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Ruobing Xiao

Brian Hjelle

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Sin Nombre Viral RNA Load in Patients with the Hantavirus

Cardiopulmonary Syndrome

Ruobing Xiao[1] and Brian Hjelle [2,3,4]

[1]Medical student, Class of 2008, University of New Mexico,

School of Medicine, Albuquerque, NM

Departments of [2]Pathology, [3]Biology and [4]Molecular Genetics and Microbiology,

University of New Mexico, School of Medicine, Albuquerque, NM.

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Abstract

To address the role of viral load in pathogenesis in patients with hantavirus cardiopulmonary syndrome (HCPS), we quantified Sin Nombre virus S segment viral RNA in plasma samples from 27 acutely-ill patients. For six patients we examined viral load in matched plasma, urine, and/or tracheal aspirate throughout the time when patients were in intensive care. Peak titers in plasma reached 1.8×10^6 copies/ml; none had viral RNA in urine. Titers in tracheal aspirates did not exceed 8×10^4/ml. We found a statistically significant association ($P < 0.005$) between plasma viral RNA levels at admission and the severity of disease. Of those with plasma viral RNA titers above the threshold for assay sensitivity (5000 copies/ml), those with mild-moderate and severe disease had an average of 27,800 and 438,545 copies/ml, respectively. These results suggest that patients with high viral loads on admission are more likely to have severe disease.

INTRODUCTION

Hantavirus (*Bunyaviridas, Hantavirus*) are rodent-borne viruses with a worldwide distribution. Hantavirus cardiopulmonary syndrome (HCPS) is the human illness associated with hantavirus infection in North and South America [1] [2]. HCPS has been reported by at least nine nations from southern Patagonia to Canada. About 500 cases have occurred in North America and several hundred cases each have been reported from Argentina, Brazil and Chile since HCPS was first recognized in 1993. While some strains of virus appear to be more virulent than others, the overall case fatality ratio for HCPS is approximately 35-45 percent. Among the more virulent of the etiologic agents are Sin Nombre virus (SNV) of the US and Canada and the "southern" or prototypical form of Andes virus, which occurs in Chile and Argentina.

The pathogenesis of HCPS, like that of many other viral hemorrhagic fevers, is little understood. The core abnormalities that define the pathogenesis generally include capillary leak syndrome that selectively involves the pulmonary bed, noncardiogenic pulmonary edema, thrombocytopenia, hypotension and/or cardiogenic shock. The long incubation period for illness, the generally well-advanced adaptive immune response at the time of onset of disease, and the apparent absence of direct lytic damage to vascular endothelium are among the findings that have been taken as evidence that HCPS pathogenesis is largely immune-mediated [3-6]. However, some workers have begun to challenge this assessment, citing, for example, the ability of hantaviruses to directly establish potentially maladaptive signaling cascades and to cause directly pathogenic

effects on endothelial cells, such as impairing their ability to migrate across substrata, at least in vitro [7-11].

A key question that would help to establish a potential role for antiviral intervention in therapy is whether viral load during acute illness can be associated with adverse clinical outcome. A study that attempted to determine whether such an association existed was able to correlate high viral RNA titers only with surrogate markers of severity such as the severity of thrombocytopenia, but its power may have been too limited to establish whether viral RNA titer is directly correlated with adverse clinical outcome [4]. Another study that used a blinded study design showed that high levels of neutralizing antibodies was associated with a favorable clinical progression in patients with HCPS [12].

The advent of sensitive real time RT-PCR assays for SNV RNA quantitation prompted us to reinvestigate the question of whether SNV RNA titer can be associated with adverse clinical outcome in patients in the acute stages of SNV infection [13,14]. Using a larger panel of plasma samples than has previously been available, we have determined that there does appear to be an association between the titer of SNV RNA and disease severity in patients in the early stages of HCPS, and that viral RNA is of low abundance or absent in tracheal aspirates and urine respectively.

MATERIALS AND METHODS

Study subjects. Case-patients with SNV infection were treated at the University of New Mexico Hospital. Informed consent was obtained from patients or their parents or guardians, and human experimentation guidelines of the US Department of Health and Human Services and the University of New Mexico Human Research Review Committee were followed in the conduct of this research.

