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Viral Load of Patients With Hantavirus Pulmonary Syndrome in Argentina

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Hantavirus causes severe illness including pneumonia, which leads to hospitalization and often death. At present, there is no specific treatment available. The hantavirus pathogenesis is not well understood, but most likely both virus-mediated and host-mediated mechanisms, are involved. The aim of this study was to correlate viral load in samples of hantavirus pulmonary syndrome cases and hantavirus infected individuals, with clinical epidemiological parameters and disease outcome. The variables that could potentially be related with viral load were analyzed. The retrospective study included 73 cases or household contacts, with different clinical evolution. Viral load was measured by reverse-transcription and real time polymerase chain reaction. There was no statistically significant association between blood viral RNA levels and severity of disease. However, viral load was inversely correlated with IgG response in a statistically significant manner. The level of viral RNA was significantly higher in patients infected with Andes virus South lineage, and was markedly low in persons infected with Laguna Negra virus. These results suggest that the infecting viral genotype is associated with disease severity, and that high viral load is associated with a low specific IgG response. Sex, age and disease severity were not related with viral load. Further investigations increasing strikingly the number of cases and also limiting the variables to be studied are necessary. ***J. Med. Virol. 9999: XX–XX, 2015.*** © 2015 Wiley Periodicals, Inc.

KEY WORDS: hantavirus; RNA load; Argentina; severity; Andes virus; pediatrics; Zoonosis; infectious disease

INTRODUCTION

Hantaviruses, belonging to the *Bunyaviridae* family, are small, enveloped viruses with a genome that consists of three single-stranded RNA segments. Most commonly, hantaviruses are transmitted to humans via aerosols of infectious excreta and secretions from chronically infected wild rodents.

In Americas, hantavirus causes hantavirus pulmonary syndrome (HPS), an acute sudden disease characterized by fever, severe myalgia and the rapid onset of respiratory distress and often, progressive non cardiogenic pulmonary edema, thrombocytopenia, hypotension, and cardiogenic shock [Duchin et al., 1994; Macneil et al., 2011b]. In Argentina, infections are caused mainly by Andes virus (ANDV) and disease severity is highly variable, with case fatality rates around 30%. In addition to rodent transmission, ANDV is the only hantavirus reported that can be transmitted from person-to-person [Padula et al., 1998; Martinez et al., 2005; Ferres et al., 2007; Martinez-Valdebenito et al., 2014].

Previous studies of viremia in HPS and hemorrhagic fever with renal syndrome (HFRS) suggested an association between initial high viral load of Dobrava (DOBV) [Saksida et al., 2008], Sin Nombre (SNV) [Terajima et al., 1999] and Puumala (PUUV) [Pettersson et al., 2014] viruses, and a severe clinical outcome. Sex-related differences in susceptibility to virus infection and disease outcome have been

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proposed but not extensively studied [Martinez et al., 2010; Klein et al., 2011; Brundin et al., 2012].

HPS has been described in pediatric patients [Pini et al., 1998; Ferres and Vial, 2004; Martinez et al., 2010] with similar presentation and outcome to that described for adult HPS cases [Ramos et al., 2001]. SNV infection in children and adolescents causes HPS with a clinical course and mortality rate similar to that described in adults. For PUUV infections, a systematic literature review showed that the frequency of benign symptoms and transient physical signs were quite similar in children and adults. However severe complications were reported only in adult patients [Huttunen et al., 2011]. In Argentina, hantavirus affects children in a high proportion [Martinez et al., 2010].

Several reports have been published, describing HPS cases in 3 regions of Argentina. Laguna Negra virus (LNV), Juquitiba genotype and ANDV-Oran, ANDV-Bermejo lineages, were reported in the North region; in the Centre ANDV-BsAs, ANDV-Lechiguanas, and ANDV-Plata were found. Only ANDV-South lineage was reported in South region [Levis et al., 1998; Padula et al., 2000a].

