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Original article

# Nucleocapsid protein-specific IgM antibody responses in the disease progression of severe fever with thrombocytopenia syndrome

Gai Wang[a,1], Haiyan Chang[b,1], Bei Jia[a], Yong Liu[c], Rui Huang[a], Weihua Wu[a], Yingying Hao[d], Xiaomin Yan[a], Juan Xia[a], Yuxin Chen[e,*], Chao Wu[a,*]

[a] Department of Infectious Diseases, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu, China
[b] Department of Infectious Diseases, Nanjing Drum Tower Hospital Clinical College of Nanjing Medical University, Nanjing, Jiangsu, China
[c] Department of Experimental Medicine, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu, China
[d] Department of Intensive Care Unit, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu, China
[e] Department of Laboratory Medicine, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu, China

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ABSTRACT

*Objectives:* Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease that is caused by the SFTS virus (SFTSV) and has a high fatality rate. SFTSV-specific antibody profiles among patients with different clinical outcomes are yet to be described. The nucleocapsid protein (NP) is the most immunogenic viral antigen of the SFTSV. This study, therefore, sought to determine NP-specific antibody responses among SFTS patients with different disease progressions.

*Methods:* In the present study, 43 patients with confirmed SFTS were enrolled in our cohort, and 9 of them deceased. The clinical presentations and key laboratory parameters associated with SFTS fatality were also recorded. Serum samples from each patient were collected every 2 days during their hospitalization. NP-specific IgM and IgG responses as well as Gn or Gc-specific IgM responses were examined by enzyme-linked immunosorbent assay (ELISA), whereas, the dynamic viral loads of SFTSV RNA were quantified *via* real-time reverse transcription polymerase chain reaction (RT-PCR).

*Results:* First, 77% of patients generated positive NP-specific IgM antibody responses within two weeks since illness onset, defined as 'N-specific IgM-positive patients', while the rest of the patients were termed as 'N-specific IgM-delayed patients'. Only 17% of the patients generated NP-specific IgG responses. The absence of NP-specific humoral responses was strongly associated with a high risk of fatality and severity of SFTS. IgM-positive patients had significantly lower levels of viral loads, less disturbed coagulopathy, and hepatic and cardiac damage compared to IgM-delayed patients. Moreover, compared to severe or fatal SFTS patients, mild SFTS patients had significantly higher magnitudes of NP-specific IgM responses, but not NP-specific IgG, Gn-specific IgM, or Gc-specific IgM responses. The abundance of NP-specific IgM responses negatively correlated with viral loads, coagulation disturbances, and hepatic injuries among SFTS patients.

*Conclusions:* Our data highlight distinct humoral profiles of NP-specific IgM responses among SFTS patients with different disease progressions and clinical outcomes.

## 1. Introduction

As an emerging hemorrhagic fever disease identified in eastern Asia, severe fever with thrombocytopenia syndrome (SFTS) is caused by a novel phlebovirus in the *Bunyaviridae* family (Kim et al., 2015; Takahashi et al., 2014; Yu et al., 2011), known as SFTS virus (SFTSV). Another new phlebovirus, Heartland virus, was isolated from two patients suffering from severe febrile illness in Missouri, USA (McMullan et al., 2012), which shares high identity with SFTSV. These two newly emerged phleboviruses have, subsequently, exerted a global public threat.

SFTSV is transmitted through a tick bite (Luo et al., 2015) and human-to-human contact *via* blood or body fluid (Bao et al., 2011; Chen et al., 2017a). SFTS has an average of 12% case fatality rate, ranging from 6% to 30% (Yu et al., 2011). The clinical symptoms of SFTS infection are variable, ranging from asymptomatic or acute self-limited

* Corresponding authors.
*E-mail addresses:* yuxin_chen2015@163.com (Y. Chen), dr.wu@nju.edu.cn (C. Wu).
[1] These authors contributed equally to this work.

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febrile condition to life-threatening illness. The typical clinical presentation is characterized by the sudden onset of fever, fatigue, gastrointestinal symptoms, leukopenia, and thrombocytopenia. Severe SFTS cases quickly develop multiple organ dysfunction (MOD) and disseminated intravascular coagulation (DIC), which might lead to fatality within 2 weeks of the disease onset (Zhang et al., 2013, 2012). Previous studies have identified a panel of risk factors associated with the fatality and severity of SFTS cases, such as old age, coagulation disturbance, and remarkable liver damage (Chen et al., 2017b; Cui et al., 2014; Ding et al., 2014; Jia et al., 2017; Zhang et al., 2012); however, the underlying immunological mechanism remains to be obscure. Although ribavirin has been proven effective in reducing the viral load of SFTSV, its usage could not decrease the case fatality ratio of SFTS patients (Liu et al., 2013; Lu et al., 2015). Currently, there is no effective treatment regimen for SFTS in clinical practice other than supportive care.