Patients were considered to have acute SNV infection on the basis of clinical considerations along with the following serological criteria: the presence of IgM-antibodies directed against the SNV N antigen and the presence of IgG antibodies against the viral G1 antigen. The IgG response to the G1 antigen is highly specific for SNV infection, enabling us to exclude patients who could potentially have been infected with hantaviruses other than SNV [15,16].

Twenty-seven patients with acute SNV infection were enrolled. Patients were categorized as having severe disease (pulmonary failure and hemodynamic compromise requiring mechanical ventilation and/or extracorporeal membrane oxygenation (ECMO)) or mild-moderate disease (no cardiopulmonary failure and did not require mechanical ventilation).

Collection and preparation of specimens. We collected plasma, tracheal aspirate and urine samples from patients with acute SNV infection at University of New Mexico Hospital. A 10-ml specimen of EDTA- anticoagulant blood was processed with Sigma Diagnostics Histopaque-1077 kit to separate plasma and peripheral blood

mononuclear cells. All samples were split into 1-ml aliquots and frozen at -80º C within 3
hours of collection.

Nested reverse transcription (RT)-PCR. We extracted RNA from body fluids
(plasma, tracheal aspirate and urine) using the Qiamp Viral RNA Mini Kit (Qiagen,
Valencia, Calif.). A 280 µl aliquot of body fluid was used for RNA extraction and the
RNA was suspended in a volume of 70 µl. Total RNA derived from 40-µl aliquots of
plasma, tracheal aspirate and urine was loaded in each PCR reaction. We carried out RT-
PCR with nested primers for the small (S) genomic segment of SNV as described by
Botten et al [13]. The coordinates of the outer primers were positions 167 and 423 on the
SNV S segment, with inner primers at 190 and 401.

Quantitative TaqMan RT-PCR. We used a Perkin-Elmer Applied Biosystems
(Foster City, Calif.) 7000 sequence detection system for triplicate RT-PCR assays. RT
was conducted with RNA derived from 20 µl of plasma or tracheal aspirate using an S
segment primer at coordinate 167. Five microliters of cDNA was removed for real-time
quantitative PCR using primers from positions 179 and 245 of the SNV S segment [14].
PCR was conducted at 95º for 10 min, followed by 40 repetitions of 95º for 10s, 50º for
10s, then 72º for 30s. In our experiments, a standard curve containing dilutions ranging
from 5 to 5×10^5 copies of template was used on each 96-well plate. All of our standard
curves produced a -0.995 or better correlation coefficient between the quantity of
template loaded and the log of the Ct value.

Spiking controls. To ensure the efficiency of our RNA extraction and nested RT-PCR processes, we spiked negative plasma and negative urine samples with supernatants of infected Vero E6 cells containing 2, 10, 100, or 1,000 copies of total S segment RNA. For experimental "spiking" of plasma or urine samples, we used particles from SNV strain SN77734 that were propagated in Vero E6 cells to most closely mimic the form of viral particles that we expected could be present in the samples from infected patients (R. Xiao, unpublished data). Known amounts of viral RNA-equivalents, as determined by TaqMan RT-PCR were added into negative-control plasma or urine samples in a volume of 200 µl before we prepared RNA. We suspended the resulting RNA into a volume of 50 µl. Total RNA derived from 40 µl of the virally-spiked samples were loaded in each RT-PCR reaction as described above.

Statistical analysis. Number of RNA copies was summarized with means and standard deviations for triplicate assays. Viral RNA copies were compared between patients with mild-moderate and severe diseases using the exact version of the Wilcoxon rank sum test. P-values of < 0.05 were considered statistically significant.

RESULTS

Nested RT-PCR. Body fluids were collected at University of New Mexico Hospital from six patients who were admitted with acute SNV infection between October 1999 and July 2000. For each patient with HCPS, we collected plasma, tracheal aspirate and urine on the day of hospital admission (day zero of hospitalization) and daily

thereafter, until the patient left the hospital's intensive care unit. Matched specimens from the six patients (patient I, II, III, IV, V and VI), including 38 plasma, 22 tracheal aspirates, and 38 urine samples, were examined for the presence of Sin Nombre (SN) viral RNA by nested RT-PCR (Table 1).