There are conflicting studies regarding hantavirus immune-mediated pathogenesis; and studies suggesting virus endothelial cell interaction contributing to vascular leak [Schonrich et al., 2008; Rang, 2010; Terajima and Ennis, 2011]. Hantavirus pathogenesis is not purely a function of organ-specific endothelial cell targeting by HPS- or HFRS-causing hantaviruses. Antigen level, host and virus genetic factors, which exert their effects prior to full development of specific immune responses, can all be critical determinants for the immediate rate of virus dissemination and establishment of infection [Mackow et al., 2014]. An innate immunity-regulating virulence determinant is uniquely encoded by the Andes virus nucleocapsid protein. However, virulence determinants that distinguish ANDV from other pathogenic hantaviruses have yet to be defined [Cimica et al., 2014]

Given the importance of predicting HPS disease severity and risk for death, the aim of this study was to measure viral load in HPS patients and its correlation with disease outcome and other clinical and epidemiological parameters.

MATERIALS AND METHODS

Samples

For viral load evaluation, 73 of 958 confirmed cases were selected considering different sex, age and occurred in different geographical regions from Argentina. The 73 blood samples of suspected HPS patients were collected at the time of hospitalization by different health care centers. The case samples were received in the laboratory and were de-identified according to legal requirements.

Days elapsed between onset of prodromal symptoms and sample collection day were defined as "disease days." Immunoglobulin responses were analyzed by enzyme-linked immunosorbent assay (ELISA) [Padula et al., 2000b], against nucleocapsid antigen. Three groups according to optical density of the reaction, at 1:400 dilution of each serum sample were established: 0–1 (low); 1.1–2.6 (medium), and more than 2.7 (high).

Cases were classified into severe and non-severe categories. Patients who met all the following signs and symptoms were considered to have a severe illness: pulmonary failure, radiologically verified pulmonary infiltrate, hemodynamic compromise, acute thrombocytopenia, treated in intensive care unit requiring mechanical ventilation and also involving other organs. Those patients with lower respiratory compromise associated with myalgia, fever, and headache were considered not-severe cases.

RNA Amplification, Nucleotide Sequencing, and Real-Time RT-PCR Assay

Total RNA was extracted as described [Padula et al., 2004]. Partial S and M-segment were amplified by reverse transcription (RT) and polymerase chain reaction (PCR) (Omniscript, QIAGEN Mississauga, Canada; Platinum® Taq DNA Polymerase, Life Technologies, 5791 Van Allen Way, Carlsbad, CA, respectively) followed by a nested PCR (RT-nPCR) and sequenced (BigDye® Terminator v3.1, Applied Biosystems, Foster City[Q5]).

RT followed by a quantitative PCR (RT-qPCR), using primers for S-segment was performed as described previously [Safronetz et al., 2009]. Two microliters of each RNA sample were amplified in duplicated assays (iCycler, Bio-Rad[Q6]) using TaqMan RT-PCR master mix (Quanta Biosciences, Gaithersburg, MD), according to manufacturer's instructions. Primer set designed to detect human GAPDH [McElroy et al., 2002] was used to ensure that samples did not contain PCR inhibitors and the RNA extractions were homogeneous.

The standard curve was constructed using in vitro-transcribed (Ribomax, Promega) RNA obtained from a cloned S-segment DNA fragment [Padula et al., 2000b]. In vitro-transcribed RNA treated with RQ1 Rnase-Free Dnase (Ribomax, Promega Corporation, Madison, WI) was purified by precipitation. Ten-fold serial dilution was tested in duplicate to obtain a standard curve according to standard RNA mass measured spectrophotometrically.

Viral load was calculated as initial copy number per mL of sample.

Statistical Analysis

Statistical analysis was performed in Graphpad Prism 5 (URL http://www.graphpad.com/prism/) or Infostat (URL http://www.infostat.com.ar) software. Principal component analysis (PCA) was used for interpretation of the complex semi-quantitative data and allowed by bi-dimensional statistics tests for the

variables of interest. Correlation between severity of disease and $\log_{10}$ transformed viral load values was assessed with a general linear model of repeated measurement data.

The χ^2 test was used to determine the relation of the categorical data. The Mann–Whitney, Wilcoxon or Student's test, were used to examine the relationship between the continuous variables. P value ≤ 0.05 was considered significant. Percentages were based on the total number of cases with available information for each tested variable.

RESULTS

Potential Predictors for Disease Severity

To explore clinical and epidemiological characteristics of HPS related to viral load, data on individual cases were collected from medical records (Table I). Samples were tested for IgM and IgG antibodies by ELISA, allowing confirmation of 69 clinical suspected HPS cases and four household contacts.

