



## Article/Artigo

# Standardization of an ELISA test using a recombinant nucleoprotein from the Junin virus as the antigen and serological screening for arenavirus among the population of Nova Xavantina, State of Mato Grosso

Padronização de um teste de ELISA utilizando a nucleoproteína recombinante de vírus Junin como antígeno e triagem sorológica para Arenavírus na população de Nova Xavantina, Estado do Mato Grosso

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### ABSTRACT

**Introduction:** Arenavirus hemorrhagic fever is a severe emerging disease. **Methods:** Considering that the levels of antibodies against arenavirus in the Brazilian population are completely unknown, we have standardized an ELISA test for detecting IgG antibodies using a recombinant nucleoprotein from the Junin virus as the antigen. This protein was obtained by inserting the gene of the Junin virus nucleoprotein into the genome of *Autographa californica nucleopolyhedrovirus*, using the Bac-to-Bac baculovirus expression system. This recombinant baculovirus was used to infect *S. frugiperda* cells (SF9). **Results:** The infection resulted in synthesis of high concentrations of recombinant protein. This protein was detected on 12.5% polyacrylamide gel and by means of Western blot. Using the standardized ELISA test, 343 samples from the population of Nova Xavantina were analyzed. We observed that 1.4% of the serum samples (five samples) presented antibody titers against arenavirus. **Conclusions:** These results show the population studied may present exposure to arenavirus infection.

**Key-words:** Arenavirus hemorrhagic fever. ELISA. Junin. Arenavirus in Brazil. Arenavirus.

### RESUMO

**Introdução:** A febre hemorrágica por Arenavirus é uma severa doença emergente. **Métodos:** Considerando que os níveis de anticorpos contra Arenavirus na população brasileira é totalmente desconhecido, nos padronizamos um teste de ELISA para detecção de anticorpos IgG usando uma nucleoproteína recombinante do vírus Junin como antígeno. Esta proteína foi obtida pela inserção do gene da nucleoproteína do vírus Junin no genoma do vírus *Autographa californica nucleopolyhedrovirus*, utilizando o sistema de expressão em Baculovirus, Bac-To-Bac. Este baculovirus recombinante foi utilizado para infecção de células de *S. frugiperda* (Sf9). **Resultados:** A infecção resultou na produção de altas concentrações de proteína recombinante. Esta proteína foi detectada em gel de poliácridamida 12,5%, e em Western blot. Utilizando o teste de ELISA padronizado, foram analisadas 343 amostras provenientes da população de Nova Xavantina. Observamos que 1,4% dos soros (5 amostras) apresentavam títulos de anticorpos contra arenavírus. **Conclusões:** Estes resultados sugerem que a população estudada pode estar sendo exposta a infecções por arenavírus.

**Palavras-chaves:** Febre hemorrágica por arenavírus. ELISA. Junin. Arenavirus no Brasil. Arenavírose.

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### INTRODUCTION

The Arenaviridae are a family of viruses whose members are rodent-borne and cause severe human diseases. Each virus usually infects and is maintained in a specific rodent species. Arenavirus hemorrhagic fever (AHF) is a severe emerging disease characterized by vascular, renal, hepatic and neurological disorders with high lethality<sup>1</sup>.

The members of the Arenaviridae family contain a single-stranded RNA genome composed of two segments, L (large) and S (Small), with average lengths of 7,100 and 3,400 nucleotides, respectively. The S RNA segment codes for the structural proteins of the virion, the nucleocapsid protein (N), two envelope glycoproteins and a signal peptide that may serve as part of the envelope glycoprotein structure and in trafficking<sup>2</sup>. The glycoproteins use a cell receptor, alpha-dystroglycan, for cell penetration<sup>3-5</sup>. The L segment codes for the viral RNA-dependent RNA polymerase and a zinc-binding protein (Z). The role of Z is poorly understood, and homologues of Z are not found in other negative RNA viruses. It has been reported that the Z protein interacts with several cell factors: promyelocytic leukemia protein and translation initiation factor 4E. Recent studies have suggested that Z may have a role in viral transcriptional regulation<sup>6-8</sup>. The viral particles are spherical to polymorphic with diameters ranging from 50 to 300nm. Ribosomes are present inside the virions and are responsible for the *sandy* appearance of the virus as seen using electron microscopy, hence the name arenavirus (*arena*: sand in Latin)<sup>9</sup>.

