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Development and evaluation of a solid-phase enzyme immunoassay based on Andes hantavirus recombinant nucleoprotein

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Hantavirus pulmonary syndrome (HPS) with high mortality rate has been reported in five countries in South America. Rapid accurate methods are important both for monitoring acute infections and for epidemiological studies. The Andes virus nucleoprotein amino acid sequence has a high identity percentage compared with other sequences of this region and has been chosen for the development of diagnostic reagents. Andes nucleoprotein expressed in Escherichia coli was applied as antigen in IgG, IgA and μ-capture IgM enzyme-linked inmunosorbent assays (ELISAs). An evaluation of this reagent was conducted to establish its usefulness for differential diagnosis of HPS and seroprevalence studies. Samples from 135 reverse transcription (RT)-PCR-confirmed HPS cases, 77 individuals with other respiratory infections and 957 healthy inhabitants from endemic and non-endemic areas were analysed. The hantavirus-infected patients had an early and strong IgM, IgG and IgA serum antibody response, in most of the cases as early as 1, 7 and 1 days following onset of symptoms, respectively. IgM and IgG detection showed a specificity and sensitivity of 100%. Andes-specific IgM antibodies were found in all patients in the first available sample, which remained detectable for at least 43 days. Specific IgA antibodies were also detected in saliva of patients with acute HPS. The short duration of the disease and the risk for contacts due to person-to-person transmission of Andes virus necessitate the use of highly sensitive tests which might lead to earlier detection of infected people and improve the treatment and management of patients with HPS.

Introduction

Hantaviruses cause >150 000 human infections worldwide annually, associated with haemorrhagic fever with renal syndrome (HFRS) [1]. In America, hantavirus pulmonary syndrome (HPS) with a high mortality rate has also been described [2]. By the first quarter of 1998, a total of 231 HPS cases had been reported in five countries in South America [3]. This infection must be differentiated from other causes of similar symptoms. Person-to-person transmission has been described in Argentina [4–6] and could not be excluded in two Andes virus-related family clusters from Chile [7], thus early diagnosis is crucial to prevent outbreaks and to perform a strict clinical follow-up of contacts. It has been considered desirable for confirmed cases of Andes virus infection to be isolated in separate care units and for 'droplet precautions' to be used [7]. In addition there are ongoing trials of antiviral therapies for known contacts.

Highly sensitive reagents are also important for assessing the seroprevalence of this virus in man and rodents. The association of hantaviruses with different rodent reservoirs in several geographic areas suggests that development of region-specific antigens should be undertaken to improve serological reactivity. As for other viruses, the use of the homologous antigen improves the sensitivity and specificity of the assays, providing earlier antibody detection in infected people [8–11]. Thus, some of the South American viruses may give higher optical density readings in enzyme-linked immunosorbent assays (ELISA) with reagents based on the local viral sequence [12].

The viral nucleoprotein (N) elicits a strong humoral

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immune response in infected patients and immunised animals [13–15] and has been used extensively to produce diagnostic reagents for hantaviruses [8, 16–18].

In phylogenetic analysis, all viruses associated with cases of HPS from Argentina [6, 19], Chile [20], Paraguay [21] and Uruguay (P. J. Padula, unpublished observations) and sigmodontine-related virus from Bolivia [22, 23], clustered together with Andes virus [24] and some North American viruses like Sin Nombre (SN) [2, 25], Bayou (BAY) [26] or Black Creek Canal (BCC) [27].

Human exposure to hantaviruses occurs principally through the respiratory tract route; thus, mucosal inmunity may confer the first line of defence against infection, as has been suggested in influenza virus infection [28] and confirmed by in-vitro studies [29]. However, secretory IgA response has not been assessed in hantavirus-infected patients.

An Andes virus sample was used to produce a recombinant antigen to detect IgM antibodies against hantavirus by capture ELISA and IgG or IgA antibodies by direct ELISA. This reagent was evaluated to establish its utility for hantavirus diagnosis in serum or saliva from acute and early convalescent patients and its application in human and rodent seroprevalence studies in affected areas of South America.