Antibodies typically play a key role in controlling viral infections, which are immune correlates contributing to the protective efficacy of many successful vaccines (Crowe, 2017). However, the role of SFTS-specific adaptive immune responses during an SFTS disease progression has to be elucidated. SFTSV-specific IgM antibody responses reportedly could be detected within an average period of 9 days, while SFTSV-specific IgG responses could only be detected within an average period of 6 weeks (Lu et al., 2015), and the neutralizing antibodies from SFTS patients could be maintained for at least 4 years (Huang et al., 2016). Nevertheless, the dynamic profile of antibody responses against specific SFTS viral proteins has not been further dissected and carefully characterized.

Nucleocapsid protein (NP), encoded by the small segment of bunyavirus, is the most conserved *Phlebovirus* genus. NP is abundantly present in viral particles and infected cells. The primary function of NP is to encapsidate the viral genome forming ribonucleoprotein complexes (RNPs) (Pekosz et al., 1999; Walter et al., 2011). Beyond its critical role in viral RNA protection, NP is actively involved in RNA transcription and replication, as well as in the formation of an inclusion body in the cytoplasm of virus-infected cells (Eifan and Elliott, 2009; Pekosz et al., 1999; Pinschewer et al., 2003; Walter et al., 2011). It has been shown that NP is highly immunogenic and that NP-specific antibodies are also readily detected early after infection in convalescent individuals, providing a robust basis for diagnostic detection of SFTS disease (Magurano and Nicoletti, 1999; Martin-Folgar et al., 2010). Interestingly, NP-specific antibodies without neutralizing activities have been demonstrated to protect animals from challenges with Rift Valley fever virus- (RVFV) and Hantavirus partially (Boshra et al., 2011b; Nakamura et al., 1985; Yoshimatsu et al., 1993). Whether viral infection-induced NP-specific humoral responses have any association with the clinical outcome of phlebovirus, including SFTSV infection *in vivo,* is rarely investigated in a real-world setting.

In the current report, we performed a prospective study on SFTS patients, including 34 survivors and 9 deceased patients. The kinetics of NP-specific IgM and IgG antibody responses during the acute infection phase was measured. Further, the clinical manifestations and the dynamic changes of clinical laboratory tests were determined to further define the clinical features among NP-specific IgM-positive *versus* IgM-delayed patients. The correlations between the magnitudes of NP-specific IgM responses and clinical laboratory parameters were analyzed. With such information, we carefully dissected the antibody profile of NP-specific humoral responses among fatal, severe and mild SFTS patients.

## 2. Material and methods

### 2.1. Human subjects

This retrospective study was performed in Nanjing Drum Tower Hospital, Nanjing, Jiangsu Province, China, from June 2016 to Oct 2018. Clinical manifestations and laboratory parameters were collected from 43 SFTS patients (9 deaths and 34 survivors). A SFTS patient was clinical defined if he or she agreed with the following criteria: (1) acute fever with body temperature of > 38 °C; (2) decline of white blood cell (WBC) and platelet count (PLT); (3) the contact history with tick bite or with the blood of SFTS patient; (4) positive detection of SFTSV by using a real-time reverse transcription polymerase chain reaction (RT-PCR) performed by Jiangsu provincial center for disease control and prevention. The day of disease onset was determined as the day the initial SFTS-like symptoms were expressed. Severe SFTS patients were defined as those SFTS patients who required hospitalization in intensive care units and met at least one of the following criteria as previously described (Deng et al., 2013; Lu et al., 2015): acute lung injury or acute respiratory distress syndrome, disseminated intravascular coagulation (DIC), encephalitis, shock, septicemia, at least one or more organ failures, such as heart failure and acute renal failure, or death. Mild SFTS patients were defined as those patients who survived SFTS and did not requir admission in intensive care units, and did not manifest any of the clinical symptoms mentioned above.

