

**Hantavirus Infection in Brazil: Development and Evaluation of an Enzyme
Immunoassay and Immunoblotting Based on N Recombinant Protein**

Running Title: Accurate detection of hantavirus in Brazil by EIA

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Abstract

The symptoms of hantavirus pulmonary syndrome (HPS) may resemble those of other febrile illnesses. The development of an accurate diagnostic test should therefore improve clinical prognosis and be useful in epidemiological studies. We evaluated the use of a recombinant antigen (rN₈₅) based on the S-segment sequences of a Brazilian hantavirus for detecting IgM and IgG antibodies against hantavirus in an indirect enzyme immunoassay (EIA). We assayed 613 serum samples (570 from humans and 43 from rodents). IgM EIA had a sensitivity of 94.1% and a specificity of 99.1%. IgG EIA had a sensitivity of 95.2% and a specificity of 98.4%. This evaluation confirms that rN₈₅ IgM and IgG EIA tests are potentially useful rapid, sensitive and cost-effective tools for detecting antibodies against hantaviruses indigenous to Brazil and other South American countries, in patients with acute or convalescent hantavirus infection, and in rodent reservoirs.

Keywords: Hantavirus, HPS; Enzyme immunoassay; Immunoblotting; Recombinant N antigen

Introduction

Hantavirus pulmonary syndrome (HPS), an emerging health problem in Brazil, has become a public health concern in North and South America. The disease is characterized by sudden fever, myalgia, asthenia, abdominal pain, high hematocrit, and thrombocytopenia, followed by coughing and dyspnea after a prodromal period of two to seven days, generally followed by acute respiratory insufficiency and heart failure (Riquelme *et al.*, 2003). Case fatality rates HPS caused by the Sin Nombre virus, the most prevalent North American, and South American hantavirus are about 30 and 50%, respectively (Custer *et al.*, 2003; Johnson *et al.*, 1997; Nichol *et al.*, 1993).

Hantaviruses are transmitted to humans principally through the inhalation of contaminated aerosols of rodent excreta (Lednicky, 2003; Schmaljohn and Hooper, 2001; Zu *et al.*, 1985). However, human-to-human transmission has also been described (Padula *et al.*, 1998).

Hantaviruses, which belong to the *Bunyaviridae* family, have a genome consisting of three negative single-stranded RNA segments: large (L), medium (M), and small (S). These segments encode the viral RNA polymerase, a precursor glycoprotein that is processed into two separate envelope glycoproteins (G1 and G2), and a nucleocapsid protein (N), respectively (Schmaljohn and Hooper, 2001).

It is difficult to diagnose HPS based on clinical examination alone, and hantavirus infections in humans result in an only very short-term viremia. Serological tests are, therefore, frequently used for the detection of HPS infections. Shortly after the onset of illness, immunoglobulin M (IgM) and G (IgG) class antibodies become detectable (Hujakka *et al.*, 2001). The nucleocapsid is the major antigenic protein. It has immunodominant, cross-reactive epitopes in the first 100 N-terminal amino acids and induces a strong, early immune response. This antigen is therefore suitable for use in assays based on recombinant antigens (Schmidt *et al.*, 2005; Elgh *et al.*, 1996; Gött, *et al.*, 1997; Yamada *et al.*, 1995; Lundkvist *et al.*, 1993).

The first confirmed cases of HPS in South America were recorded in the São Paulo State of Brazil in 1993. Data to June 2006 indicate that 716 cases of HPS have been reported in Brazil (Brazilian Ministry of Health/SVS), with a mortality rate of about 40%. (Elkhoury *et al.*, 2005). Hantavirus infection is diagnosed serologically, by Brazilian Public Health Laboratories, using in-house tests with recombinant Andes (Padula *et al.*, 2000) or Sin Nombre virus nucleoprotein as the antigen, these proteins being supplied by ANLIS 'Dr Carlos G Malbrán' Institute (Buenos Aires, Argentina) and the Centers for

Disease Control and Prevention (CDC/Atlanta), respectively. However, these reagents have not been validated for the serological diagnosis of hantavirus infections in Brazil, despite performing well in bench assays. Previous reports have recommended the development of region-specific antigens, to improve serological reactivity (Padula *et al.*, 2000; Schmidt *et al.*, 2005).

We evaluate here the use of a recombinant antigen based on the S-segment sequences of Brazilian hantavirus (Araucaria group) for the detection in an indirect enzyme immunoassay (EIA) of IgM and IgG antibodies against hantavirus (Raboni *et al.*, 2005a). We tested 570 human and 43 rodent serum/clot samples from different regions of Brazil and other South American countries, to optimize and validate this reagent and to establish its use for the diagnosis of hantavirus infection in patients in the acute and convalescent stages of infection, and its use in human and rodent seroprevalence studies in affected areas.

Materials and Methods

Cloning and expression of a truncated N gene (rN Δ_{85}).

