of type 2 found outside blue regions, identified at S RNA positions GPC_{168}, GPC_{427}, GPC_{446} and N_{47}, and L RNA positions L_{76}, L_{936}, L_{1156}, could be more confidently involved in the virulence attenuation process.

Furthermore, we searched for the presence of some of the type 2 mutations in a set of field samples. These Junín virus strains were isolated in the endemic area from human cases and captured rodents from the period of 1963-1991. Four genomic regions were selected for the analysis: i) G fragment: comprises the nucleotide positions 303 to 941 (in the glycoprotein precursor coding sequence), ii) N fragment, located between nucleotides 1632 and 2095 (in the nucleoprotein coding sequence), iii) L1 fragment, comprise between 1086 and 2005 nucleotide residues, covering the totality of RNA polymerase-RNA dependent IV motif, and iv) L2 fragment, covering the residues comprises between the 4234 and 4890 positions (C terminal of motif II). These regions include some of the type 2 mutations detected in previously. For 13 field JUNV strains, these regions were amplified and sequenced.

In the alignments of the different *in silico* translated proteins from sequenced fragments we search for the positions in which it was possible to identify variations between strains. However, for the positions included in this analysis, the variation observed between the attenuated JUNV strains was also present in some of the new field samples, taken from both rodents and human cases. As example, in Table 3 we showed some of the positions with type 2 mutations (potentially related to the virulence attenuation process) within the G fragment. The analyzed region spanned positions 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). As a consequence, the relative importance of these variations in the attenuation process should be re-evaluated.

3.2 Non coding regions

It has been previously suggested that changes in the intergenic region could play a role in the attenuation processes of arenaviruses (Wilson & Clegg, 1991). However, our sequence

analysis of the intergenic regions from XJ13, XJ#44 and Candid#1 revealed 100% conservation in both genomic RNAs. If nucleotide changes were not tolerated in this region, it could suggests that a major evolutive constraint is operating, perhaps related to the calculated secondary structure conformation and its proposed function in the transcription regulation process (Franze-Fernández *et al*., 1993; Tortorici *et al*., 2001a). Furthermore, the non-coding regions at the genomic ends are highly conserved among analyzed strains, varying between 93% and 97% of nucleotide sequence homology. The 3′ non-coding region, which in the virions shows a high degree of complementarity with the 5′ non-coding region, exhibits very few differences in independent clones of each strain and varies only slightly from one strain to another.

On the other hand, when infected cell-derived RNAs were sequenced, a high degree of sequence variability has been observed at the 5′ and 3′ non-coding regions among RNAs derived from the same strain. We did a RACE analysis to observe specifically the genomic or the antigenomic forms of Junín virus RNAs. For example, after RACE analysis, a series of non-template bases were found at the 5´end of Candid#1 L and S RNAs. In the comparison between the 5´end of genomic RNAs and the 3´end of antigenomic RNAs (which are used as a template for the former), at least one additional guanine is present in all 5′ end genomic clones comprising extra bases, similarly as detected for other arenavirus (Polyak *et al*., 1995). The 3´ RACE analysis of genomic S and L RNA obtained from Candid#1 infected cells rendered several clones harboring short deletions. RNA secondary structure analysis from Candid#1 S and L RNA predicted a panhandle structure between 5´ and 3´ ends of both genomic RNAs. Deletions at the 3´ end were localized inside the panhandle (Figure 8).

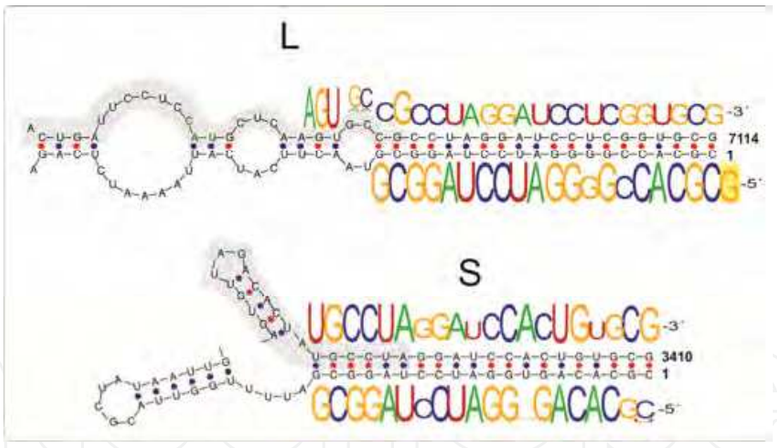


Fig. 8. End sequence determination of Junín virus, Candid#1 strain RNAs. Panhandle structures predicted for Candid#1, L and S RNAs. Shadowed with gray is the region present in all 3´-end clones. Sequence logos representing the distribution in the last nucleotides of 3´-end and 5´-end sequence of L and S RNA clones as determined by a genome specific RACE technique. Shadowed box corresponds to an additional extended G of the genomic sequence.