PCA may be used to reduce the dimensions of multivariate data, creating a smaller number of uncorrelated synthetic factors (components), and also to explore the relationship between variables. To evaluate factors associated with HPS severity, the relationship with viral load was examined. A PCA analysis was performed over 9 parameters: viral load, outcome, disease severity, IgM and IgG responses, disease days, sex, age, and region (Fig. 1). Each resulting component was an association of variables that best encompasses the variation found in the whole data set. First principal component (PC1) could distinguish fatal from non-fatal cases. Second principal component (PC2) displayed large loadings for items: "viral load," "disease days," and "region." Taking into account that "region" is closely related to the viral genotype, PC2 may be interpreted as a measure of virus-related factors. The relationship, suggested by PCA, between viral load and the other 8 parameters were evaluated using univariate statistics.

Viral Load Testing

From 73 RNA samples, 64 (88%) had RNA quantities above the cut-off value for RT-qPCR sensitivity. The correlation coefficient of the calibration curve was 0.99 and the limit of detection was 520 copies/ml. The intra-assay variability ranged from 0% to 1.8% and 0% to 5.5% for inter-assay. Samples with results that exceeded that limit were rejected. Primers and probe detected all genetic variants with the same efficiency. Median RNA copy number was 2.7×10^6 ranging from 2.8×10^3 to 7.1×10^7 copies/ml ($n = 64$). Viral load showed stability during the early days of the disease, and declined after days 3–6 (Fig. 2).

To rule out RNA extraction bias, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA copies were quantified in parallel in the same extracts. GAPDH mRNA quantitites did not differ between cases, suggesting high integrity and quality of the RNA.

Viral Load in Severe and Non-Severe Cases

To assess whether there was an association between viral load and disease severity or outcome, the 73 RNA samples were studied. Four of them were obtained from different household contacts of index cases. Contact samples had IgM and IgG antibodies, but did not report symptoms. Moreover, viral genomes were amplified and LNV was identified in 3 out of 4 samples by sequencing. None of these three samples tested were positive for RT- qPCR, presumably due to low viral load. The fourth sample was characterized as ANDV-South and was RT-qPCR positive (2.4×10^7copies/ml).

Of the 69 symptomatic infected individuals, 30 were considered non-severe and 35 severe cases. For the remaining four cases, medical records were not enough to determine severity and were considered indeterminate.

For non-severe and severe cases, mean viral load values were 8.6×10^6 and 5.7×10^6 copies/ml respectively (Fig. 3). Statistical analysis showed no significant differences between severe and non-severe or between fatal and non-fatal cases (Mann Whitney-U Test $P = 0.76$ and $P = 0.73$, respectively). This result was not significant even when the 9 samples that were positive only by RT-nPCR (and not by RT-qPCR) were considered (χ^2 test; $P = 0.21$ and $P = 0.08$, respectively).

Taking into account the existence of regional ANDV lineages or even different viruses across the country, selected RNA samples of each geographical region were amplified by RT-nPCR and sequenced. The alignments with published sequences allowed us to indentify: ANDV-South, ANDV-Oran, ANDV-BsAs, ANDV-Lechiguanas, and LNV. Compared with all other ANDV lineages, ANDV-South samples showed the highest viral load values ($P = 0.03$; $n = 64$). Five LNV samples were not detected by RT-qPCR but they were RT-nPCR positive. The comparison between ANDV-South and LNV samples showed significant differences ($P = 0.0009$; $n = 28$) in viral load (Fig. 4).

Viral Load According to Sex and Age

From 958 Argentine HPS reported cases, 750 men were distinguished (78%). Death rate was significantly higher in women (51% vs. 38%; $P = 0.001$; nfemales = 146; nmales = 436). This difference was given especially by North region, where the lethality was 49% and 30% for women and men respectively ($P = 0.02$; nmales = 208 nfemales = 46). However viral load between sexes, had no significant differences ($P = 0.15$; $n = 64$) neither for cases across the country nor for patients of the north region ($P = 0.75$; $n = 18$).