Hantavirus RNA (BR/02-72) was isolated from the serum of an HPS case (Raboni *et al.*, 2005a; Raboni *et al.*, 2005b), using a QIAmp Viral RNA Mini Spin kit (Qiagen Inc, Ontario, CA), essentially following the manufacturer's instructions. One primer corresponding to nucleotides 1051-1071 of the N gene (ATGCGCAACACCATCATGGCT) was used to produce a cDNA (1 h at 42°C), using ImProm Reverse Transcriptase (Promega Corporation, Madison, WI, USA), as previously described (24). The resulting cDNA (5 μ l) was then amplified by PCR (94°C for 3 min, then 40 cycles of 94°C for 30s, 52°C for 30s, and 68°C for 2 min), using the TripleMaster PCR System (Eppendorf, Hamburg, GE) and the following primers: forward - CGGGATCCATGAGCAACC and reverse - GGGTACCCAGCCATGATT (*Bam*HI and *Kpn*I sites are underlined). This PCR product generated a truncated protein corresponding to the first 343 amino acids of the N protein.

Plasmids were constructed by standard protocols (Sambrook *et al.*, 1989). The resulting PCR product was digested by *Bam*HI and *Kpn*I, and ligated with T4 DNA ligase (New England Biolabs Inc.) into pQE30 vector (Qiagen Inc, Ontario, CA), which had previously been linearized with the same restriction endonucleases. The ligation product was used to transform competent M15 bacteria. Selected clones were amplified, and recombinant plasmids (prN Δ_{85}) were analyzed after digestion with restriction enzymes and by sequencing on an ABI 3100 device using the BigDye® Terminator method (Applied Biosystems Inc, USA).

E. coli strain M15 cells were induced by incubation at 30°C for 4 h with isopropyl- β -D-thiogalactopyranose (IPTG; 0.5mM). The cells were then harvested and stored at -20 °C until use. The frozen cells were suspended in 10 ml of lysis buffer (100 mM NaHPO₄, 10 mM Tris-HCl, pH 8.0) per liter of original culture. They were disrupted by sonication (five 30s pulses) on ice and centrifuged at 10,000 g for 20 min at 4°C. The recombinant protein was detected principally in the insoluble fraction. The encoded fusion protein contained six amino-terminal histidine residues, facilitating affinity-based purification.

The recombinant N protein (rN Δ_{85}) was purified under denaturing conditions, using the following protocol: the insoluble fraction of the clarified cell lysate was suspended in buffer B (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 8.0) and immediately

purified by passage through a 1 ml Ni⁺²-nitrilotriacetic acid-agarose (Qiagen Inc, Ontario, CA) or Co⁺²-TALON Metal Affinity Resin (Clontech, Palo Alto, CA, USA) column equilibrated with buffer B. The column was washed extensively with buffer C (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 6.3) and the resin-bound protein was eluted with elution buffer (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 7.0, supplemented with 500 mM imidazole; 2 x 250 µl fractions). Samples were stored at -20°C in the final elution buffer. The identity, concentration and purity of the purified protein fractions were analyzed on 13% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), stained with Coomassie blue. Fractions containing the recombinant protein were pooled and dialyzed for 2 h each against solution I (4 M urea, 100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 7.0) and solution II (0.5 M urea, 100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 7.0), and then quantified, using the Micro BCATM Protein Assay kit (Pierce Inc., Rockford, IL, USA). The specific reactivity of the purified recombinant antigen was analyzed by immunoblotting, using either a monoclonal anti-histidine antibody (Invitrogen Inc, USA) or serum samples from HPS patients, according to standard procedures (Towbin *et al.*, 1979; Yoshimatsu *et al.*, 1996).

Samples from patients and rodents

The analyzed samples were assigned to five groups, as described in Table 1. This study was approved by local and national ethics committees.

Enzyme immunoassay (EIA)

Polystyrene microtitration plates (Nunc, Roskilde, Denmark) were coated with 100 µl per well of rN_A85 (2 µg/ml) in coating buffer (0.05M sodium carbonate, pH 9.6) by incubation overnight at 4°C. The plates were then washed five times with phosphate-buffered saline supplemented with 0.1% Tween 20 (PBST) and stored at -20°C until use.

IgM EIA. A volume of 100 µl was used for all assays. Serum samples were diluted 1:400 in dilution buffer (phosphate-buffered saline supplemented with 0.5% Tween 20 and 2% non fat milk powder) and incubated at 37°C for 30 minutes. One positive and four negative control serum samples were included on each plate. Plates were washed five times with PBST, and 100 µl of peroxidase-labeled affinity-purified goat anti-human IgM (Sigma-Aldrich Inc, Saint Louis, Missouri, USA), at a pre-tested dilution of 1:15,000, was added to each well, and the plate incubated at 37°C for 30 minutes. The plates were washed five times with PBST, and specific antibody binding was detected by incubation with

tetramethylbenzidine (Dako® TMB+, Dako Corporation, Carpinteria, CA, USA) as the substrate, the reaction being stopped by the addition of 100 µl of 2M H₂SO₄.

