MAJOR ARTICLE

Hemorrhagic Fever Caused by a Novel Bunyavirus in China: Pathogenesis and Correlates of Fatal Outcome

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Background. Hemorrhagic fever–like illness caused by a novel Bunyavirus, Huaiyangshan virus (HYSV, also known as Severe Fever with Thrombocytopenia virus [SFTSV] and Fever, Thrombocytopenia and Leukopenia Syndrome [FTLS]), has recently been described in China.

Methods. Patients with laboratory-confirmed HYSV infection who were admitted to Union Hospital or Zhongnan Hospital between April 2010 and October 2010 were included in this study. Clinical and routine laboratory data were collected and blood, throat swab, urine, or feces were obtained when possible. Viral RNA was quantified by real-time reverse-transcriptase polymerase chain reaction. Blood levels of a range of cytokines, chemokines, and acute phase proteins were assayed.

Results. A total of 49 patients with hemorrhagic fever caused by HYSV were included; 8 (16.3%) patients died. A fatal outcome was associated with high viral RNA load in blood at admission, as well as higher serum liver transaminase levels, more pronounced coagulation disturbances (activated partial thromboplastin time, thrombin time), and higher levels of acute phase proteins (phospholipase A, fibrinogen, hepcidin), cytokines (interleukin [IL]–6, IL-10, interferon-γ), and chemokines (IL-8, monocyte chemotactic protein 1, macrophage inflammatory protein 1b). The levels of these host parameters correlated with viral RNA levels. Blood viral RNA levels gradually declined over 3–4 weeks after illness onset, accompanied by resolution of symptoms and laboratory abnormalities. Viral RNA was also detectable in throat, urine, and fecal specimens of a substantial proportion of patients, including all fatal cases assayed.

Conclusions. Viral replication and host immune responses play an important role in determining the severity and clinical outcome in patients with infection by HYSV.

Recently, a novel tick-borne Bunyavirus related to the *Phlebovirus* genus, which was named Huaiyangshan virus (HYSV) [1], was identified in farmers with unexplained hemorrhagic fever–like illnesses in the Huaiyangshan mountains and other parts of China [1, 2]. The clinical illness caused by HYSV is characterized by nonspecific symptoms and signs, including high fever, severe malaise, nausea, vomiting, and diarrhea, with manifest bleeding tendencies in some patients. Laboratory abnormalities share several features with other viral hemorrhagic fevers, such as leukopenia, severe thrombocytopenia, and coagulation abnormalities. The case-fatality ratio in laboratory-confirmed patients was about 15%. Causes of death were cerebral hemorrhage or multiorgan failure [3, 4].

To identify correlates of severity and clinical outcome of this novel disease, we measured viral loads as well as

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cytokine and chemokine levels in blood and related those levels to routine laboratory abnormalities and fatal outcome in this study. We also investigated the detection of viral RNA outside the bloodstream, such as in urine, throat, and feces. Finally, we studied the natural course of viremia in sequential blood specimens. These observations indicated that high levels of viral replication and subsequent innate immune responses, as well as liver and coagulation disturbances, determine the pathogenesis of this novel viral disease and can be used as prognostic predictors of poor outcome.

METHODS

Patients

In this prospective study, patients from Huaiyangshan mountain areas who were admitted to Union Hospital or Zhongnan Hospital in Wuhan City during the period from 20 April 2010 to 31 October 2010, and who presented with acute onset of high fever (>37.5°C), leukopenia ($<4.0 \times 10^9$/L), and thrombocytopenia ($<100 \times 10^9$/L) were tested for HYSV by reverse-transcriptase polymerase chain reaction (RT-PCR) of blood at admission. All patients who tested positive for HYSV were included in this study. Admission blood samples were used for correlation of viral load to other laboratory markers. Positive RT-PCR results were subsequently confirmed by sequencing. Serial blood, throat swab, urine, and feces were collected when possible. Clinical history and physical examination, as well as routine clinical biochemical and hematological laboratory results, were collected [5]. This study was approved by the institutional review board of the Authority of National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention (CDC).