TABLE I. Viral Load of Patients With HPS in[Q7] Argentina

Dis. Days	Region	Sex/age, y	Outcome/severity	Viral load (copies/ml)	Dis. Days	Region	Sex/age, y	Outcome/severity	Viral load (copies/ml)
as.	South	F/3	as./–	2.4×10^7	5	North	F/10	non-fatal/mod	2.7×10^6
1	North	F/9	fatal/SE	4.7×10^5	5	Centre	M/26	non-fatal/SE	1×10^7
1	Centre	F/0.3	non-fatal/NA	1.6×10^5	6	South	M/10	non-fatal/mod	4.5×10^6
2	Centre	M/21	non-fatal/mod	1.2×10^7	6	North	M/62	non-fatal/mod	No detected
2	Centre	M/38	fatal/SE	6.1×10^6	6	South	F/44	non-fatal/mod	2.1×10^4
2	South	M/26	NA/mod	8.4×10^5	6	South	F/53	fatal/SE	7.3×10^5
2	South	M/52	NA/mod	3.4×10^7	6	South	M/14	non-fatal/mod	9×10^6
2	North	M/9	fatal/SE	4.2×10^6	6	Centre	F/6	NA/SE	No detected
2	North	F/54	non-fatal/mod	No detected	6	South	F/27	NA/SE	1.6×10^6
2	North	M/12	non-fatal/mod	4.7×10^5	6	South	M/22	fatal/SE	1.1×10^7
3	North	M/33	non-fatal/mod	4.2×10^6	6	South	F/64	non-fatal/mod	9.5×10^5
3	North	F/38	non-fatal/SE	2.8×10^3	7	Centre	M/9	fatal/SE	1×10^7
3	South	F/14	non-fatal/mod	1.7×10^6	7	South	M/55	fatal/SE	1.3×10^5
3	South	F/41	fatal/SE	1.3×10^7	7	North	F/30	NA/SE	1.5×10^5
3	Centre	M/22	fatal/SE	No detected	7	North	M/11	NA/SE	3.9×10^6
3	Centre	F/22	non-fatal/SE	2.5×10^5	7	North	M/2	fatal/SE	2.7×10^5
3	Centre	F/21	non-fatal/mod	1.6×10^5	7	South	M/37	fatal/SE	3.5×10^5
3	Centre	F/22	NA/SE	8.2×10^6	7	Centre	M/42	NA/ SE	3.9×10^6
4	Centre	F/23	fatal/SE	1.1×10^5	7	South	F/33	non-fatal/SE	5.4×10^7
4	North	M/24	fatal/SE	2.7×10^6	7	Centre	M/27	fatal/SE	8.2×10^6
4	South	M/13	non-fatal/SE	4.7×10^6	7	North	M/NA	fatal/mod	1.7×10^6
4	South	M/40	fatal/SE	4×10^5	8	South	F/64	non-fatal/mod	2.5×10^7
4	North	F/25	fatal/SE	2×10^7	8	North	F/18	non-fatal/SE	8.6×10^4
4	Centre	F/29	NA/mod	1.7×10^4	8	Centre	M/28	non-fatal/mod	8.2×10^6
4	South	F/70	fatal/SE	2×10^7	9	North	M/23	NA/SE	8.6×10^4
4	North	F/16	NA/mod	1.3×10^7	10	North	M/9	NA/NA	1.3×10^4
4	Centre	F/2	NA/mod	1.9×10^6	11	South	M/58	non-fatal/mod	7.1×10^7
4	Centre	M/25	non-fata/mod	1.7×10^5	>11	Centre	M/31	NA/mod	5.9×10^6
5	North	M/15	non-fatal/mod	No detected.	>11	Centre	F/50	NA/mod	1.3×10^7
5	South	M/22	non-fatal/mod	9.4×10^6	NA	Centre	M/68	fatal/SE	8.2×10^6
5	Centre	M/43	non-fatal/mod	3.7×10^6	as.	North	M/19	as./–	No detected
5	Centre	F/8	non-fatal/mod	7.4×10^6	as.	North	M/46	as./–	No detected
5	South	M/18	non-fatal/ND	2.4×10^7	as.	North	F/78	as./–	No detected
5	Centre	M/14	fatal/SE	4.7×10^5	NA	North	M/46	NA/NA	1.3×10^6
5	Centre	F/28	NA/mod	9.3×10^5	NA	South	M/13	NA/SE	No detected
5	North	M/14	fatal/SE	2.9×10^5	NA	Centre	M/54	NA/mod	2.1×10^5
					NA	North	F/50	fatal/SE	7.2×10^6

ND, not determined, not detected, not detectable by RT-qPCR; NA, not available; SE, severe case; mod, moderate case; as, asymptomatic, without symptoms of HPS.
North: Salta, Jujuy provinces; South: Río Negro, Chubut, Neuquén provinces and Centre: Buenos Aires and Entre Ríos provinces. Dis. days: days between onset of symptoms and sample collection. Viral load: copies/mL of sample.