Materials and methods

Samples from patients and rodents

Routine serum samples from patients with suspected HPS from Argentina, Chile, Paraguay and Uruguay were received over a 2-year period. One hundred and seventy-three serum samples from 135 hantavirus-specific reverse transcription (RT)-PCR-positive patients were tested for hantavirus antibodies by μ-capture IgM ELISA, and IgG and IgA ELISA. Eight saliva samples were studied by IgA ELISA. Seroprevalence studies from an endemic region (Salta, north Argentina; $n = 57$ subjects) and from a non-endemic city (Buenos Aires; $n = 210$ subjects) were also conducted.

Hantavirus antisera developed in rodents live-trapped primarily in rural and peri-domestic habitats in northern and southern regions of Argentina were investigated for Andes-specific IgG.

Control samples

Fifty saliva samples and 190 sera collected from apparently healthy adults and 45 sera from healthy children not living in endemic areas were used to investigate the specificity of the assays. Also, 455 HPS contacts without disease were studied. Serum samples from people who had been infected with: Chlamydia spp. ($n = 14$), Leptospira interrogans (11), Legionella spp. (5), Mycoplasma pneumoniae (9), Junin virus (Argentine haemorrhagic fever) (20), influenza A and B (18), were also tested. Sera were kept at -20°C until analysis.

Preparation of antigens

Based upon the Andes S segment sequence, PCR primers were designed flanked by appropriate restriction endonuclease sites, to allow the cloning and expression of the Andes N gene. Total RNA extraction, RT-PCR amplification and sequencing were performed as described previously [20].

The Andes N gene was amplified in a 'hemi-nested' fashion with the following primers: +5 (5′-AGTAGACTCCTTGAGAAGCTAC-3′), +22 (5′-GCTACTACGACTAAAGCTGGAATGAG-3′), −1554 (5′-TATAGACTAACCCACCTCCC-3′).

The DNA fragment containing the N protein ORF was cloned in pRSET A vector according to the manufacturer's instructions (R&D Systems Europe, Oxford). The recombinant clone was used to transform Escherichia coli BL21 (DE3) cells. Expression was obtained without induction. Cells were harvested by centrifugation at 5000 g for 20 min and were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.3 M NaCl, lysozyme 0.01% and Triton X-100 0.1%), centrifuged and resuspended in a denaturation buffer (50 mM Tris-HCl, pH 7.5, 6 M urea, β-mercaptoethanol 1% and SDS 1%). The pellet was recovered and processed on a nickel resin affinity column (Ni-NTA agarose provided by Quiagen, Gmbtt Hilden, Germany).

A recombinant opposite sense-encoding N protein was expressed in the same manner and was used as negative control antigen.

Amino acid comparison

The following published S segment sequences were used for amino acid comparisons:

Andes virus (AND) [20], AF004660; Chile (CH-1/96) [20], AF005941, AF005945, AF005947; Uruguay (URU-3) (submitted to Gene Bank); Laguna Negra virus (LN) [21], AF005727; Rio Mamore virus (RIOM) [23], U52136; Caño Delgadito virus (CDG) [11], AF000140; Rio Segundo virus (RIOS) [30], U18100; Bayou virus (BAY) [26], L36929; Black Creek Canal virus (BCC) [27], L39949; Sin Nombre virus (SN) [31], L37904.

Multiple sequence alignment and comparison of deduced amino-acid sequences were performed with CLUSTAL V, programs of the PCGENE 6.8 software, Intelligenetics (Mountain View, CA, USA).

Immunisation procedure

Rabbits, weighing an average of 2 kg, were inoculated subcutaneously with multiple injections three times at 15-day intervals with 200 μg of Andes N purified protein. Serum was obtained 20 days after the last immunisation. The reactivity of the serum was checked by IgG ELISA. Immunoblotting was used to characterise the antigen population against which specific antibodies had been raised [32].