All six patients exhibited RNA viremia (defined here as the presence of viral RNA, not infectious virus) in plasma samples. We found that viral RNA was present in plasma samples from all six patients during at least the first four days of hospitalization. Patient VI, who was in the intensive care unit for thirteen days and was the only patient monitored after d5, was positive for SNV RNA from d0 to d7 in her plasma samples, with no detectable viral RNA in her plasma samples on subsequent days (Table 1).

A 5-ml sample of tracheal aspirate was taken daily through the endotracheal tube from the patients who required mechanical ventilation during hospitalization. Tracheal aspirate samples from the four patients on mechanical ventilation were screened for viral RNA by nested RT-PCR (Table 1). We were able to screen nine tracheal aspirate samples from d0 to d10 while patient VI was in the intensive care unit. We found that her tracheal aspirate samples were positive for viral RNA from d0 to d4, and there was no detectable viral RNA in her tracheal aspirate starting at d5 (Table 1). We also examined three tracheal aspirate samples collected within the first 48 hours after admission from another three patients whose plasma samples exhibited detectable viral RNA. Of the three samples examined by nested RT-PCR, all three tracheal aspirates displayed viral RNA (data not shown).

To examine the sensitivity of our nested RT-PCR, we spiked human plasma and urine samples from healthy individuals with known quantities of cell-free virus particles.

With nested RT-PCR, our sensitivity thresholds were no more than 2 copies of viral RNA from healthy human plasma and urine samples (Figure 1).

A panel of 38 urine samples from the six patients was examined for viral RNA by nested PCR. We found that there was no detectable viral RNA present in any of the urine samples. Attempts to produce amplification products from centrifuged urinary sediment were also unsuccessful (N=3).

Quantitative RT-PCR. To examine the changes of viral RNA load as a function of time in a patient during the acute phase of disease, we quantified viral RNA in plasma and tracheal aspirate samples of the six patients with HCPS throughout the entire time course (day zero of hospitalization to at least day 5 in the intensive care unit). Our quantitative RT-PCR system was less sensitive than nested RT-PCR, but allowed us to track viral RNA load at levels of $\geq$ 5,000 copies per ml.

All six patients had viral RNA in plasma, with peak titers ranging from 1.5 x 10^5 to 1.8 x 10^6 per ml, in all cases occurring within the first three days of hospitalization (Table 1) (Figure 2). Four patients had viral RNA in tracheal aspirates with titers up to 8 x 10^4 per ml (Table 1). Peak vRNA titers for tracheal aspirates were also found in the first three days of admission (Figure 2). Starting on day 2 or day 3 after hospital admission, the number of viral RNA copies began to decrease in both plasma and tracheal aspirate samples (Figure 2). We found that the number of viral RNA copies in tracheal aspirate never exceeded the level of viral RNA load in plasma sample from a patient at any time point examined, nor were there any instances in which viral RNA in tracheal aspirate was detectable and undetectable in plasma (Figure 2).

We also screened three tracheal aspirates taken within the first 48 hours after admission from another three patients with HCPS and found that the number of viral RNA copies in each of these specimens did not exceed the titer of 2×10^4 per ml (data not shown).

Viral load in severe and mild-moderate cases. To ascertain whether there is an association between the viral RNA load and the disease severity in patients with HCPS, we studied 27 patients during the acute phase of the disease, including 17 patients with severe disease and 10 patients with mild-moderate disease. Table 2 shows the geographic distribution, age range, sex and clinical characteristics of the 27 patients. Plasma samples were collected within the first 24 h of hospital admission (day zero) for 22 patients and on day one for the remaining 5 patients.

By screening for the presence of negative-strand viral RNA by quantitative RT-PCR, we examined the earliest available plasma samples in the mildly ill patients (class 0 and I), in comparison with those patients with severe illness (class II and III) (Figure 3). Of those whose sample had levels of S segment negative-strand RNA above the cutoff for assay sensitivity of 5000 copies/ml, those with mild-moderate and severe disease had an average of 27,800 and 438,545 copies/ml, respectively. Looking at all patients, we observed that the median number of viral RNA copies was higher for patients with severe disease as compared to those with mild-moderate disease ($p < 0.005$).