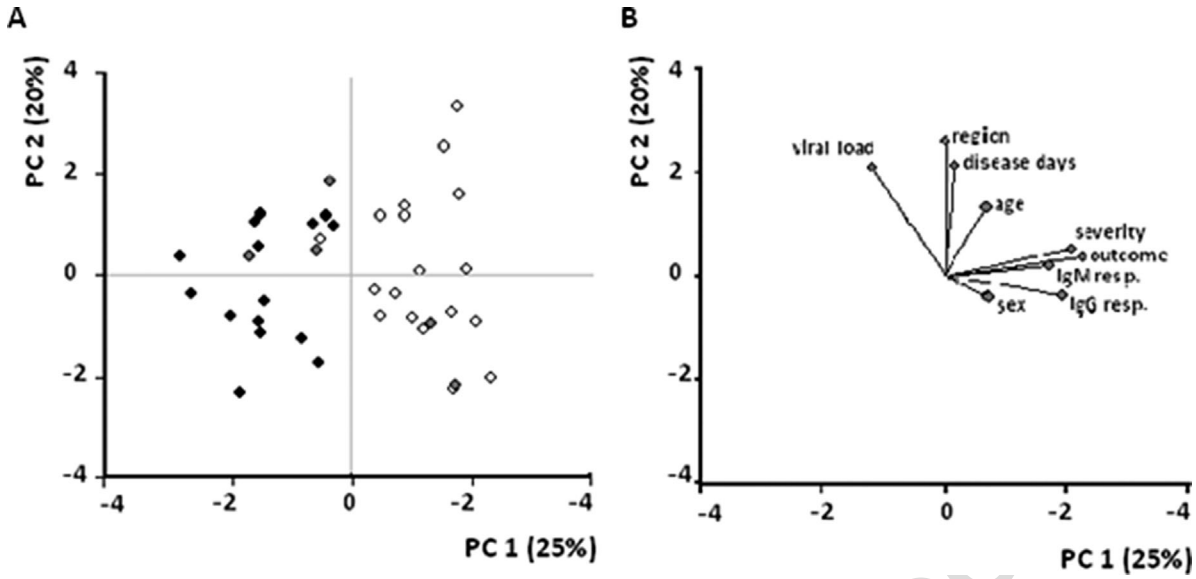

Fig. 1. Principal components analysis of: 8 clinical and epidemiological parameters, disease outcome and viral load. The ordinate and abscissa represent the first and second principal components (PC) from the PCA, which explain 45% of the variance. Each dot represents an HPS case, black: severe and fatal; gray: severe and non-fatal and white: moderate and non-fatal. PCA scatter plot was obtained using INFOSTAT software. Only cases with information about the nine variables were evaluated (n = 38).