μ-Capture IgM ELISA technique

Serial dilutions of all reagents were tested in various combinations to achieve the clearest separation of Andes IgM seropositive and negative samples. All reagents were added in 0.1-ml volumes. Highly activated flat-bottomed polystyrene microtitration plates (Certified Maxisorp, Nunc) or U-bottomed polyvinylchloride microtitration plates (Falcon) were coated with a 1 in 1000 dilution of an affinity-purified goat anti-human IgM (μ) antibody (Kierkegaard and Perry) in 0.1 M carbonate buffer, pH 9.5. All the following incubations were done at 37°C for 1 h and the plates were washed six times in wash buffer (Tween-20 0.1% in PBS) between each step.

Serum samples diluted 1 in 100 and four-fold up to 1 in 6400 in dilution buffer (skimmed milk powder 5% in PBS-Tween-20) were allowed to react with the anti-human IgM (μ) antibody-coated wells. Recombinant Andes and control antigen diluted in dilution buffer at a concentration of 2 μg/ml were allowed to react with the specific binding antibodies. Subsequently, a 1 in 25 000 dilution of rabbit hyperimmune serum in dilution buffer was added. The addition of a 1 in 5000 dilution of hantavirus antibody-negative human serum to each antigen preparation and to the conjugated serum before they were added to the plate served to block any open capture antibody sites and kept background signals low. Goat anti-rabbit serum conjugated to alkaline peroxidase (Kierkegaard and Perry) (1 in 5000 in dilution buffer) was then added. Finally, ABTS (2.2′-azino-di [3-ethyl-benzthiazoline sulphonate (6)] substrate (Kierkegaard and Perry) was added and absorbance at 405 nm was measured with a BioRad 550 microplate reader. The values were expressed as the optical density (OD) obtained with Andes antigen substracted from the OD of the control antigen.

IgG and IgA ELISA techniques

Polystyrene microtitration plates (Polysorp, Nunc) or U-bottomed polyvinylchloride microtitration plates (Falcon) were coated with N Andes antigen and control antigen, at concentrations of 4 μg/ml and 0.4 μg/ml, respectively, diluted in 0.1 M sodium carbonate buffer, pH 9.5. Patient and control sera diluted 1 in 100 in dilution buffer and four-fold up to 1 in 6400 or saliva diluted 1 in 16 and two-fold up to 1 in 64 were added to the antigen-coated wells. Sera were diluted up to 1 in 102 400 for titration tests. The plates were incubated at 37°C for 1 h and washed as for μ-capture IgM ELISA. Peroxidase-labelled affinity-purified goat anti-human IgG (Fc) or peroxidase-labelled affinity-purified goat anti-human IgA (α) antibody (Kierkegaard and Perry) (1 in 3000 in dilution buffer) were added and specific antibody binding was detected by ABTS substrate. To remove IgG present in sera for performing the IgA test, 25 μl of each serum were mixed with 100 μl of a 50% suspension of Gamma-bind G agarose beads (Protein G Sepharose, Pharmacia) and incubated as recommended by the manufacturer.

Preparations of 1 in 200 and 1 in 400 in dilution buffer and peroxidase-labelled affinity-purified antibody to Peromyscus leucopus IgG (H+L) (Kierkegaard and Perry) (1 in 2000 in dilution buffer) were used for rodent serum assays.

The OD values were expressed as for the IgM technique.

Results

Amino-acid sequence comparison of hantavirus N proteins

To evaluate the convenience of using Andes N recombinant protein as an immunodiagnostic antigen, the study compared the amino-acid identity between putative N proteins of representative HPS viruses.

Although the amino-acid sequences of 10 HPS hantaviruses from the Americas showed a degree of identity >79.1%, when Andes N protein virus was compared with others from South America the values ranged from 84.3% with CDG from Venezuela to 98% with Chilean virus (Table 1). Comparison between partial N protein sequences belonging to Argentine central region genotypes and Andes AH-1 strain showed amino acid identities of nearly 94% (data not shown).