DISCUSSION

Our study demonstrates a statistically significant association (P<0.005) between the titer of SNV RNA and the disease severity in patients in the early stages of HCPS. This finding is in contrast to a previous study by Terajima et al in 1999 where it was not possible to show an association between viremia and disease severity, although it was possible to show an association between load and surrogate lab markers for severity [4]. In this study, we studied plasma viral RNA load by quantitative TaqMan RT-PCR, which is a relatively robust and sensitive technique that has been used extensively in rodent SNV infection models with reproducible results [13,14,17]. Samples sizes were too small to be certain that the results of Terajima et al in 2003 are in conflict with those report here, and there were other important methodologic differences [5].

Our findings on the association between viral load in plasma and disease severity indicate that those patients with high viral loads on admission are more likely to have a severe course of disease. Previously we showed that a high-titer neutralizing antibody response early in the course of hospitalization may be a predictor of a non-fatal outcome in SNV infection [12]. It is possible that variations in viral load are related to variations among titers of neutralizing antibody, but additional studies will be needed to further clarify this relationship. The premise that the pathogenesis of HCPS is immune-mediated is widely cited by workers in the field [2]. However, the present study suggests that ongoing in vivo replication of SNV might still be an important determinant of disease

severity. Therefore, level of viral load in patients during the early stages of HCPS might eventually be used to monitor and/or predict the severity of clinical manifestations, which vary from mild disease with stable hemodynamic status and minimal or no oxygen requirement to fulminant progression and death due to cardiogenic shock. Markers indicative of prognosis for patients with HCPS are needed to guide clinicians in deciding whether triage to tertiary care centers or ECMO is appropriate. Currently only current clinical status and the rate of change of clinical-laboratory findings are available as guides. We anticipate the possibility that prompt determination of SNV viral load upon admission may eventually guide clinicians toward more or less aggressive therapy.

Hantaviruses have been very difficult to isolate from human sources despite many past attempts to do so. Using six plasma/tracheal aspirate specimen collected from HCPS patients during the first day of hospitalization, we applied 1-ml of sample, either neat or diluted at 1:5 in media, onto subconfluent Vero E6 cells and blind-passaged the cells for 4-6 wk, after which we screened the culture supernatants for the presence of viral RNA. None was positive.

Our data on SNV load in body fluids demonstrated that SNV or SNV RNA appear to be liberated into tracheal aspirates but not into urine in the patients with plasma viremia. This finding has parallels with studies involving deer mice, where viral RNA could be detected in the rodents' saliva but not in urine [17]. These findings are undertaken in part to allow comparisons between SNV, which is not transmitted nosocomially, and the closely-related Andes virus which has been transmitted in hospitals

by an unknown route [18], and person-to-person transmission of Andes virus likely occurs during the prodromal phase or shortly thereafter [19].

Whether ongoing replication of SNV is an important factor in the pathogenesis of HCPS is not well understood. Using a panel of plasma samples and a real time RT-PCR assay, we have shown an association between the titer of SNV RNA and disease severity in patients in the early stages of HCPS, and that viral RNA is of low abundance or absent in tracheal aspirates and urine respectively. By contrast, preliminary studies with Andes hantavirus infections in Chile suggest that plasma viral loads are too often below the limit of detection of the assay to be useful in prediction of disease severity (M. Ferres, personal communication). In order to place our present findings in context, further studies are needed to determine the mechanisms by which SNV replication is regulated during acute SNV infection, define the cell-mediated immune response, and gain more understanding on how SNV is recognized by both the innate and acquired immune systems.

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ACKNOWLEDGMENTS

Figure Legends:

Figure 1: Viral RNA spiking experiments. Viral supernatants of SN77734-infected cultures that contained known numbers of copies of SNV S segment RNA were added into negative serum samples before extraction of viral RNA. These RNAs were then used in nested RT-PCR reactions.

Figure 2: SN viral RNA titers in plasma and tracheal aspirate samples of six patients with HCPS. Negative-strand viral S segment RNA levels are expressed as copy numbers per milliliter of plasma and tracheal aspirate. Error bars indicate the standard deviation for triplicate. Threshold sensitivity was 5,000 copies/ml. Data is obtained from six patients "patient I – VI". Day of hospitalization is listed on the X-axis. Top panel shows results for plasma samples. Bottom panel shows results for tracheal aspirates. "E" indicates that the time points when the patient was on extracorporeal membrane oxygenation (ECMO). NA, not available.