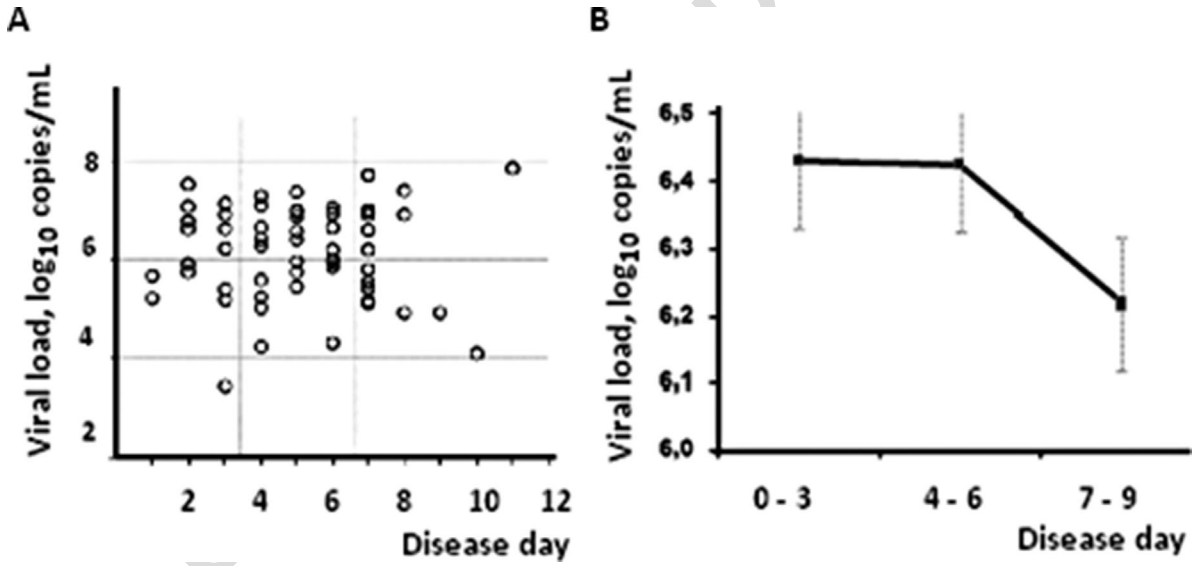

Fig. 2. Correlation between viral load and disease days. **A**: Case viral load by disease day. **B**: Median of grouped days. Viral S-segment RNA levels are expressed as log copies/ml of sample.

Of 958 total HPS cases, 110 (11%) were pediatrics; among them 41 were girls (37%). Ages ranged from 4 months to 14 years old (median: 9 years). Child death was 26% (n = 29). Similar to adults, children had sex differences in mortality (girls 44%; boys 31%), but it was not statistically significant (n = 80; $P = 0.34$). Children under 11 had no significant differences in mortality according to sex. However for the 11–14 age range, there was a significant inequality (n = 35; $P = 0.01$) as for adult cases (Table II).

The mean viral load values for adults and children were 7.8×10^6 copies/ml (range: 2.8×10^3–7.1×10^7; n = 45) and 4.3×10^6, (range: 1.3×10^4–2.4×10^7; n = 18) respectively, without significant differences between groups (n = 63; $P = 0.51$). Mean viral load values were similar for both sexes (girls = 5.5×10^6 ; boys = 3.5×10^6; $P = 0.98$).

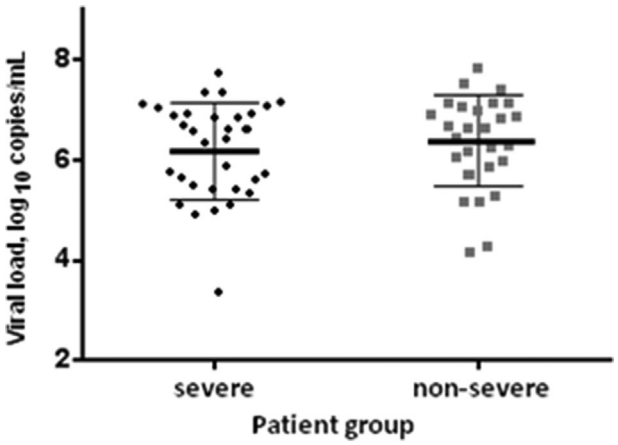

Fig. 3. Comparison of viral load between patients with severe or not-severe course of disease. Viral S-segment RNA levels are expressed as log copies/ml of sample.