The most important viral antigens are those of nucleoproteins and glycoproteins, and nucleoprotein antigens present better conservation among arenaviruses<sup>10</sup>.

This family consists of 23 recognized viruses that have been classified according to their antigenic properties into two groups: the Tacaribe serocomplex (or New World group), including viruses indigenous to the Americas; and the Lassa-lymphocytic choriomeningitis serocomplex (LCM) (or Old World group), which includes viruses indigenous to Africa and the ubiquitous LCM virus<sup>11-14</sup>.

In South America, several arenaviruses are known to cause human disease: the Junin virus (JUNV), Machupo virus (MACV), Flexal virus (FLEV), Guanarito virus (GUAV) and Sabia virus (SABV). The last of these was isolated from a fatal case of hemorrhagic fever (HF) in São Paulo, Brazil; subsequently, two cases of nonfatal laboratory infections have occurred<sup>15-17</sup>. Recently, a new arenavirus was discovered following a fatal hemorrhagic fever case in Bolivia<sup>18</sup>. This new arenavirus was called the Chapare virus.

Over recent years, the potential use of hemorrhagic fever viruses as biological weapons has been highlighted<sup>19,20</sup>. Therefore, development of diagnostic systems for these diseases is important even in countries that are free from or have few cases of AHF.

Constant monitoring of arenavirus exposure plays an important role in controlling disease outbreaks, especially in Brazil, where the specific antibody levels in the population are completely unknown. However, manipulation of infectious SABV is necessary for detection of specific antibodies. This implies the need for a high-containment laboratory (Biosafety Level 4; BSL4) to handle infectious SABV and, therefore, preparation of SABV antigens cannot be implemented without a BSL4 facility<sup>21</sup>. Thus, it is important to develop sensitive and specific diagnostic systems for HF without manipulation of infectious SABV.

Because of the need to handle SABV and other arenaviruses, and the unavailability of the genome for SABV, we used a construct previously established by Dr. Mario Lozano, which contained the gene for the nucleocapsid protein. Thus, in the present study, a recombinant nucleoprotein from JUNV was expressed and tested as the ELISA antigen for detecting arenavirus antibodies in a serological survey of the population of the municipality of Nova Xavantina, Brazil. The present study presents an alternative strategy for diagnosing and serologically screening for arenavirus in Brazil.

## METHODS

### Production, expression and semi-purification of the Junin virus nucleoprotein

The recombinant nucleoprotein (NP) of the Junin virus was expressed in the baculovirus system (Bac-to-Bac system, Invitrogen, USA). In order to construct the transfer vector, a cDNA clone of NP from Junin strains was used. The nucleoprotein gene was amplified by means of PCR using specific primers and cloned into Topo TA plasmid. The Topo TA-JUNV-NP was digested using a restriction enzyme, and the selected gene was cloned into pFastBac donor plasmid. The plasmid pFastBac-JUNV-NP was used to transform DH10Bac-competent *Escherichia coli*, in order to generate a recombinant bacmid. This bacmid was transfected into a *Spodoptera frugiperda* insect cell line (SF9) to generate a recombinant baculovirus. The SF9 cells were cultured in SF900II culture medium at a temperature of 27°C. The recombinant baculovirus (Bac-JUNV-His-NP) was purified and was kindly provided by Dr. Mário Lozano from Quilmes University, Argentina.