Quantitation of Viral Load With Real-Time RT-PCR

In brief, RNA was extracted from 200 μL of whole blood or other clinical specimens using the QIAamp MinElute Virus Spin Kit (Qiagen) according to the manufacturer's protocol. Primers and probes targeted at the S segment were designed based on the published sequences (HQ179729-HQ179750). The forward and reverse primer sequences are 5′-ACCTCTTTGACCCTGAGTTWGACA-3′ and 5′-CTGAAGGAGACAGGTGGAGATGA-3′, respectively. The sequence of the TaqMan MGB (minor groove binder) probe was 5′-TGCCTTGACGATCTTA-3′, coupled with HEX (hexachloro-6-carboxyfluorescein) as the reporter dye at the 5′ end, and a nonfluorescent quencher and MGB, which served as a Tm enhancer, at the 3′ end. Reverse transcription, amplification, and detection were performed in an automated Rotor-Gene Q Analyzer using QuantiTect virus + ROX vial kits (Qiagen). The 20-μL PCR mixture consisted of 2 μL of RNA, 400 nM primers, and 200 nM probes. Thermal cycling conditions were as follows: 20 minutes at 50°C and 5 minutes at 95°C, followed by 45 cycles at 95°C for 15 seconds and 60°C for 45 seconds. The standard curve for HYSV quantitation was based on 10-fold dilutions of in vitro transcribed target RNA at concentrations ranging from 10^0 to 10^{10} copies/μL. The limit of detection of this assay was 100 copies of RNA genome equivalents per milliliter.

Detection of Cytokines and Other Host Response Markers in Serum

Levels of cytokines and chemokines in serum were measured using the Bio-Plex Pro Human Cytokine 17-Plex Panels (Bio-Rad) on the Bio-Plex platform. The cytokines and chemokines tested included interleukin (IL)–1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). Levels were analyzed using Bio-Plex Manager 6.0 software.

Serum levels of acute phase proteins, including C-reactive protein, fibrinogen, alpha-2-macroglobulin, serum amyloid P, haptoglobin, procalcitonin, ferritin, tissue plasminogen activator, and serum amyloid A, were measured using the Bio-Plex Human Acute Phase 4 + 5 Plex Panels (Bio-Rad), following the manufacturer's protocol. Nitric oxide levels were detected using the Griess assay (Promega) following the manufacturer's protocol. Serum levels of hyaluronic acid, hepcidin, and phospholipase A2 were measured using enzyme-linked immunosorbent assay (Uscn Life Science) following the manufacturer's instructions [6].

Statistical Analysis

Results were analyzed using the statistical software package SPSS 15.0 for Windows (SPSS, an IBM Company). Continuous variables were analyzed after logarithmic transformation. For the purpose of analyses, lower limits of detection were used in cases of negative assay results. Statistical analyses were performed using the Student unpaired *t* test or rank-sum test where appropriate. The correlation between variables was assessed using the Pearson test. Fisher exact probabilities in 2 × 2 tables were used to test relationships between clinical presentation and virus shedding from throat, urine, and feces. *P* values of $< .05$ were considered statistically significant.

RESULTS

Demographic and Clinical Characteristics in Relation to Outcome

During the period April 2010 to October 2010, 49 hospitalized, laboratory-confirmed HYSV-infected patients from 54 suspected cases were identified and enrolled in the study. Five suspected patients who satisfied the inclusion criteria/parameters were negative by PCR and excluded from further analyses. Of the 49 confirmed cases, 8 patients (16.3%) died within 1–4

Table 1. Differences in Clinical and Laboratory Characteristics Between Fatal and Nonfatal Cases of Huaiyangshan Virus

Characteristic	All Patients (N = 49)	Nonfatal (n = 41)	Fatal (n = 8)	*P* Value[a]
Age, mean (SD)	54.4 (9.1)	52.9 (8.9)	62.1 (9.6)	.011
Days of hospitalization	10 (7–14)	11 (9–15)	3 (1–4)	<.001
Aspartate aminotransferase, U/L	237 (106–438)	224 (97–343)	558 (288–1815)	.037
Thrombin time, s	21.5 (16.8–40.8)	20.5 (16.3–28.0)	75.8 (65.1–101.7)	.021
Activated partial thromboplastin time, s	55.4 (47.0–67.2)	55.2 (46.0–64.4)	103.4 (62.6–133.4)	.041

Data are median (interquartile range) unless otherwise specified.