We calculated the cutoff A_{450} value as the mean absorbance of the negative samples ($n = 4$) plus three standard deviations. We calculated an index value for each sample (the ratio of specimen absorbance to the cutoff value) to facilitate comparisons of the results between the groups tested. Index values greater than 1.1 were considered positive, index values lower than 0.9 were considered negative, and index values between 0.9 and 1.1 were considered equivocal. This index is also known as the signal-to-cutoff (S/CO) ratio (Schubert *et al.*, 2001). Multiple assays for the detection of IgM and IgG in group 3 (healthy blood donors), using the rN₁85 EIA system, gave cutoff values of about 0.300. Based on this result, we decided that a cutoff value of at least 0.300 should be considered for index calculation.

The IgM EIA was performed in two steps: a screening test in which S/CO was calculated, and a confirmatory test, in which samples giving positive results in the screening assay were retested as serial dilutions (1:100, 1:400, 1:1,600 and 1:6,400). The sum of all net absorbance values had to exceed 1,100 for a sample to be considered positive (Frey *et al.*, 2003).

IgG EIA. IgG EIA was carried out as described for IgM EIA, except that the secondary antibody was a peroxidase-labeled affinity-purified goat anti-human IgG (Sigma-Aldrich Inc, Saint Louis, Missouri, USA) used at a pretested dilution of 1:20,000 for HPS samples, and a peroxidase-labeled affinity-purified goat anti-mouse IgG (Sigma-Aldrich Inc, Saint Louis, Missouri, USA) used at a pretested dilution of 1:5,000 for rodent samples. Samples were tested at a dilution of 1:400 only and positive samples were not titrated.

Comparative Methods

Samples giving positive results, but with low absorbance values ($A < 0.300$) or discordant results were retested in duplicate, using two independent EIA tests: a commercially available IgM and IgG kit (Focus Technologies, California, USA) or an in-house test. The Focus kit uses a pool of baculovirus-recombinant N-truncated protein from several hantaviruses as the antigen. The protocol described in the kit manual was followed. Briefly, serum samples from patients were diluted 1:100 and incubated for 1 h at room temperature in antigen-coated, 96-well plates. Peroxidase-coupled anti-human IgG or IgM was used as the secondary antibody, and was incubated with the plates for 30 minutes at room temperature. The substrate was reacted and color development was assessed by

measuring absorbance at 450 nm. In the case of “in-house” μ -capture IgM, and indirect IgG EIA, using the native Maciel antigen provided by Silvana Levis (from the “Dr Julio I. Maiztegui Institute”), samples were tested in serial dilutions (1:100, 1:400, 1:1600, 1:6400). The sum of all net absorbance values had to be $\geq 1,100$ for a sample to be considered positive.

As no gold standard test is available, the alternative for validation purposes was the use of several assays with different antigens (recombinant and native antigens) and formats (indirect EIA, μ -capture and immunoblotting) (Fletcher *et al.*, 1989).

Immunoblotting

Purified rN_A₈₅ fusion protein (3.4 μ g) preparations were separated by SDS-PAGE (Lammler, 1970) in a 13% acrylamide gel and blotted onto nitrocellulose strips (Towbin *et al.*, 1979). Individual nitrocellulose strips were incubated for 30 minutes at 37°C with serum samples from patients diluted 1/100 in Tris-buffered saline (TBS) (500 mM NaCl, 100 mM Tris-HCl, pH 8.0) supplemented with 5% non fat milk powder. The strips were then washed with TBS to remove unbound material. Antibodies bound specifically to the rN_A₈₅ fusion protein were visualized, using a series of reactions with goat anti-human IgG or IgM conjugated with alkaline phosphatase (Promega Inc., Madison, WI, USA) and 50 mg/mL of the substrate BCIP/NBT (Promega Inc., Madison, WI, USA). Human sera containing reactive and non reactive antibodies against *Araucaria* hantavirus were used as positive and negative controls, respectively. The presence or absence of anti-hantavirus antibodies was determined by comparing each nitrocellulose strip with strips corresponding to positive and negative control reactions. Seroreactivity was classified as positive (pattern identical to positive control), indeterminate (reactivity, but with a pattern different from that of the positive control), or negative (no reactivity) (Yoshimatsu, 1996). We assessed the specificity of the test, by including a non-related recombinant antigen (rGFP- green fluorescent protein) expressed in the same system.

Nested RT-PCR

Viral RNA was extracted from blood clots or serum collected from HPS patients, using either a QIAmp Viral RNA Mini Spin kit (Qiagen Inc, Ontario, CA) or a High Pure Viral RNA Kit (Roche Inc, Mannheim, GE), essentially according to the manufacturers' instructions. RNA was eluted with RNase-free water and stored at -70°C . RNA samples (10 μ l) were used as a template for nRT-PCR.

The cDNA was synthesized and used for PCR, using the partial S genome segment of the N-encoding region which was amplified by nRT-PCR, using specific primers designed to detect Araucaria hantaviruses, as previously described (Raboni *et al.*, 2005b).

RESULTS

1. Amino-acid sequence comparisons for hantavirus N protein

In a previous study, we characterized the S segment of hantaviruses from 12 HPS patients in Brazil and found that the first 343 amino acids of the nucleocapsid coding sequence were extremely similar in all cases (Raboni *et al.*, 2005a).