The JUNV rNP was expressed by infecting SF9 cells with Bac-JUNV-HIS-NP and incubating at 27°C for 96 hours. The cells were then washed twice with cold phosphate-buffered saline (PBS) solution and centrifuged at 5,000 x g for 10 minutes. The pelleted fractions were then collected. The cell pellet was dissolved in lysis buffer (50mM NaH<sub>2</sub>PO<sub>4</sub>, 300mM NaCl, 1.5M urea, 1% Tween 20, pH 8.0, protease inhibitors), incubated on ice for 120 minutes and centrifuged at 5,000 x g for five minutes. The pellet fractions containing the insoluble JUNV rNP were then collected. To this pellet was added 2ml of lysis buffer with 8M urea, and this mixture was incubated on ice for 30 min and centrifuged at 12,000 x g for one minute. The supernatant fractions were then collected.

The expression efficiency of JUNV rNP was analyzed by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) after staining with Coomassie blue.

The antigenicity of JUNV rNP was confirmed by means of the Western blot test (Invitrogen, USA) using rabbit serum anti-Amapari arenavirus and mouse serum anti-JUNV, in accordance with the manufacturers' instructions.

### IgG-ELISA

Immunoglobulin G (IgG)-ELISA was performed as previously described except for the antigen preparation<sup>22</sup>. The amount of JUNV rNP used in the test was determined by using the micro-BCA protein assay kit (Pierce, USA). Thus, 20ug/ml of purified JUNV rNP/well was used in the IgG-ELISA. One hundred microliters of the antigen was added to 96-well microplates and incubated overnight at 4°C. Protein extracts of SF9 cells without infection were used as negative control and added at the same concentration as the antigen. Then, 250ul of the blocking buffer containing 10% milk and 0.05% Tween 20 in PBS (PBS-M-T) was inoculated into each microplate well. After two hours of incubation, the plates were washed three times with 0.05% Tween 20 in PBS (PBS-T), and the microplates were inoculated with the test samples diluted in PBS-M-T at 1:100, 1:500 and 1:1,000 dilutions (100 ul/well), and incubated for one hour at 37°C. After one hour of incubation, the plates were washed three times with PBS-T and then inoculated with goat anti-human IgG peroxidase conjugate (Sigma, USA) at 1:2,000 dilution, in PBS-M-T, 100ul/well, and incubated for one hour at 37°C. After this period, the plates were washed with PBS-T three times, and 100ul of ABTS solution (KP, USA) was added to each well. The plates were incubated for 30 minutes at 37°C, and were read in a spectrophotometer at 405nm. The means and standard deviations were calculated with 30 negative control serum samples. The cutoff value for the assay was defined as the mean plus three standard deviations.

### Serological screening

*Municipality of Nova Xavantina.* This municipality is located in the northeast of the state of Mato Grosso, Brazil (**Figure 1**) and has a population of approximately 18,000 inhabitants, of which 20% live in the rural zone. Arenavirus cases had never previously been reported in Nova Xavantina. However, hantavirus pulmonary syndrome, a disease also transmitted by wild rodents, has been notified to the Brazilian Ministry of Health as present in the region of Nova Xavantina.

*Human serum.* Serum from 343 individuals living in the municipality of Nova Xavantina who presented fever with unidentified etiology had previously been analyzed in serological screening using hantavirus IgG-ELISA, and all these samples had



FIGURE 1 - Location of the municipality of Nova Xavantina.

proven to be negative. Because of this, the 343 samples were analyzed in the present serological screening for arenavirus using IgG-ELISA with JUNV rNP as the antigen. Among the 343 individuals studied, 152 were women with an average age of 34 years and 191 were men with an average age of 32 years. The procedures followed among the participants in this serological screening included provision of information about the aims of the study and obtaining of their signed agreement.

#### Ethical

The study had previously been approved by the Ethics Committee for Human Research of the General Hospital of the Ribeirão Preto School of Medicine, University of São Paulo, in the City of Ribeirão Preto.