Abbreviations: S, seconds; SD, standard deviation; U/L, units/liter.

[a] Fatal versus nonfatal.

days of admission. Surviving patients were admitted for a median of 11 days (range, 4–23).

The median age of fatal cases was significantly higher than that of nonfatal cases (62.1 vs 52.9 years, respectively; $P = .011$) (Table 1); however, there were no differences in sex, illness day since onset at admission, or presenting symptoms. None of the patients, including those who died, had evidence of preexisting comorbidities. Of the clinical laboratory parameters tested, blood coagulation times (activated partial thromboplastin time [APTT] and thrombin time [TT]) were significantly longer in fatal cases. Plasma aspartate aminotransferase (AST) levels were significantly higher, whereas a trend toward higher alanine aminotransferase (ALT) and lactic acid dehydrogenase (LDH) levels in fatal cases was also observed. Peripheral blood white cell and platelet counts were low in all patients and did not differ between fatal and nonfatal cases (Table 1).

Viral Markers and Clinical Outcome

At admission, the HYSV RNA load in blood of the 49 patients studied ranged from 1.0×10^2 copies/mL to 8.2×10^7copies/mL. Admission viral loads were significantly higher in fatal cases when compared to those who survived (median, 10E-5.6 vs 10E-3.1 copies/mL, respectively; $P = .001$; Table 2). The 3 patients with admission viral loads of $\geq 10^7$ copies/mL and 5 of the 7 patients with levels between 10^5 and 10^6 copies/mL died (Figure 1). In contrast, all 39 patients with admission blood viral loads of $<10^5$ copies/mL survived (Figure 1).

Viral RNA could be detected in throat swabs from 28 of 46 (61%) patients, urine from 20 of 39 (51%), and feces from 13 of 23 (57%). Of note, all throat and urine specimens from fatal cases were viral RNA positive. There was no correlation between the type of specimen in which viral RNA was detected and specific clinical symptoms, for example, respiratory or gastrointestinal abnormalities or laboratory abnormalities (eg, renal dysfunction) (data not shown)

Host Response Markers and Clinical Outcome

Of the tested cytokines, serum IL-6, IL-10, G-CSF, and IFN-γ levels were increased in HYSV-infected patients and were significantly higher in fatal cases than in survivors (Table 2, Figure 1; online only). Although some patients had increased levels of TNF-α (at levels up to 239 pg/mL in a fatal case and up

Table 2. Viral and Host Biomarkers in Patients Infected With Huaiyangshan Virus and Healthy Controls

	Fatal Cases (n = 8)	Nonfatal Cases (n = 41)	*P* Value	All Patients (N = 49)	Healthy Individuals (n = 16)	*P* Value
Viral load (log_{10})	5.6 (5.2–7.2)	3.1 (2.2–4.3)	.001	3.5 (2.4–4.9)	Nondetected	
IL-6, pg/mL	143.8 (80.5–397.8)	22.9 (2.8–45.0)	.001	28.1 (2.8–70.7)	Undetectable	.001
IL-8, pg/mL	80.2 (38.5–198.4)	8.3 (2.9–33.5)	.002	11.7 (2.9–49.1)	38.6 (35.1–63.6)	.015
IL-10, pg/mL	86.3 (35.4–121.2)	0.3 (0.3–24.9)	.001	1.7 (0.3–60.0)	Undetectable	.001
Granulocyte colony-stimulating factor, pg/mL	98.4 (47.1–248.2)	19.9 (3.5–57.2)	.005	36.2 (3.5–77.4)	Undetectable	.001
Interferon-γ, pg/mL	331.5 (31.0–479.0)	3.7 (3.7–68.2)	.002	3.7 (3.7–175.9)	Undetectable	.001
Fibrinogen, μg/mL	298.3 (67.3–371.6)	12.1 (6.0–213.6)	.021	14.4 (9.1–312.8)	1.8 (1.4–2.0)	.001
Hepcidin, ng/mL	3704.2 (3191.5–3815.0)	2617.4 (525.1–3649.3)	.024	3065.3 (874.5–3798.5)	120.3 (59.0–171.7)	.001
Phospholipase A2, ng/mL	10 886.4 (838.7–37 443.4)	508.7 (167.8–2174.7)	.005	734.0 (210.6–3655.6)	154.5 (125.5–173.3)	.001

Data are median (interquartile range).