Cloning expression and purification of Andes virus N ORF frame

Nucleotide sequence of the expression vector constructs identified the corresponding published sequence. The predicted fusion protein expressed by this vector should have a mol. wt of 54 kDa, which includes six amino-terminal histidine residues for convenient affinity purification. Gel electrophoresis on SDS 10% polyacrylamide gels revealed highly purified recombinant protein of the expected size (Fig. 1, lane 1). The yield of recombinant proteins ranged from 16 to 24 mg/L of culture for four different batches and that of the purified antigen ranged from 9 to 14 mg/L.

Table 1. Percentage amino-acid (aa) sequence identity between N proteins of South and North American sigmodontine hantaviruses

	AND* Argentina	URU-3* Uruguay	CH-1/96* Chile	LN* Paraguay	RIOM Bolivia	CDG* Venezuela	RIOS Costa Rica	BAY* USA	BCC* USA	SN* USA
AND Argentina		95.3	98.3	90.4	91.1	84.3	81.8	88.3	86.4	86.0
URU-3 Uruguay			92.0	86.4	87.4	82.1	79.1	85.7	83.1	85.0
CH-1/96 Chile				87.0	88.3	81.3	79.7	86.3	84.3	83.7
LN Paraguay					93.2	83.2	80.6	87.1	85.5	85.3
RIOM Bolivia						83.5	81.5	88.1	86.7	84.6
CDG Venezuela							79.8	81.6	80.9	83.2
RIOS Costa Rica								83.6	92.0	83.2
BAY USA									92.3	86.9
BCC USA										83.9
SN USA										

All compared sequences were 428 aa in length, except for CH-1/96 (300 aa), URU-3 (301 aa) and CDG (376 aa).
*Hantaviruses associated with human disease.

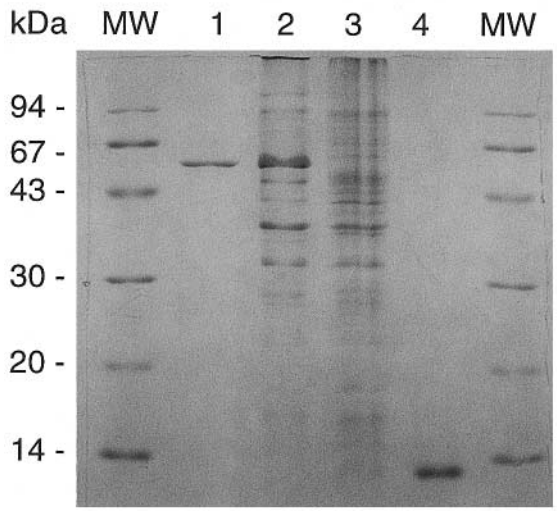

Fig. 1. Polyacrylamide gel electrophoresis of Andes virus fusion proteins produced in E. coli. Recombinant Andes N protein (land 1) and recombinant opposite sense-encoding N protein (4) after purification by Ni-NTA column chromatography; total cellular proteins of the N protein expression clone (2); crude cell lysate of untransformed cells (3). The numbers correspond to the mol. wt (MW).

The identity of the protein expressed by the transformants after purification by chromatography was confirmed by immunoblot analysis with sera from Andes virus-infected patients and negative control sera where the unique expected band was observed. The recombinant opposite sense-encoding N protein expressed by the transformants showed no reactivity with these sera (data not shown).

Hantavirus IgG, IgM and IgA responses of HPS from South America

A total of 173 sera from 135 patients with confirmed HPS was studied to evaluate the reagent. All specimens were tested in duplicate with N Andes antigen and also with negative control antigen. Hantavirus RNA detection by PCR and the broad detectable reactive antibodies specific for the Andes virus nucleocapsid protein by μ-capture IgM ELISA showed 100% correlation of diagnosis in the patients studied.