## RESULTS

#### Expression and semi-purification of JUNV rNP

The recombinant baculovirus JUNV rNP, was successfully multiplied into SF9 cells for creation of a  $2 \times 10^7$  PFU/ml viral stock. Two multiplicities of infection were used for protein expression (Bac-to-Bac, Invitrogen, USA). We used MOI values of 2 and 7, such that MOI values of 7 expressed a greater quantity of recombinant proteins. SF9 cells infected with the recombinant baculovirus Bac-JUNV-HIS-NP and presenting cytophlyactic effect were dissolved in lysis buffer with 1.5M urea, and the pellet was redissolved in lysis buffer with 8M urea. The solution was analyzed using SDS Page. A band of approximately 62KDa was observed that was not seen with the negative control (Figure 2). The yield of recombinant protein ranged from 15 to 20mg/l of culture.

#### Western blotting analysis

The antigenic properties of both the expressed protein extract and the purified protein were confirmed by immunoblot analysis with rabbit serum anti-AMAV. Negative control serum did not detect the N protein in the immunoblot assay (Figure 3).

#### Development of JUNV rNP-based IgG-ELISA

The purified JUNV rNP (20ug/ml) was used in the IgG-ELISA. Anti-AMAV produced in rabbits and JUNV rNP produced in mice were the positive control samples. Thirty human serum samples from individuals unaffected by the arenavirus were used as negative

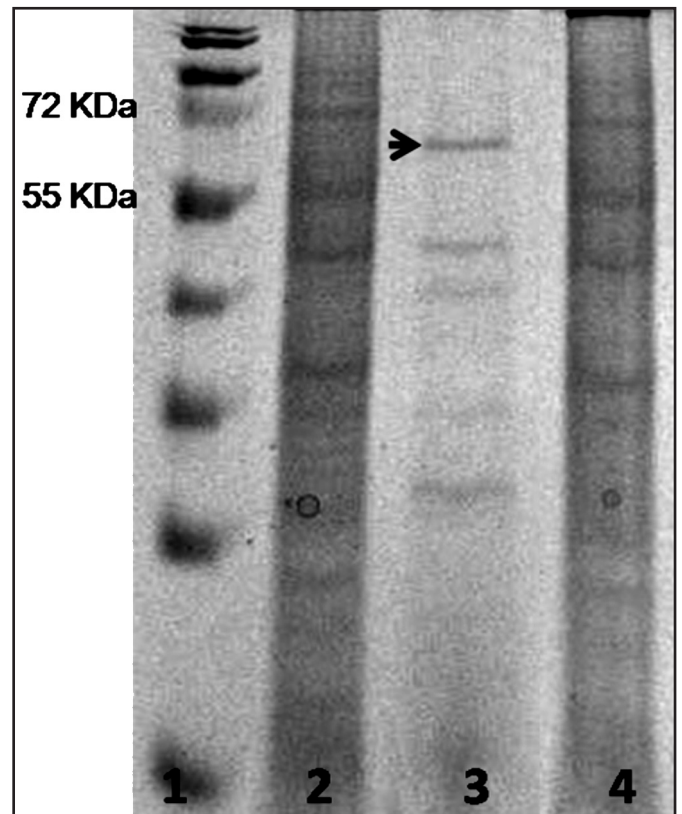


FIGURE 2 - Expression of N in insect cells. SDS Page. SF9 cells infected with recombinant baculovirus overexpressing N (96 h p.i., MOI 7); 1) molecular weight; 2) uninfected cells, negative control (-); 3) JUNV rNP (62 KDa); 4) uninfected cells, negative control (-).