Abbreviation: IL, interleukin.

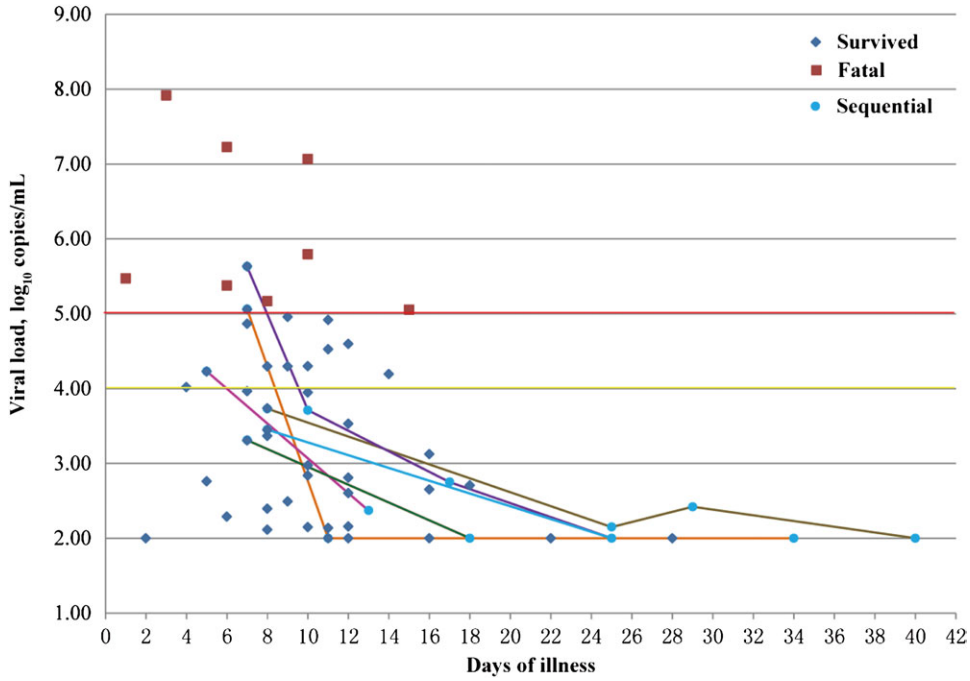

Figure 1. Natural course of viral load in blood. Viral RNA loads in blood specimens of fatal (red squares) and nonfatal (blue diamonds) cases collected at admission are depicted in relation to illness day. The dots (light blue) represent viral loads in sequentially obtained specimens from 6 patients.

to 26 pg/mL in a surviving case), overall there were no significant differences between groups. Levels of IL-8, MCP-1, and MIP-1b were lower or similar in surviving HYSV-infected patients when compared to healthy controls. Significantly higher levels were observed in fatal cases. Similarly, levels of fibrinogen, hepcidin, and phospholipase A2 (PLA2) in blood were significantly higher in fatal than in nonfatal cases (Table 2, Figure 2).

Clinical and Host Response Markers Correlate With Viral Load

Levels of laboratory parameters described above that were associated with fatal outcome, including liver enzymes, coagulation parameters, cytokines, and acute phase proteins, all correlated with levels of HYSV RNA in blood (Figure 2, Figure 1; online only).

The Natural Course of Viral and Host Markers During Infection

To estimate the natural course of viral RNA load in blood during infection, admission viral loads from individual patients were plotted against the day since onset of illness (Figure 1). Viral loads in available serially obtained blood specimens from 6 patients were also included to strengthen the estimation. Viral RNA levels seemed to gradually decline over 3 weeks after illness onset but remained detectable up to 30 days at low levels. The decline in viral load in the 6 patients from whom serial specimens were available was accompanied by resolution of symptoms and normalization of liver enzyme levels and peripheral blood white cell and platelet counts (Table 1; online only). Furthermore, levels of cytokines and acute phase proteins also reduced as levels of viral RNA decreased.