These results are consistent with a model that suggests the use of cellular RNAs to prime the viral RNA synthesis and the use of 5´ end sequences from viral RNA into a non-completed panhandle structure, as template for the 3´ end sequence completion. This heterogeneity could have arisen from different transcription-related editing of the subgenomic RNAs,

reported previously for other arenaviruses (Garcin & Kolakofsky, 1992). However, the involvement of these regions in the attenuation process remains to be evaluated.

Interestingly, comparison between 5´ and 3´ ends sequences from both genomic RNAs (S and L segments), show highly conserved positions. These positions could be related with the minimal promoter sequence. The 5′ and 3′ non-coding sequences from Candid#1 S RNA have approximately 80 nucleotides in length, similarly to the 5′ end from L RNA non-coding sequences. However, 3′ non-coding sequences from L RNA have only 30 nucleotides in length. When comparing the 80 nucleotides from non-coding sequences, the homology between 5´ end sequences of the L and S RNAs was 60% while that from 3′ end sequences was only 50%. However, if we compare only 30 nucleotides from the 3′ end of both genomic RNAs the homology of this region ascends to 71%. Because both, genomic or antigenomic, non-coding regions must be recognized by the viral RNA polymerase to complete the viral replicative cycle, the promoter region should be present in the first 30 nucleotides of the antigenomic L RNA. Genomic ends of arenaviruses comprise a highly conserved region of 19 nucleotides, called arena region. Outside this region the 3′ end of Junín virus L RNA comprises only 11 nucleotides (GCTCAAGTGCC). These nucleotides show a high degree of homology with two regions of S RNA 3′ end sequences. Thus, two boxes at the S RNA appear to match with a unique box at the L RNA. The S RNA boxes (positions 1-12 and 35-45 in the alignment) could be related to translation or transcription processes. Other arenaviruses have similar characteristics at their genomic ends. For example, an extensive analysis using the genomic sequences from other New World arenaviruses belonging to B1 subclade (Machupo, Junín and Tacaribe viruses; Flanagan *et al.*, 2008), show that the 38-46 box (GCUCAAGUG for the Junín virus L RNA and GCUCAGUG for the Junin virus S RNA) was conserved in the group (Goñi *et al.*, 2010). Consequently, it is possible that a sequence motif could be present at the 3′ end of both genomic RNAs of arenaviruses. Furthermore, for Junín, Machupo and Tacaribe viruses, this motif, described by the sequence GSYC$(A)_{1-2}$GUR, shows a relative degree of conservation in position in the RNA secondary structure calculated by bioinformatic tools.

3.3 Phylogeny

An independent parsimony analysis was performed for each arenavirus gene (GPC, N, Z and L). An additive tree-file was constructed adding the obtained parsimony tree-files from each gene in order to obtain a consensus tree for the four genes. Clades and subclades of Old World and New World arenaviruses are in accord with Charrell *et al.* (2008). Phylogenetic analysis show that all Junín virus strains (Candid#1, XJ13, XJ#44, MC2 and Romero) grouped together with other hemorrhagic New World arenaviruses. Furthermore, the phylogeny correlates with the genealogy of the vaccine strain, Candid#1, and a small set of nucleotide changes seems to be central to define the phenotypic variation from virulence to attenuation. If this is confirmed in a more extensive study, any surveillance program designed to monitor the natural vaccine variations should search for possible point mutations at those positions related with attenuation.

4. Conclusions

Candid#1, the most attenuated Junín strain, has a set of putative attenuation markers into the GPC, N and L protein ORFs. Some of these changes could be associated more

confidently with the attenuated phenotype. Initially, we focussed on changes detected into those genomic regions harboring a low wild type mutation frequency index (GPC_{168}, GPC_{427}, GPC_{446}, N_{47}, L_{76}, L_{936}, L_{1156}).