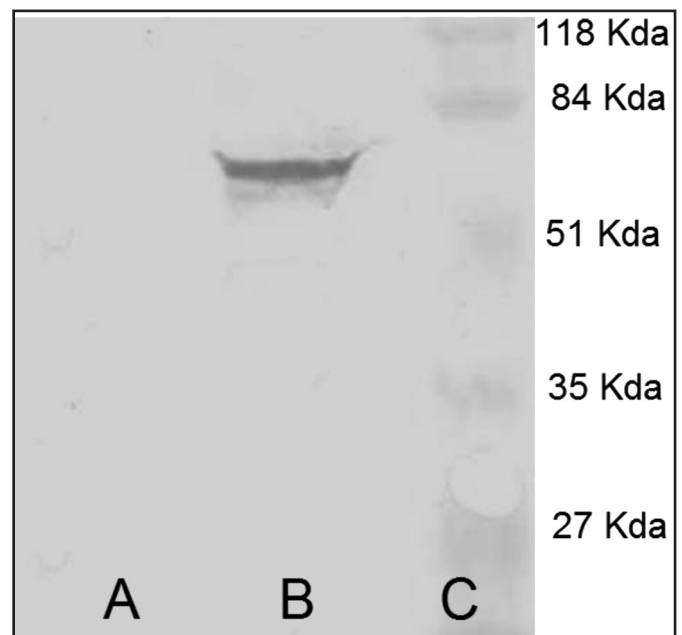


FIGURE 3 - Antigenic properties confirmed by immunoblot analysis with rabbit serum anti-AMAV: A) negative expression control; B) detection of JUNV rNP with antibodies against AMAV, concentration 1:1,000; C) molecular weight.

controls. The serum pool from mice immunized with JUNV rNP and the serum pool from rabbits immunized with AMV were both positive in the IgG-ELISA, showing high titers such as 16,384. The thirty arenavirus-negative control serum samples were all negative in the test. Pools of rodent serum containing anti-hantavirus antibodies were also tested for arenavirus using this IgG-ELISA

and were all found to be negative. To test the possible cross-reaction with LCMV (arenavirus causing lymphochoriomeningitis), three known positive human serum samples (IgG) were analyzed using JUNV-IgG-ELISA. These samples showed cross-reactions with the recombinant Junin protein, but with absorbance values lower than those found in samples that were positive for JUNV and AMAV (Figure 4). The JUNV rNP-based IgG-ELISA was able to clearly detect IgG antibodies for arenavirus.

#### Serological screening for arenavirus in the municipality of Nova Xavantina

The JUNV rNP-based IgG-ELISA was used for serological analysis on 343 serum samples from the population of the municipality of

Nova Xavantina. IgG antibodies for arenavirus were observed in five samples (1.4%). Three adult males and two adult females were thus found to be seropositive for arenavirus (Table 1 and Figure 4).

TABLE 1 - Nova Xavantina positives IgG patients for arenavirus.

| Patient | Gender | Age | Absorbance Level |
|---------|--------|-----|------------------|
| 1       | M      | 29  | 0,812            |
| 2       | F      | 35  | 0,761            |
| 3       | M      | 32  | 0,930            |
| 4       | M      | 30  | 0,651            |
| 5       | F      | 30  | 0,861            |

M: male, F: female.