We compared the similarity of the amino-acid sequences of N proteins from Brazilian and representative hantavirus strains (HPS cases and rodents) from South, Central and North America: the degree of identity was 93.6% for South American strains, 87.6% for Central American strains and 63.7% for North American strains (Raboni *et al.*, 2005a).

2. Cloning, production and purification of hantavirus nucleoprotein

Analysis of the nucleotide sequence of the prN_{Δ85} construct showed that it encoded the expected protein, with a predicted molecular weight of 48 kDa, including the six amino-terminal histidine residues. A purified recombinant protein of the expected size was detected on SDS-PAGE (Fig.1A). Anti-hantavirus antibodies present in the sera of HPS patients and anti-histidine monoclonal antibodies specifically recognized the recombinant antigen on immunoblots (Fig.1B). No reaction was detected with an unrelated antigen (rGFP) (result not shown).

3. Evaluation of diagnostic applicability: enzyme immunoassay (EIAs)

3.1 IgG and IgM detection

We investigated the sensitivity and specificity of the rN_{Δ85} antigen for detecting IgG and IgM in indirect enzyme immunoassays on human sera. We tested five groups for both IgM and IgG antibodies: patients with suspected HPS, healthy individuals living in an endemic area, healthy blood donors, a blind-panel of human and rodent serum samples provided by Brazilian and South America Public Health Laboratories and patients with acute infections other than HPS. The S/CO ratios obtained for the five groups are shown in Fig 2.

Despite the clinical signs of HPS in all patients of group 1 (Table 2), four patients were excluded because they tested negative for both IgM and IgG in *Focus Technologies* IgM/IgG EIA, Brazilian Health Public Laboratory EIA and rN_{Δ85} EIA. IgM antibodies were found in the remaining 22 of the 26 (84.6%) subjects. All patients were tested for hantavirus RNA by nRT-PCR and 70% concordance was observed between the serological and molecular diagnosis. The 30% non concordant diagnoses may be accounted for by unsuitable sample transport and storage conditions. IgG anti-hantavirus antibodies were found in 22 of the 26 (84.6%) of with suspected HPS. Although a delay in the hantavirus-

specific IgG response has been reported in patients infected with Puumala and Dobrava viruses, which cause hemorrhagic fever with renal syndrome (HFRS) (Kallio-Kokko *et al.*, 1993; Lundkvist *et al.*, 1993; Lundkvist *et al.*, 1995; Lundkvist *et al.*, 1997), we nonetheless detected IgG antibodies during the early phase of infection. The mean time between the onset of symptoms and serum sampling was four days, and it was therefore possible to use the combined detection of IgG and IgM to confirm acute hantavirus infection.

Group 2 comprised 107 serum samples from individuals living in an endemic area but with no history of HPS. All samples were tested in duplicate with the rN_{Δ85} antigen and the *Focus Technologies* kit for IgM and IgG EIA. As expected, all samples were IgM-negative in both tests. IgG EIA gave discordant results for eight samples. The results obtained in immunoblotting assays were 100% consistent with the EIA rN_{Δ85} results (Table 3).

For the healthy blood donors (group 3), 305 serum samples were analyzed, to assess the specificity of the rN_{Δ85} antigen in EIA tests. For the IgM EIA, the mean cutoff value was 0.300. Absorbance values were close to the cutoff value for seven samples (2.3%). One sample that tested positive in EIA with rN_{Δ85} as the antigen gave a negative result on immunoblotting, giving a false positive frequency of 0.32%. One sample was equivocal in EIA, with an S/CO ratio of about 1.0, and tested negative by immunoblotting (data not shown). In the EIA for IgG antibody detection, the mean calculated cutoff value was 0.353. Positive results were obtained for five samples (1.6%), only one of which was confirmed by immunoblotting (Fig.S1). These samples were tested in duplicate using the *Focus Technologies* kit for IgG EIA (Table 4).

Samples displaying EIA discordant results were analyzed by immunoblot and positive results are showed in Fig. S1.

3.2 Hantavirus IgG antibody responses of HPS patients and rodent sera from different regions of Brazil

The sensitivity of the rN_{Δ85} IgG EIA was evaluated by the blind testing of 80 serum samples (45 from humans and 35 from rodents) obtained from Brazilian Public Health Laboratories (group 4). The recombinant antigens used for indirect IgG enzyme immunoassay by these laboratories, were provided by CDC (SNV) or by *Instituto Nacional de Enfermedades Infecciosas*, Dr Carlos G Malbran, Argentina (ANDV). The results of the two tests were concordant for 94.2% of rodent samples and 95.5% of human samples. The discrepant results (n=4) concerned samples with very low absorbance values, possibly due to prolonged storage and the effects of consecutive freeze/thaw cycles. Two samples that

previously tested negative gave equivocal results with the rN₈₅ antigen, with one of these samples providing a negative result on immunoblotting. One of the positive samples tested negative by EIA and was equivocal on immunoblotting, whereas another positive sample gave equivocal results on EIA.