At present, we have made bioinformatic and phylogenetic analysis on the putative attenuation markers. For L proteins, the group of Vieth (Vieth *et al.*, 2004) describe four conserved regions among all arenaviruses. Inside of the region III, Lan and coworkers (Lan *et al.*, 2008), found the polymerase domain, and proposed the presence of four motifs: A, B, C and D. In this region we detect only one change (R→K→K), at the 1156 position. Although this change is classified as conservative, it could be related to the attenuation process because this region seems not to sustain mutations in nature. Other L changes are located between region II and III (L_{936}, L→L→P) or inside region I ($L_{76,}$ H→Y→Y). Change L_{936}, in spite of being present in a region of high index for type 2 mutations, could only be associated with a structural change of the Candid#1 derived L protein based on the structural characteristics of the Proline residue. L_{76} change falls near the putative ATP/GTP binding site (P-loop) predicted using the Expasy web site (www.expasy.ch). Although our results, suggesting the involvement of the RNA polymerase in attenuation of virulence are preliminary, they are consistent with reports on other viruses (Endres *et al.,* 1991; Lan *et al.*, 2008; Riviere *et al.,* 1985a). Furthermore, some changes in the structural proteins, nucleoprotein and both mature glycoproteins, could be related with the attenuation of virulence. The carboxyl-terminus of N protein, which contains a zinc binding domain (Tortorici *et al.*, 2001b) is highly conserved, and the found N_{47} change (V→V→E) falls outside this region and would be associated with another characteristic of the protein, as the ability to oligomerize (N-N interactions, Levingston Macleod *et al.*, 2011). Therefore, N protein has a dual function during the virus life cycle. First, it is involved in essential steps of genome replication, promoting the synthesis of the full-length antigenomic copy of S RNA, and second, it associates with the genomic RNA to form the nucleocapsid. For the glycoprotein precursor, we found a mutation in the carboxyl-terminus of G1 (GPC_{168}, T→A→A). This change affects directly the conserved sequence ($N_{166}R_{167}T_{168}K_{169}$) for the principal N-glycosylation site predicted by NETNGLYC 1.0. G2 has three domains, the outer (caboxi-terminus) domain located outside the virion, the transmembrane domain and the inner (amino-terminus) domain, located inside the virion. The outer domain interacts with G1, the transmembrane domain interacts with the signal peptide, and the inner domain could interact with Z or the nucleocapsid (Capul *et al.*, 2007; York *et al.*, 2004). G2 changes fall inside the transmembrane domain (GPC_{427}, F→F→I) or at the cytoplasmic tail (GPC_{446}, T→T→S) and could affect such important interactions.

In terms of distribution of the potential attenuation markers, we find a lower level of nucleotide sequence conservation in the S RNA than in the L RNA, indicating a faster rate of evolution in the S polypeptides. Lan and colaborators (Lan *et al.*, 2008) showed the genome comparison of virulent and avirulent strains of the Pichinde arenavirus, and found a lower number of attenuation related mutations, but at comparable genomic regions. If we compared only the field strains of Junín virus (MC2, Romero, IV4454 and XJ13), the mutations distribute markedly differently among large and small segments. We considered specific changes for MC2, Romero or IV4454 when their sequences differed from XJ13 sequence, and specific changes for XJ13 when its sequence differed from all three Junín virus strains. Analyzing the protein sequences of the field strains, there were 45 divergence sites for the S RNA ORFs and 48 for the L RNA ORFs. Interestingly, 96% (46/48) of L RNA-

derived divergence sites were XJ13-specific changes, and only 9% (4/45) of this type of changes were present when S RNA-derived divergence sites were analyzed.

The increasing sequence information from complete arenavirus genomes, especially of the JUNV species, contributed to the identification of putative markers of virulence attenuation. In this sense, Goñi *et al.* (2010) and the recent paper of Albariño *et al.* (2011a) publish sequence data involving seven members of the vaccine strain family (XJ13, XJ17, XJ34, XJ39, XJ44, XJ48 and Candid#1 strains). To investigate the role of different point mutations in the virulence attenuation process Albariño *et al.*, (2011a) used a reverse genetics system for JUNV they developed (Albariño *et al.*, 2011b). They show that point mutations located at the Candid#1 GPC ORF, conferred the attenuated phenotype.

Furthermore, with the objective of analyzing the mutation distribution in nature, Goñi *et al.* (2011) designed a molecular technique of RT-Nested-PCR and afterwards used with rodent and human samples from the endemic area. A previous study, performed on different genomic regions with another group of strains (García *et al.*, 2000), suggested a high degree of homology for the S RNA derived ORFs. The protein sequences we obtained by *in silico* translation of the four fragments were analyzed with different tools.