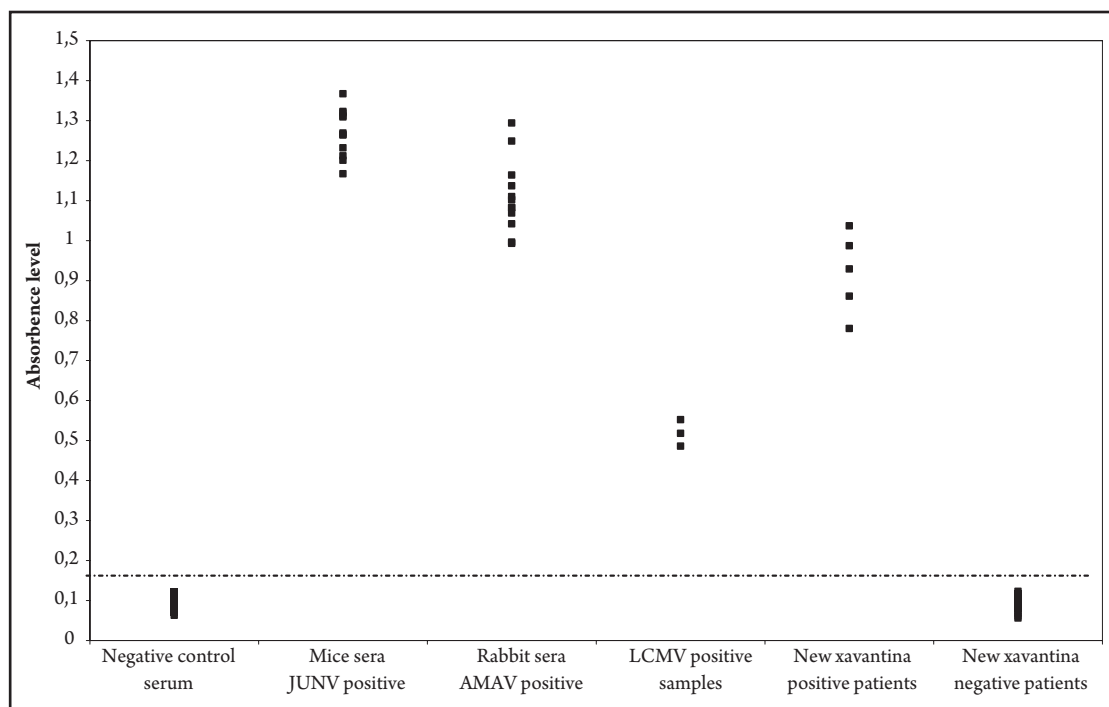


FIGURE 4 - Dot diagram showing absorbance values obtained with the ELISA test described here. Horizontal dotted line shows the cutoff value.

## DISCUSSION

We expressed the nucleoprotein of the Junin virus through baculovirus in insect cells (SF9 cells) and used it as a diagnostic tool. A large amount of JUNV rNP was obtained from SF9 cells (15 to 20mg/l). Production of the recombinant antigen was rapid (one week) and purification of the protein was not required, since cells infected with the Baculovirus without the insert (native baculovirus) served as a negative control. This recombinant antigen appeared to be very sensitive and specific in the Western blot and ELISA tests. Finally, JUNV rNP was used in ELISA assays to detect IgG antibodies in serum samples from participants living in the municipality of Nova Xavantina.

Human infection with arenavirus in Brazil has been diagnosed by means of virus isolation in mice, in cases of patients with severe disease<sup>1</sup>. Serological diagnosis of arenavirus has not been performed, mainly because of a lack of arenavirus antigens, which are difficult to obtain. Considering that extensive cross-reactivity occurs in serological tests performed on all arenaviruses within the Tacaribe complex, this recombinant N protein from the Junin virus was found to be a useful tool for serological diagnosis of human infections

due to arenaviruses in Brazil. This JUNV rNP-based IgG-ELISA combines the broad cross-reactivity of the antigen to antibodies against South American arenaviruses with the high sensitivity of the enzyme immunoassay<sup>23</sup>. The test was able to detect antibodies in serum diluted by a factor of more than 10,000, which suggests that it is highly sensitive. The test was also reproducible, rapid and easy to perform. We observed that cross-reaction occurred with the lymphochoriomeningitis virus (LCMV), but with a level of absorbance lower than has been found for other arenaviruses such as JUNV, AMAV and SABV.

Five individuals in the municipality of Nova Xavantina, in western Brazil, were infected by arenavirus. This locality is not far away from the Bolivian border, where Machupo virus occurs. Moreover, the same rodent reservoirs of Machupo and other arenaviruses are present in Brazil, thus suggesting that these zoonotic viruses also circulate in this country. However, the arenavirus-seropositive individuals observed in this study denied that they had had any previous severe disease such as Bolivian hemorrhagic fever. Only three cases of hemorrhagic fever due to arenavirus have been reported in Brazil, and subclinical infections have not previously

been reported<sup>24,25</sup>. This is the first report on the possible occurrence of arenavirus infections in Brazil without producing hemorrhagic fever. Therefore, the levels of antibodies to arenavirus in the Brazilian population are completely unknown. Thus, this JUNV rNP-based IgG-ELISA could be used for determining arenavirus antibody levels in populations living in different regions in order to ascertain the importance of arenaviruses in Brazil.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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