Specific IgM antibodies were detected in acute- and early convalescent-phase specimens in sera from Argentina, Chile, Uruguay and Paraguay in the first sample available as early as 1 or 2 days after the onset of symptoms. An IgG response was detected mostly after the first week, although some sera were reactive at the onset (Table 2). Increase of IgG antibody titres was observed between the first sample (days 1–9) and the

Table 2. μ-capture IgM and direct ELISA IgG and IgA antibody detection in serum samples from patients with HPS

Days after onset of symptoms	Number of patients (positive/total) in ELISA for		
	IgM	IgG	IgA
1	4/4	1/4	2/2
2	12/12	8/12	7/7
3	11/11	6/11	6/8
4	20/20	13/20	12/15
5	14/14	10/14	8/10
6	16/16	12/16	9/9
7	17/17	14/17	10/11
8	7/7	7/7	6/6
9	2/2	2/2	1/1
10	6/6	4/6	3/5
11	4/4	3/4	4/4
12	2/2	2/2	2/2
13	2/2	1/2	1/1
14	6/6	6/6	2/3
15	2/2	2/2	1/1
16	6/6	5/6	4/5
17	3/3	3/3	4/4
18	1/2	2/2	1/1
20	2/3	3/3	3/3
21	2/2	2/2	1/1
22	2/2	2/2	2/2
23	3/3	3/3	1/1
24	1/1	1/1	1/1
30	1/1	1/1	0/1
31	2/2	2/2	1/1
32	1/1	1/1	1/1
36	1/1	1/1	1/1
42	1/1	1/1	1/1
44	0/1	1/1	0/1
45–87	4/13	13/13	6/7
124–195	0/6	6/6	2/4
Total	155/173	138/173	103/120

Sera with OD >0.3 were considered positive.

second sample (days 18–40) in 29 of 30 individuals from whom paired sera were obtained. Seroconversion was observed in at least 15 cases. Thirty days after the onset, IgG titres were >25 600 in most of the cases. No decrease in IgG antibody titres was observed during the study period.

Some early Andes IgG-ELISA seropositive samples were negative with SN antigen; however, subsequent samples from these patients reacted with both reagents (N. Pini, unpublished results).

In contrast, 455 case contacts, without HPS symptoms, were negative for μ-capture IgM and IgG antibodies when analysed within 7 days (345 serum samples) and 60 days (217 serum samples) after the onset of symptoms in the HPS case.

The IgA response peaked at c. 1–2 weeks after onset of symptoms. Ninety-one (87.5%) of 104 samples obtained up to 30 days after onset had specific serum IgA antibodies. No significant differences were found when latex was used for IgG adsorption. Seven of nine saliva samples from eight patients with HPS had detectable specific saliva IgA antibodies from day 5 and within a month after the onset of symptoms (Table 3).

The reagent was used to study seroprevalence in three Argentine regions. In a non-epidemic area (Buenos Aires City), none of 210 sera was positive. In the southern region where person-to-person transmission was demonstrated [6], the seropositive percentage was 0.6% (n = 470) [33]. In workers living in a rural sugar refinery in the Salta province of Argentina, seroprevalence was 5.1% (n = 57). These latter two were both endemic areas. No equivocal test result was found in this seroprevalence study, although lower IgG titres (1400–6400) were obtained when compared with sera from HPS patients.

Specificity and sensitivity of the ELISA assays

To evaluate the specificity of the assays, sera and saliva samples from healthy adults and children not living in endemic areas and from subjects with other respiratory infections and haemorrhagic diseases were tested for Andes virus-specific IgM, IgG and IgA antibodies.

All sera were tested with Andes antigen and control antigen and none was reactive. A malaria parasite-positive individual with compatible symptoms of hantavirus infection gave a high signal with the recombinant opposite sense-encoding N protein, although the final result was negative.

The specificity and sensitivity of the IgM and IgG assays in early and convalescent samples were 100%.

The coefficients of inter-assay variation were 11.6, 9.1 and 13.0 for IgM, IgG and IgA, respectively. The negative media OD values were 0.06, 0.03, 0.04 and the positive OD values were 2.7, 2.8, 2.8 for IgM, IgG and IgA ELISA, respectively, at 1 in 400 serum dilution.

Protein Andes N reactivity of animal sera

Sera from several species of rodents were examined in this study. ELISA antibodies were detected most frequently in sera from Oligoryzomys longicaudatus with an overall antibody prevalence rate of 4.15% (n = 193). However, antibody was not restricted to this species. O. chacoensis and O. flavescens were also frequently positive. Sera from individual animals of these two species had high antibody titres by ELISA (6400) and were clearly true positive RT-PCR reactions. However, RT-PCR performed on lungs of 20 seronegative O. longicaudatus selected at random gave negative results.