Figure 3: SN viral RNA titers in plasma samples of patients with severe and mild-moderate infections at hospital admission. Negative-strand viral S segment RNA levels are expressed as copy numbers per milliliter of sample. Error bars indicate the standard deviation for triplicate analyses. Threshold sensitivity was 5,000 copies/ml. Data were obtained from 27 patients within 48 hours of admission.

Table 1. Detection of viral RNA by nested RT-PCR and TaqMan RT-PCR in samples of plasma and tracheal aspirate from six HCPS patients.

Patient[a] ID	Status	Post admission day	Presence of viral RNA by nested RT-PCR / Copies of RNA per ml (Mean ± standard deviation) by TaqMan RT-PCR in indicated specimen[b]	
			Plasma	Tracheal aspirate
I	Severe	d0	+[c] / (151910 ± 24510)	NA[d]
		d1	+ / (39820 ± 6420)	NA
		d2	+ / (34900 ± 1300)	+ / (33310 ± 3302)
		d3	+ / (17860 ± 1500)	+ / (5090 ± 2065)
		d4	+ / ND[e]	-[f] / ND
		d5	+ / ND	- / ND
II	Severe	d0am	+ / (738150 ± 33070)	NA
		d0pm	+ / (324730 ± 40950)	NA
		d1	+ / (441770 ± 34790)	NA
		d2	+ / (112680 ± 4340)	NA
		d3	NA	NA
		d4	+ / (32100 ± 1440)	NA
III	Severe	d0	+ / (374950 ± 14120)	NA
		d1	+ / (303370 ± 11730)	+ / (80430 ± 13116)
		d2	NA	+ / (10960 ± 2798)
		d3	+ / (33140 ± 640)	NA
		d4	+ / (28060 ± 3340)	- / ND

		d5	+ / (13520 ± 400)	- / ND
IV	Severe	d0	+ / (667720 ± 36450)	NA
		d1	+ / (228620 ± 3860)	- / ND
		d2	+ / (274720 ± 6650)	+ / ND
		d3	+ / (243440 ± 28840)	- / ND
		d4	+ / (230280 ± 39020)	- / ND
V	Mild-moderate	d0	+ / (19110 ± 1040)	NA
		d1	+ / (265400 ± 15010)	NA
		d2	+ / (58490 ± 5150)	NA
		d3	+ / (29510 ± 1830)	NA
VI	Severe	d0	+ / (1815920 ± 66010)	+ / ND
		d1	+ / (1623410 ± 7310)	+ / (8740 ± 926)
		d2	+ / (281530 ± 640)	+ / ND
		d3	+ / (71590 ± 10260)	+ / ND
		d4	+ / (17060 ± 2680)	+ / ND
		d5	+ / ND	- / ND
		d6	+ / ND	- / ND
		d7	+ / ND	NA
		d8, d9 and d10	- / ND	- / ND
		d11 and d12	- / ND	NA

<superscript>a</superscript> Age range of patients, 23- 46. Sex ratio M: F, 3: 3.

<superscript>b</superscript> No viral RNA detected in any of the urine samples.

<superscript>c</superscript> Indicates the presence of viral RNA detected by nested RT-PCR.

<superscript>d</superscript> NA, not available.

<superscript>e</superscript> ND, not detectable by TaqMan RT-PCR.

<superscript>f</superscript> Indicates no viral RNA detected by nested RT-PCR.

Table 2. Demographic and clinical characteristics of 27 patients with acute SNV infection.