DISCUSSION

Our study of a cohort of hospitalized patients admitted to 2 hospitals provides important insights into the pathogenesis of a newly emerged disease caused by HYSV [1, 2]. A fatal outcome of HYSV infection was associated with high viral RNA levels in blood at admission, suggesting that high levels of viral replication are central to disease pathogenesis. Estimation of the natural course of viral load suggested that viral RNA levels gradually decline over 3–4 weeks after onset of illness but may remain detectable for up to >30 days. Reductions in viral load over time were associated with clinical recovery and normalization of clinical laboratory parameters.

Viral RNA was also frequently detected in urine, feces, or throat specimens. However, because there was no association with specific symptomatology or laboratory abnormalities, this likely reflects high levels of viremia and likely originates from blood through transudation or bleeding. Although the extent to which detected viral RNA reflects the presence of infectious virus is unclear, infection control measures should take the potential infectivity of these specimens into account.

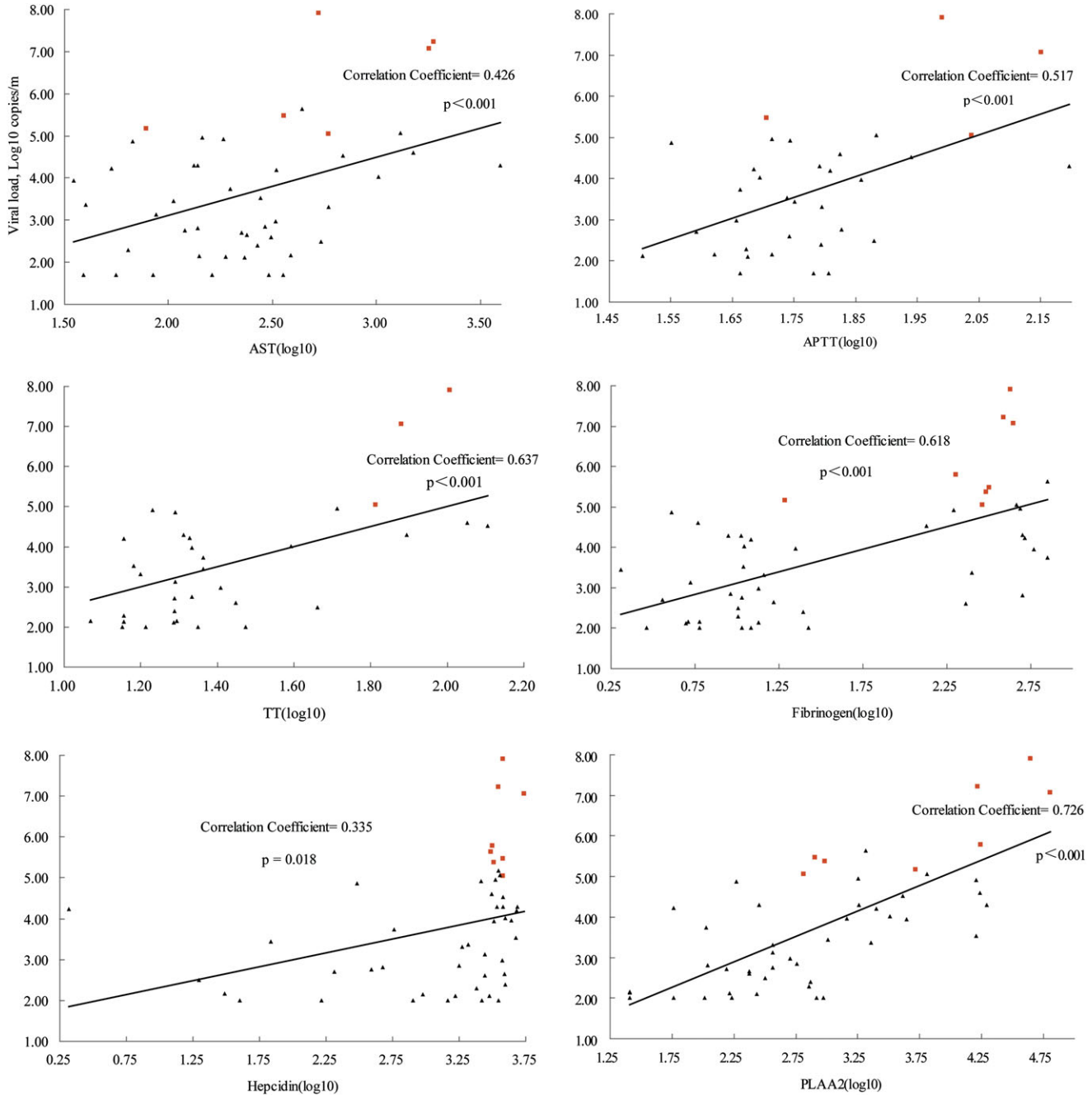

Figure 2. Correlation between viral load and host biomarkers; logarithmic values are used. Correlations between variables were assessed using the Pearson test. *P* values of <.05 were considered significant. Symbols and colors: red square, fatal patients; blue diamonds, nonfatal patients.

The coagulation disturbances, such as increased TT and APTT, and markers of liver damage, such as AST, ALT, and LDH, were observed in all patients but were most pronounced in fatal cases and correlated with blood viral loads. The serum fibrinogen levels were high, particularly in fatal cases [6, 7], and the serum levels of hepcidin, which is produced in the liver in response to acute inflammation, possibly induced by cytokines [8, 9], were also increased and highest in fatal cases, suggesting a central role of the liver in disease pathogenesis.

These observations suggest that the extent of liver damage and coagulation disturbances are related to levels of viral replication and may play an important role in determining clinical outcome. However, the coagulation disturbances may be secondary to endothelial damage and diffuse intravascular

coagulation, similar to other viral hemorrhagic fevers such as Crimean-Congo hemorrhagic fever (CCHF), or to decreased production of coagulation factors due to acute hepatic damage [4, 5].