We compare the data collected for the GPC derived sequences (G fragment) after the results included in our studies and in the Albariño *et al.* (2011a) paper. In the Table 3 we show the positions with residue differences between G fragments encoded proteins from JUNV strains belonging to the vaccine family and JUNV strains collected from field samples. As noted above, the G 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. Some of the changes observed in the G fragment (63% of G1), could be related to the host jump process of a particular strain from rodents to humans (probably derived from the receptor affinity variation in the human isolates). These changes could be related to changes in the virulence (attenuation or increase) trough modification of the cellular tropism. 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 65% of the field strains have the target sequence, while the other 35% have an alanine residue (A$_{168}$).

The bioinformatics studies did not show important changes in the protein properties at this region (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). Interestingly, all XJ13-derived attenuated strains have an A$_{168}$ mutation.

Residue Position	XJ13	XJ17	XJ34	XJ39	XJ#44	XJ48	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
107	S	S	S	S	S	S	S	S	**T**	S	S	S	S	S	S	S	S	S	S	**T**	**T**	**T**	S
109	Q	Q	Q	Q	Q	Q	Q	**K**	**M**	**M**	Q	Q	Q	**M**	Q	Q	**M**	**M**	Q	**M**	**M**	**M**	Q
111	S	S	S	S	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	A	A	A	A	E	A	A	A	A	A	A	A	A	A	A	A	A	A
121	Q	Q	Q	Q	Q	Q	Q	E	Q	Q	Q	Q	E	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
125	I	I	I	I	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	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	W	W	W	W	**R**	**R**	**R**	W
151	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	**V**	**V**	**V**	A
157	H	H	H	H	H	H	H	**Y**	**Y**	H	H	H	H	H	H	H	H	H	H	H	H	H	H
168	T	**A**	**A**	**A**	**A**	**A**	**A**	T	T	T	**A**	**A**	T	T	**A**	**A**	T	T	**A**	T	T	T	**A**
184	V	V	V	V	V	V	V	**I**	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
186	E	E	E	E	E	E	**G**	E	E	E	**G**	**G**	E	E	**G**	**G**	E	E	**G**	E	E	E	**G**
206	S	S	S	S	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**	**L**	**L**	**L**	**L**
209	N	N	N	N	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	Q	Q	Q	Q	**H**	Q
245	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	**F**	L

Letters in bold indicate the residues that are different from the residue present in the reference XJ13 strain. The amino acids are shown in the one letter code. The type 2 mutations between vaccine family strains are shadowed in grey.

Table 3. Positions with residue differences between G fragment encoded proteins.

We hypothesized that those mutations found among field strains are of minor importance in the phenomenon of virulence attenuation. None of the type 2 mutations here analyzed, were absent in natural samples. In this context, the data presented in Table 3, could indicate that none of the mutations found in G1 is important to define the attenuated phenotype. This result is in accordance with those published by Albariño *et al.*, (2011a), who located the most important mutations for attenuation in G2.

In summary, the present work shows a set of mutations that could be related to the virulence attenuation phenomenon. Furthermore, most of both described types of mutations (type 1 and type 2) could be grouped into a few regions (Figure 7). In order to analyze genomic variability in Junín virus and to search for the presence of mutations of type 1 or type 2 among field strains, we designed a set of primers that are used in a rapid method, based on RT-PCR and nucleotide sequencing. This method could be useful in order to observe the biodiversity in nature or to develop an epidemiologic surveillance program of vaccinated people. The information accumulated by sequence analysis of viral genomes with different degrees of virulence will certainly serve as a starting point to study this biological phenomenon. 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 survival mechanisms and are a potential target for mutagenesis studies.

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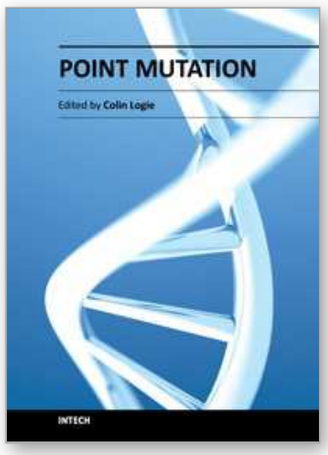

Point Mutation

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This book concerns the signatures left behind in chromosomes by the forces that drive DNA code evolution in the form of DNA nucleotide substitutions. Since the genetic code predetermines the molecular basis of life, it could have been about any aspect of biology. As it happens, it is largely about recent adaptation of pathogens and their human host. Nine chapters are medically oriented, two are bioinformatics-oriented and one is technological, describing the state of the art in synthetic point mutagenesis. What stands out in this book is the increasing rate at which DNA data has been amassed in the course of the past decade and how knowledge in this vibrant research field is currently being translated in the medical world.

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