Two seropositive rodents from other genera, one Akodon various and one Abrothix longipilis, were negative by RT-PCR reactions.

Discussion

At present, the optimal test for diagnosis of acute HPS is the μ-capture IgM ELISA [12]. Serological

Table 3. ELISA IgA antibodies in saliva from HPS patients by day of disease

HPS patient no.	Days after onset of symptoms	OD values* at dilution		
		1 in 16	1 in 32	1 in 64
1	3	0.19	0.13	0.08
2	5	0.97	0.51	0.24
3	7	1.78	0.67	0.14
	16	3.14	2.33	1.3
4	10	0.83	0.48	0.22
5	16	1.65	1.41	0.76
6	17	1.02	0.52	0.32
7	31	0.99	0.63	0.29
8	36	0.13	0.09	0.06
Negative control	–	0.049 (0.06)†	0.019 (0.02)	0.015 (0.009)

*Mean OD from three independent tests.
†Mean OD (SD) of saliva samples from 50 healthy subjects.

confirmation with this assay can usually be made with a single serum sample, as specific IgM antibodies are detectable shortly after onset of clinical HPS symptoms.

The outbreak of HPS cases in South America was associated with the Andes and closely related viruses. The choice of Andes N protein provided the opportunity to express a good predictive recombinant specific antigen to improve the diagnostic capabilities for South American cases of HPS. Comparison of HPS virus N protein sequences of the region with the corresponding sequences of representative sigmodontine hantaviruses showed the highest degree of similarity with that of Andes virus in most of the cases.

IgM ELISA results from the first available sample from HPS cases showed 100% sensitivity and specificity, as well as excellent reproducibility. Specific IgM antibodies were present for >1 month following the onset of disease. Sera from 190 healthy individuals were examined and there were no false positive samples by IgM capture or IgG ELISA. Specific IgG antibodies were detected in all sera from convalescent patients. The consistent IgG antibody detection obtained in late samples from all HPS case and from seroprevalence studies may show that this test is helpful for epidemiological studies.

Although native hantavirus antigens are being used widely as diagnostic reagents, their production requires expensive containment laboratory conditions for virus propagation. Recombinant protein technology is less expensive and proteins are generated in larger amounts and with higher purity than native viral antigens. The successful use of recombinant N antigen in the IgM capture assay, with high sensitivity and specificity, for diagnosis of HPS in different regions and countries shows that this approach is feasible in practice.

IgG antibody detection is especially useful for rodent serosurveys and has played an integral part in establishing the reservoir host and studying the natural ecology of the virus. Detection of antibodies against Andes N protein in sera from different rodent species in our region showed that this study may be used with high efficiency in rodent seroprevalence assays. A significant number of rodent sera gave positive reactions in ELISA with Andes antigen and negative reactions when SN antigen was used (N. Pini, personal communication).

Recently it has been reported that the ratio of the ELISA values obtained with SN versus Puumala virus protein as antigen increased with time after onset of disease in Puumala-infected patients [34]. Thus the use of homologous antigens becomes more important when acutely infected patients are being studied. Careful optimisation and validation of this test will ensure that there is no loss of sensitivity or specificity when used in cases from distant regions.

The widespread distribution of Andes-related viruses in different rodent species [19] raises the possibility that HPS may well exist undiagnosed in regions of South America beyond its traditionally recognised enzootic boundaries.

Determination of specific antibodies in saliva samples makes this test even more useful for isolated communities and, especially among some native populations of South America who, due to cultural reasons, do not always accept venepuncture. As human infection occurs via inhalation of contaminated aerosol, secretory IgA could provide effective protection against hantavirus infection.

The high mortality rate observed in infected patients [2, 12, 35], the short duration of the disease and the risk to contacts caused by person-to-person transmission necessitate the use of more sensitive tests which might lead to earlier detection of infected people and improve the treatment and management of HPS patients.

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