3.3 Hantavirus IgG and IgM antibody responses of HPS patient and rodent sera from other South American countries: Argentina and Uruguay

We broadened the study by investigating the applicability of the rN₈₅ antigen to diagnosis for samples from other South American countries with confirmed hantavirus circulation (n=33). We analyzed 15 human samples from Pergamino (Argentina), 10 of which had been diagnosed as IgM-positive and five as IgM-negative. We assayed all the samples for anti-hantavirus IgM and IgG antibodies with the rN₈₅ antigen and obtained identical results for IgM. For IgG, non concordant results were obtained for four samples. Three sera previously considered negative (P10, P11 and P12) reacted with the rN₈₅ antigen, on either EIA or immunoblotting (Fig.1S). Conversely, one sample that previously tested positive with the Maciel antigen did not react with the rN₈₅ antigen in EIA, and another (P8) gave an equivocal result on EIA and a positive result on immunoblotting with the rN₈₅ antigen (Fig.1S).

The results obtained for EIA using the rN₈₅ antigen were identical to the results originally obtained for 18 rodent samples from Uruguay (11 positive and 7 negative).

3.4 EIA with non HPS acute disease samples

We investigated cross-reactivity, by testing 68 serum samples from patients with non HPS acute disease (group 5, Table 1) for anti-hantavirus IgM, using the rN₈₅ antigen (Table 5). In the screening tests (dilution 1/400), 88.3% of the samples gave negative results, with seven samples (10.3%) testing positive and one (1.4%) giving equivocal results. These samples were retested, using a titration range for serum dilution of 1/100 to 1/6,400. The sum of the absorbance values was greater than 1,100, indicating a positive result, for only two samples (acute Chagas' disease and acute EBV), giving a false positive frequency of 2.9%. Identical results were obtained with the commercially available *Focus Technologies* kit (data not shown). Both samples tested negative for anti-hantavirus IgG on EIA and immunoblotting with the rN₈₅ antigen.

The overall results for IgM and IgG EIA sensitivity, specificity and reproducibility for the groups five are shown in Tables 6 and 7.

DISCUSSION

HPS symptoms are not specific and are common to other infectious diseases, such as leptospirosis and influenza. There is no specific treatment for HPS and rapid, accurate diagnosis early in the course of disease is essential for successful supportive care. Improvements in diagnosis should decrease the risk of missing HPS infections, thereby helping to decrease the mortality rate.

Despite previous reports that of low sensitivity and specificity for indirect EIA based on *E. coli*-expressed hantavirus N antigen (Elgh *et al.*, 1996; Zöller *et al.*, 1993), we observed no background or loss of sensitivity or specificity for the use of the rN₈₅ antigen in indirect IgM and IgG EIA at serum dilutions of 1/400. Furthermore, combining IgG and IgM made it possible to improve diagnostic conditions.

The results of IgM and IgG EIA using the rN₈₅ antigen for all groups demonstrated a high degree of sensitivity and specificity, and excellent reproducibility, demonstrating that this test could be used as a simple, rapid diagnostic test (sensitivity and specificity for IgM EIA of 91.6% and 99.3%, respectively, with a positive predictive value of 88% and a negative predictive value of 99.5%, and a sensitivity of 93.9% and specificity of 98.9% for IgG with positive and negative predictive values of 86.1% and 99.5%, respectively).

Discrepancies were observed in the results for four patients from group 1, all previously diagnosed with IgM-positive HPS by the Brazilian Public Health Laboratory. All tested negative by nRT-PCR (Raboni *et al.*, 2005b), whereas two were positive and two were negative for both IgG and IgM in immunoblotting assays, with rN₈₅ used as the antigen (Table 2 and Fig.S1). Our EIA test failed to detect IgM and IgG in patient BR/01-60, and patient BR/01-69 could have been incorrectly diagnosed as IgM-positive. Sample number 6 (BR/02-67) gave conflicting results and, when retested with the *Focus Technologies* IgM/IgG EIA kit, the results for both IgM and IgG were consistent with those for our test (results not shown). In both tests, a high S/CO index was obtained for IgG. For this sample, negative results were obtained for IgM and IgG immunoblotting assays. Unfortunately, due to the intense hemolysis and small sample volume, we were unable to repeat the assays. All these samples had been stored for two years and had undergone several freeze/thaw cycles, which may have compromised their quality. Furthermore, these samples were not simultaneously tested by us and by the Brazilian Public Health Laboratory. Since 2003 (samples 11 to 30), diagnoses have been carried out

simultaneously by the Brazilian Public Health Laboratory and our laboratory, with 100% concordant results (Table 2).

In group 2, the IgG rN₈₅ EIA results revealed a seroprevalence of 8.4% (9/107) for a specific IgG anti-hantavirus antibody. Several serological surveys carried out in different Brazilian regions have reported a high prevalence of IgG antibodies in the population, independent of sex, profession, or history of contact with rodents (Campos *et al.*, 2003; Figueiredo *et al.*, 2003; Mendes *et al.*, 2001; Mendes *et al.*, 2004). None these individuals had a history of HPS. These findings suggest that a less-virulent hantavirus species may also be circulating in this region, causing undetected infections.