HYSV infection was associated with marked induction of innate immune responses, as reflected by high levels of cytokines such as IL-6, IL-10, and IFN-γ in the blood of infected patients. Increased levels of these cytokines are commonly associated with severe viral infections including those caused by other hemorrhagic fever viruses such as CCHF and Ebola [10, 11]. In HYSV-infected patients, cytokine levels were significantly higher in those who died than in survivors and correlated with blood levels of HYSV RNA. High levels of TNF-α were reported in other viral hemorrhagic fevers such as CCHF, Dengue hemorrhagic fever, and Ebola hemorrhagic fever [10, 12–15]. Although some patients had increased levels of TNF-α, overall there were no significant differences between groups in this study. Further studies are needed to investigate whether the role of TNF-α in HYSV infection is different from that in other viral hemorrhagic fevers.

Although serum levels of chemokines such as IL-8, MCP-1, and MIP-1b in nonfatal cases were similar to those in healthy individuals (even lower for IL-8), levels were significantly high in fatal cases, similar to those in the previous study [10]. These observations suggest that an intense inflammatory response to high levels of virus, perhaps specifically involving recruitment of inflammatory cells to infected tissues, contributes to disease pathogenesis [16].

Limitations of our study include the relatively small number of patients studied. However, this study does represent the largest cohort of HYSV-infected patients reported to date and, despite the low number of fatalities, did identify laboratory correlates of poor outcome. Additional limitations are the low number of patients from whom serial specimens could be collected, preventing a more reliable estimate of the natural history, and the fact that no tissue specimens or peripheral blood mononuclear cells were collected, which would enable further detailed studies of replication sites and immune responses. Further investigations in additional patient cohorts, which should include autopsy studies of fatal cases, are needed to overcome the limitations of the current study. In addition, efforts should be made to investigate whether mild or subclinical HYSV infections occur outside the hospital.

In summary, high levels of viral replication are indicative of severe and progressive infection and may be central to the pathogenesis of disease caused by HYSV infection. A fatal outcome is also associated with more pronounced coagulation abnormalities, markers of liver damage, and innate immune responses [17]. Because tissues were not available for immunohistochemical analysis, further studies, including animal models, are needed to investigate the exact sites of viral replication and the relative roles of virus-induced and immune-mediated pathology.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/cid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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