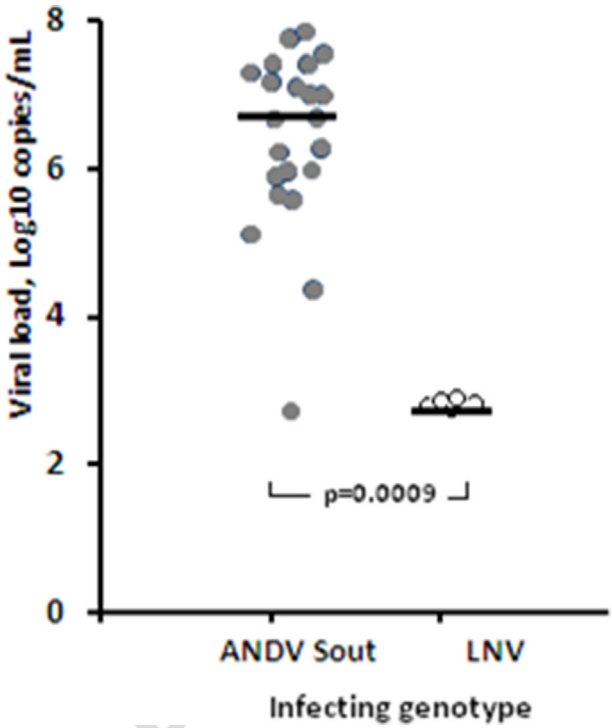

Fig. 4. Comparison of viral load between patients infected with ANDV South or with LNV. Viral S-segment RNA levels are expressed as log copies/mL of sample. Viral load of the undetectable samples was recorded as the mean value between the detection limit (520 copies/ml) and zero.

Association Between Aantibody Response and Viral Load

At diagnostic day, the 69 patients and four contacts had anti-ANDV IgM and for 70 of them IgG antibodies were also detected. There was a statistically significant inverse association between IgG antibodies and viral load ($P = 0.01$; $n = 66$). No such association could be seen for IgM ($P > 0.16$; $n = 68$) (Fig. 5).

There was a correlation between antibody IgG responses and the clinical course of the disease in the studied patients (Fig. 6).

DISCUSSION

In a comprehensive analysis during the acute phase of HPS cases, the blood ANDV RNA load revealed to have significant differences when assessed in cases with different IgG response or hantavirus genotype, but not with disease severity, disease days, sex or age.

Previously have been reported mortality differences between men and women HPS patients [Martinez et al., 2010], however in these results, the viral load would have no influence on the differences. (para cambiar el anterior x este)

However it has been reported that hormonal factors could exert influence on the case outcome [Klein et al., 2011].

The differences in mortality by sex were also observed for prepubertal children (11–14 years). However, children under 11 had no significant differences in mortality according to sex (Table II), contributing to the idea that the hormonal or biochemical differences related to sex may play a role in the pathophysiology of HPS [Klein et al., 2011].

Asymptomatic infected individuals with IgM, IgG, and RT-nPCR positive were detected, similar to those reported in Brazil [de Borba et al., 2013], Panamá [Armien et al., 2013] and northwestern Argentina [Levis et al., 2004]; suggesting an underestimation of hantavirus infection.

In this study, clinical samples from HPS cases across the country were included to cover the wide viral genetic variability. This is the first analysis of viral load between different co-circulating hantavirus

TABLE II. Outcome of Pediatric HPS Cases by Age Group

	0–14		11–14		0–11	
Years old	Non-fatal (%)	Fatal	Nnon-fatal (%)	Ffatal	Non-fatal (%)	Fatal
Girls	56	44	62	38	45	54
Boys	69	31	50	50	88	12
Fisheŕs exact test/n	0.34/80		0.55/45		0.01/35	