We analyzed healthy blood donor serum samples (group 3) to evaluate the specificity of the test. For the IgM rN₈₅ EIA, we obtained 0.3% false positive results. All results were confirmed with the commercially available *Focus Technologies* kit. The false positive rate for the IgG test was 1.3%. The S/CO values for all these samples were around the cutoff value, suggesting that samples with low positive values should be retested on a second sample to check for seroconversion.

Hantaviruses have been poorly identified in Brazil, due to difficulties in investigating suspect cases in the laboratory. Little is known about the epidemiology, genetic diversity and epizootiology of this virus. The development and distribution of region-specific antigens and a rapid, carefully standardized and validated test that can be performed by regional laboratories should increase diagnostic capacity with no loss of sensitivity or specificity. We assessed the feasibility of using rN₈₅ EIA for IgG detection in human and rodent sera from all regions of Brazil. Only four samples gave discordant results. The strong correlation between the results obtained in the different tests shows that, despite the large size of the various territories and rodent diversity in Brazil, the rN₈₅ antigen EIA can be used for rodent and human serum surveys with no loss of sensitivity and specificity, for identifying possible reservoir hosts, and for studying the dynamics of hantavirus transmission, even from distant Brazilian regions. The IgM rN₈₅ EIA test is currently being validated in association with the Brazilian Ministry of Health.

We assessed the applicability of the test we developed to other South American countries, by evaluating the cross-reactivity of the recombinant Araucária N protein with sera from patients and rodents infected with other species of hantavirus from Argentina and Uruguay. The results obtained for the IgM test were 100% concordant for the Argentinean samples. For IgG, 100% and 85% concordance was observed for the Uruguayan and

Argentinean samples, respectively. Thus, the IgM and IgG rN₈₅ EIA tests are potentially useful for hantavirus diagnosis in the border regions of Brazil and other South American countries.

We assessed interference due to non specific IgM, by IgM rN₈₅ EIA of a panel of serum samples from patients presenting acute infections other than HPS. Two samples tested positive: one from a patient with acute Chagas' disease and the other from a patient with infectious mononucleosis. These diseases cause polyclonal activation, which may in turn lead to nonspecific IgM reactions (Alpers *et al.*, 1994; Schubert *et al.*, 2001). Both samples tested negative for hantavirus-specific IgGs. Careful clinical evaluation and a combination of IgM and IgG tests should be considered as an aid to diagnosis (Schubert *et al.*, 2001). We also suggest that the number of equivocal and false-positive results in IgM EIA tests could be reduced by titrating samples from 1:100 to 1:6,400, with results considered positive if the sum of the absorbances of the dilutions is greater than 1,100 (Frey *et al.*, 2003). Serological diagnosis (detection of anti-hantavirus IgM alone or in combination with anti-hantavirus IgG) in individuals with signs/symptoms and a history of exposure consistent with HPS is essential for early, appropriate supportive care.

Our results show that the truncated N protein of Araucária hantavirus is a valid antigen for detecting hantavirus-specific IgM and IgG antibodies in sera from patients with acute or convalescent hantavirus infection, and in rodent reservoirs. This rapid, sensitive and cost-effective test is valid for many regions of Brazil and for other South American countries.

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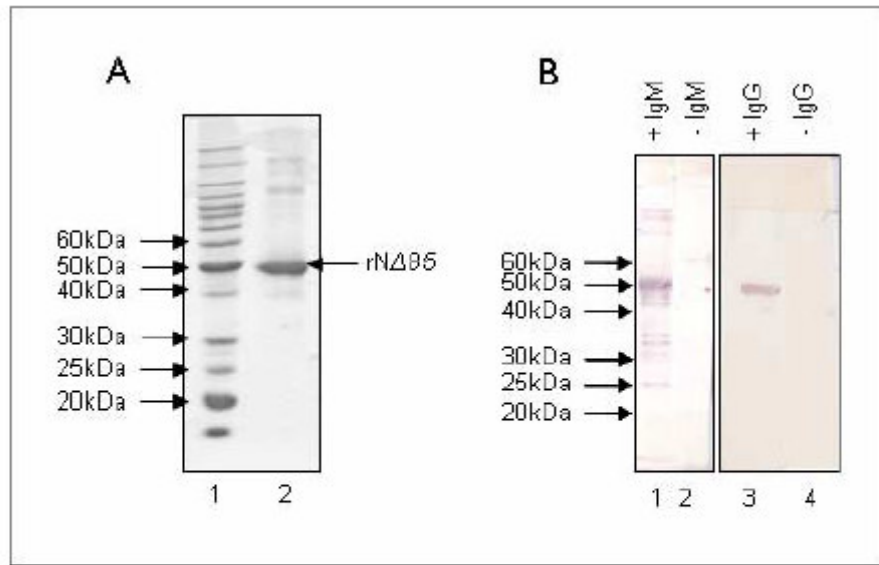


Fig 1. (A) Coomassie Blue stained 13% SDS-PAGE. 1. Molecular mass marker. 2. Purified recombinant antigen. (B) Immunoblot strips for IgM and IgG detection using $rN\Delta 85$. Lane 1 and 3: HPS patient sera (BR 03/97). Lane 2 and 4: negative control sera.