State of infection: number of patients	Severity [a]: number of patients	Date of collection : number of samples	Age (year)	Sex: Number of male (M) and female (F) patients
NM: 18 AZ: 5 CO: 3 KS: 1	Class 0: 1 Class I: 9 Class II: 9 Class III: 8	Day 0: 22 Day 1 : 5	Range: 11 – 63 Mean: 39	M: 11 F: 16

[a] Severity scale: Class 0, no cardiopulmonary manifestations; Class I: pulmonary edema not requiring endotracheal intubation; Class II: survived, but required endotracheal intubation; Class III: died. For the purpose of this study, patients in classes 0 and I were considered mild-moderate cases, and those in classes II and III were considered severe cases.
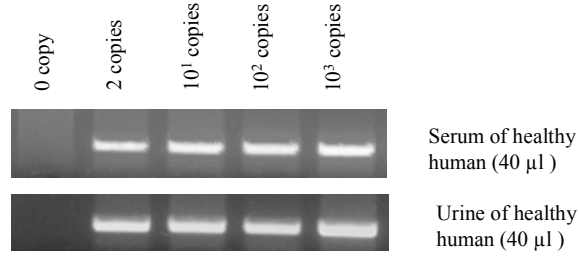
Figure 1.

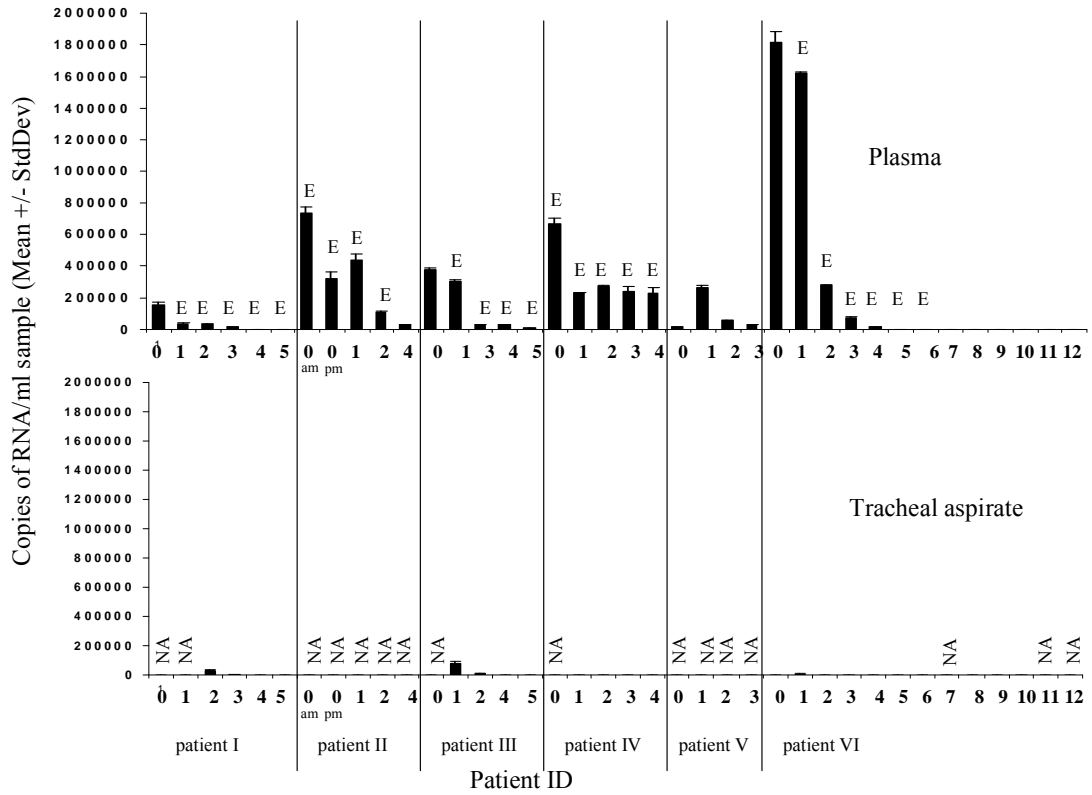|				
0 copy	2 copies	10^1 copies	10^2 copies	10^3 copies

Serum of healthy
human (40 µl)

Urine of healthy
human (40 µl)

Figure 2.

Plasma

Tracheal aspirate

Copies of RNA/ml sample (Mean +/- StdDev)

Patient ID

patient I   patient II   patient III   patient IV   patient V   patient VI

Figure 3.

X-axis: Number of plasma/serum at admission
Y-axis: Copies of RNA /ml sample (Mean +/- Std.Dev.)
Labels: Severe cases | Mild cases