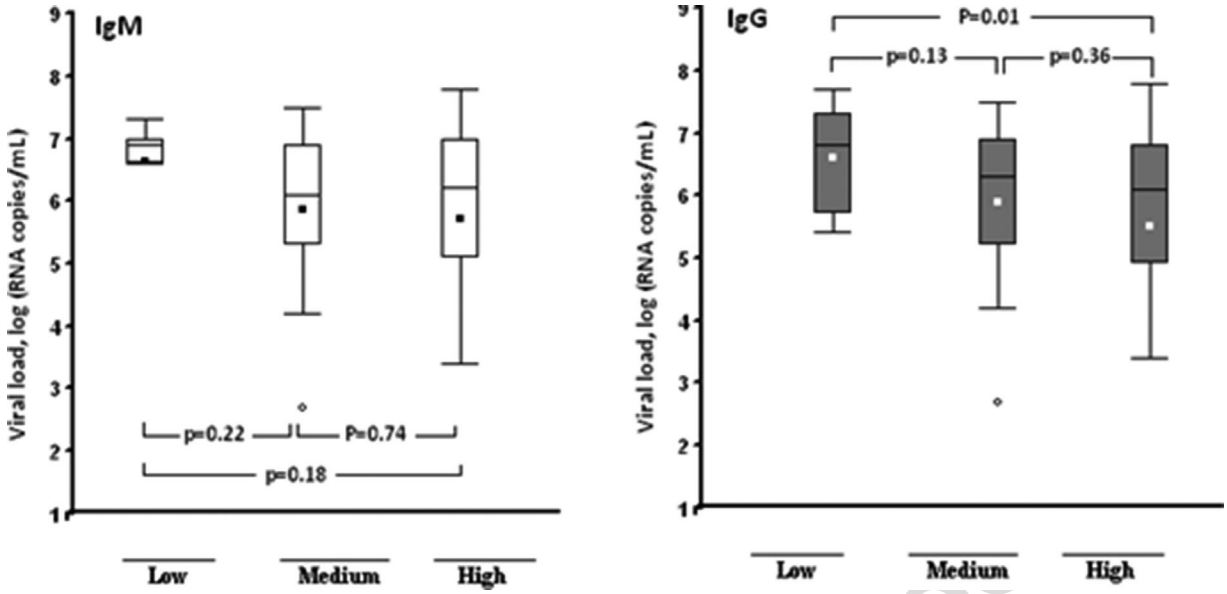

Fig. 5. Comparison of ANDV viral load by IgM or IgG titer groups.

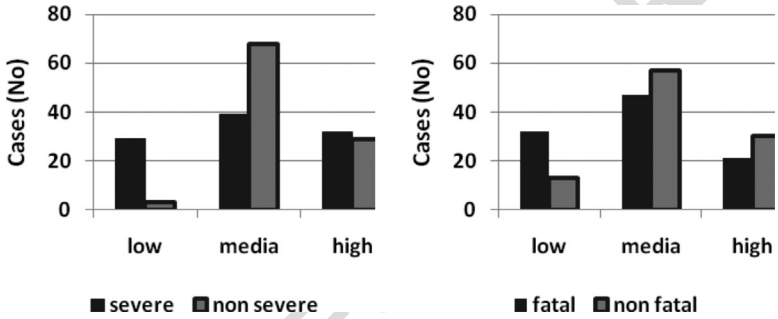

Fig. 6. Relationship between ANDV load and disease severity of HPS. Comparison of viral load between the severe/ non-severe group and the fatal/ non fatal group.

lineages or virus, in Argentina. Patients infected with ANDV-South had significant higher levels of viral load; on the contrary, LNV cases or infected contacts had viral quantities only detectable by a more sensitive technique like RT-nPCR. High level in viral load of ANDV-South samples could be related to the person-to-person transmission. Highly variable viral load values were observed among cases, suggesting that host–virus interaction could be a factor in HPS pathogenesis as was proposed previously [Dalrymple and Mackow, 2014; Guichard et al., 2014; Hepojoki et al., 2014; Baigildina et al., 2015].

Only one asymptomatic HPS contact infected with AND-South was reported. High viral load for this sample could be due to infecting genotype but also because the sample was taken immediately after her mother was confirmed as an acute HPS case (the case could be detected for being the daughter of an acute case of HPS).

The results show that a low IgG response in the acute HPS samples was significantly associated with high viral load, similar to those reported for DOBV virus [Saksida et al., 2008; Korva et al., 2013].

A correlation between high levels of viremia and severity of the disease has been described in previous studies for SNV Virus [Xiao et al., 2006] and DOBV virus [Saksida et al., 2008]. A recent report [Pettersson et al., 2014] asserts that there was a tendency for a higher PUUV RNA load in patients with non-severe/ severe illness during the first 0–3 days. There were no significative differences between non-severe/ severe cases or between fatal/ non-fatal cases analyzed in the present study. However, it is important to consider that the analyzed cases had a wide range (0–11) of days between onset of symptoms and sample collection.

Disease severity or high mortality were previously associated with low IgG response [Bharadwaj et al., 2000; Padula et al., 2000a; MacNeil et al., 2011a] and ANDV-South lineage infection [Tager Frey et al., 2003; Martinez et al., 2010]. Interesting in the present report, ANDV-South lineage and low IgG response were associated with high viral load.

Since HPS is a relatively rare disease but it is among the most pathogenic of human viral infections, more efforts will be necessary to recognize and identify risk factors for severe outcomes.

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