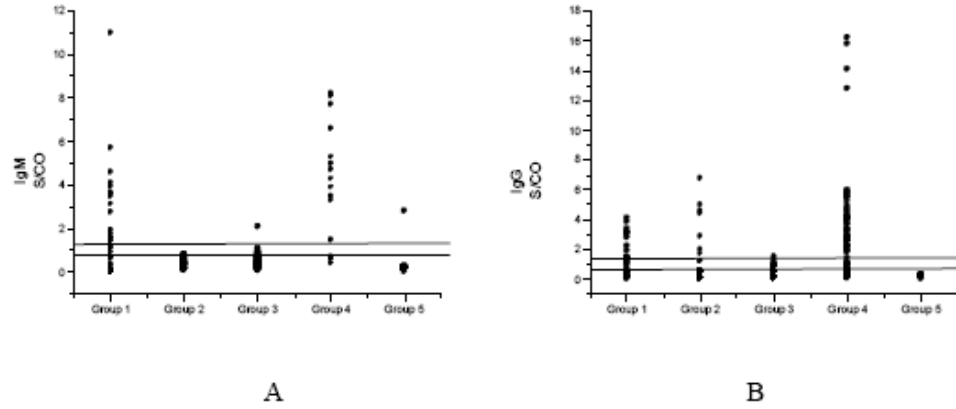


Fig 2. Results of hantavirus EIA using the rN₈₅ antigen: (A) IgM S/CO ratios of samples from patients with suspected HPS (group 1), healthy individuals living in an endemic area (group 2), healthy blood donors (group 3), samples from Brazilian and South American Public Health Laboratories (group 4), and samples from patients with acute infections other than HPS (group 5) (B) IgG S/CO ratios of samples from patients with suspected HPS (group 1), healthy individuals living in an endemic area (group 2), healthy blood donors (group 3), samples from Brazilian and South America Public Health Laboratories (group 4), and samples from patients with acute infections other than HPS (group 5).

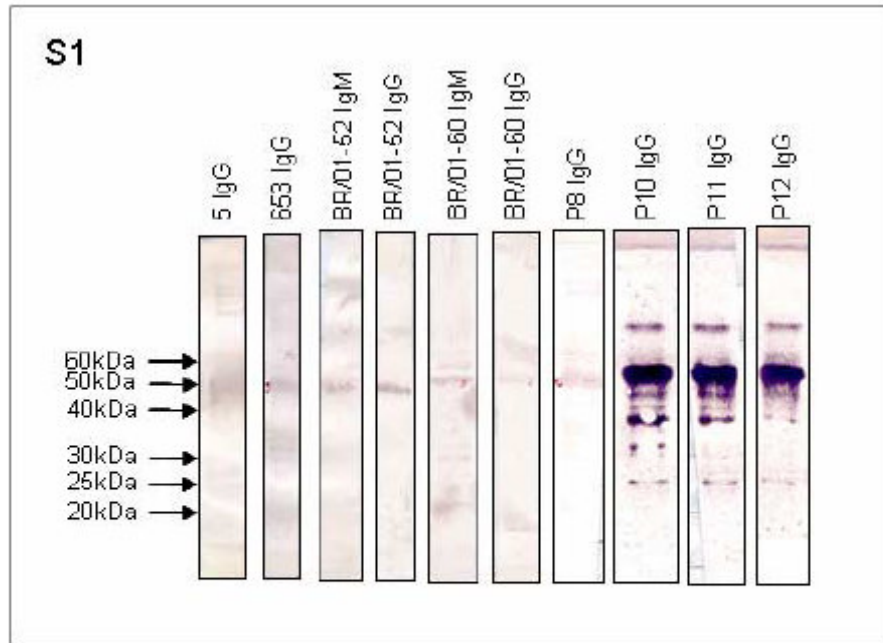


Fig S1 - Positive immunoblots strips from samples displaying discordant EIA results. Samples 5 (Group 2), 653 (Group 3), BR/01-52 and BR/01-60 (Group 1) and P8, P10, P11 and P12 (Group 4).

TABLE 1. Serum samples from humans and rodents

Group Identification	Number	Description
Group 1	30	Serum samples from patients with suspected HPS, provided by Local Public Health Laboratory (LACEN-PR)
Group 2	107	Serum samples from healthy individuals living in an endemic area (General Carneiro, Paraná, Brazil)
Group 3	305	Serum samples from blood donors from a non-HPS end
Group 4		Serum samples from patients and rodents from different Brazilian geographic areas, provided by the Brazilian Public Health Laboratories*, and from other South America countries#.
Humans	60	
Rodents	43	
Group 5	68	Serum samples from patients with acute infections other than HPS: EBV (n=17); CMV (n=13); EBV and CMV (n=2); VDRL-positive (n=27); acute Chagas' disease (n=1); toxoplasmosis (n=8).
Total	613	

*Brazilian Public Health Laboratories– Adolfo Lutz Institute, São Paulo, Evandro Chagas Institute, Pará and Oswaldo Cruz Institute, Rio de Janeiro.

#Uruguay – Virology Laboratory, Universidad de la Republica, Montevideo; Argentine - Dr J Maztegui Institute, Pergamino.

EBV – Epstein Barr virus. CMV – cytomegalovirus. VDRL - Venereal Diseases Research Laboratory.

All samples had been stored at –20°C until testing.

TABLE 2. Reactivity of human sera (group 1/ suspected HPS) in IgM and immunoblotting and nRT-PCR

No	Patient*	nRT-PCR Brazil-specific primers ^a	IgM Brazilian Public Health Laboratory ^b	IgM rN ₈₅ antigen	IgG rN ₈₅ antigen	I
1	BR/01-50	+	+	+	+	
2	BR/01-51	-	+	+	+	
3	BR/01-52	-	+	-	+	
4	BR/01-55	+	+	+	+	
5	BR/01-60	-	+	-	-	
6	BR/02-67	-	+	-	+	
7	BR/01-69	-	+	-	equivocal	
8	BR/02-71	+	+	+	+	
9	BR/02-72	+	+	+	+	
10	BR/02-85	+	+	+	equivocal	
11	BR/03-91	-	+	+	+	
12	BR/03-92	-	+	+	+	
13	BR/03-95	+	+	+	+	
14	BR/03-97	+	+	+	+	
15	BR/03-98	+	+	+	+	
16	BR/03-99	+	+	+	+	
17	BR/03-100	+	+	+	+	
18	BR/03-101	+	+	+	+	
19	BR/04-102	+	+	+	+	
20	BR/04-104	+	+	+	+	

21	BR/04-105	+	+	+	+
22	BR/04-106	+	+	+	-
23	BR/04-107	+	+	+	+
24	BR/04-108	+	+	+	+
25	BR/04-109	-	+	+	+
26	BR/04-111	-	+	+	+
27	BR/04-A ^c	-	-	-	-
28	BR/04-B ^c	-	-	-	-
29	BR/04-C ^c	-	-	-	-
30	BR/04-D ^c	-	-	-	-

^a Raboni *et al.*, 2005a. ^bAdolfo Lutz Institute. ^c Negative samples.

TABLE 3. Group 2 (healthy individuals living in an HPS-endemic area):
IgG serology for discordant results

No.	EIA $rN\Delta_{85}$ antigen	EIA <i>Focus kit</i>	Immunoblotting IgG $rN\Delta_{85}$ antigen
	S/CO*	S/CO*	
5	2.9	0.5	Positive
13	0.7	1.8	Negative
14	0.3	1.6	Negative
17	0.4	3.9	Negative
27	0.2	1.5	Negative
43	0.2	1.1	Negative
61	0.3	1.1	Negative
70	0.2	1.4	Negative

*S/CO: signal-to-cutoff ratio

TABLE 4. Group 3 (blood donors):

IgG serology for patients with positive or equivocal results

No.	EIA <i>rND₈₅</i> antigen S/CO*	EIA <i>Focus kit</i> S/CO*	Immunoblotting IgG <i>rND₈₅</i> antigen
646	1.1	1.3	Negative
653	1.7	0.3	Positive weak
775	2.9	0.9	Negative
784	1.5	0.1	Negative
785	2.5	0.4	Negative
835	1.0	0.2	Negative
853	2.9	0.3	Negative
857	1.1	0.9	Negative

*S/CO: signal-to-cutoff ratio

S/CO > 1.1 = positive, S/CO < 0.9 = negative, $1.1 \geq \text{S/CO} \geq 0.9$ = equivocal.

TABLE 5. Group 5 (patients with acute infections other than HPS): Hantavirus enzyme immunoassay for difficult samples

Samples (n)	Hantavirus IgG EIA			Hantavirus IgM EIA			Hantavirus IgM EIA	
	rN ₂ antigen			rN ₂ antigen (Screening)			Sum of titration*	
	Positive	Equivocal	Negative	Positive	Equivocal	Negative	Positive	Negative
Difficult samples, subgroups:								
Acute CMV (13)	-	-	13	1	-	12	-	13
Acute EBV (17)	-	-	17	3	-	14	1	16
CMV/EBV-positive IgM (2)	-	-	2	-	-	2	-	2
Acute Chagas' disease (1)	-	-	1	1	-	-	1	-
Acute toxoplasmosis (8)	-	-	8	2	-	6	-	8
VDRL-positive (27)	-	-	27	-	1	26	-	27

N: number of samples. *Sum of titration $\geq 1,100$ positive and $<1,100$ negative

Table 6. Calculation of sensitivity, specificity and predictive values for IgM rN₈₅ EIA

Group	N	Positive	Negative	Equivocal [#]
Group 1	30	22	8	0
Group 2	107	0	107	0
Group 3	305	1	303	1
Group 4	103*	10	05	0
Group 5	68	2	66	0
Sensitivity			94.1%	
Specificity			99.1%	
PPV			88.8%	
NPV			99.5%	

*Only 15 samples tested for IgM

[#]Equivocal results were excluded from the analysis

PPV: positive predictive value; NPV: negative predictive value