### 2.2. Study design

Clinical characteristics and the dynamic data of the laboratory results of SFTS patients were extracted from the first day of hospitalization to the day of discharge. Laboratory parameters that were routinely tested and considered to be related to the prognosis of SFTS patients were evaluated, including white blood cell counts (WBC), lymphocyte percentage, PLT, alanine transaminase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK), albumin (ALB), thrombin time (TT), and activated partial thromboplastin time (APTT). Sera were also collected from SFTS patients in our cohort every other day during their hospitalization and immediately frozen at −80 °C for later experiments.

### 2.3. The expression and purification of nucleocapsid protein (NP), glycoprotein N (Gn) and glycoprotein C (Gc) from SFTSV

The gene segment coding for NP from the SFTSV strain HB29 was synthesized and subcloned into the expression vector pET-28(a) + (Novagen). The recombinant vector pET-28(a)-N was transformed into the chemically competent *Escherichia Coli* BL21 for expression. The expression of NP was performed overnight in an LB broth with isopropyl-1-D-thiogalactopyranoside (IPTG) at 37 °C. The culture pellets containing the recombinant protein were re-suspended in the binding buffer and further sonicated. Furthermore, the SFTSV Gn ectodomain (GenBank accession no. JF906057.1, residue 20–452) and SFTSV Gc ectodomain (UniProt accession no. R4V2Q5, residue 563–996) with a C-terminal six-histidine purication tag was subcloned into a pcDNA3.1 vector (Thermo Fisher Scientifc), respectively. The transfection was conducted in 293 F cells using 293fectin (Thermo Fisher Scientific) for Gn and Gc protein expression. The cell lysates mentioned above were centrifuged, and the supernatants were loaded into the nickel ion affinity column (GE Healthcare). After washing, the $^{6}$His-labeled NP was eluted from the column with an elution buffer. The purified NP was analyzed using SDS-PAGE and visualized with comassie staining, and results were further confirmed by western blotting analysis using anti-His tag monoclonal antibody (mAb).

### 2.4. Determination of antibody response specific to NP, Gn and Gc for SFTS patients at acute infection phase

Recombinant desired viral antigens, such as NP, Gn and Gc, were coated onto 96 well microtiter plates (Costar #3369) at 1 μg/mL in 100 μL of Bicarbonate-Carbonate Buffer (PH 9.4) for 1 h at room temperature. Plates were then washed 5 times in wash buffer (PBS containing 0.1% Triton X-100) and blocked overnight at 4° in PBS

containing 0.5% Tween-20 and 5% BSA. The following morning, plates were washed 5 times in wash buffer and serially diluted human sera (from 1:1000 to 1:256,000) was added to the wells in a volume of 100 µL. Then plates were washed 5 times and 100 µL of HRP anti-human IgM or IgG at 0.5 µg/mL was incubated on the plate for 1 h at room temperature. Plates were washed 5 times and developed for 3 min in 100 µL mixture of chromogen solution A and chromogen solution B. The reaction was stopped with addition of 50 µL of stop solution. Endpoint titers as reported are defined as the last dilution of a serially diluted serum sample with greater than double the background optical density of a healthy person serum sample.

### 2.5. Quantitation of viral loads using quantitative real-time reverse transcription polymerase chain reaction

SFTSV viral load was detected by using the quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). The SFTS viral RNA was extracted from the serum samples using RNA extraction kit (Qiagen, Hilden, Germany) and the detection of SFTS virus was conducted by using a quantitative RT-PCR kit (BGI, Shenzhen, China). Sera from healthy donors were used as negative control. The RT-PCR reaction system was performed in 30 µl volume. The temperature and time were as follows: firstly 50 °C for 30 min with 1 cycle; 95 °C for 15 min with 1 cycle; 95 °C for 15 s and 60 °C for 45 s with 40 cycles. Fluorescent signals were collected by FAM channel. The cut-off cycle threshold ($C_t$) value less than 35 cycles was considered as positive.

### 2.6. Statistical analysis

All statistical analyses were performed with the statistical software SPSS 22.0 (Chicago, USA) and $p$ value $< 0.05$ was considered statistically significant. Continuous variables with normal distribution were compared with t test; whereas continuous variables with non-normal distribution were compared with nonparametric test and described as median and interquartile range (IQR). Categorical variables were compared with $\chi 2$ test. Figures were drawn by using GraphPad Prism 5 (Graph Pad Prism, La Jolla, CA, USA).

### 2.7. Ethics statement

The research protocol was approved by the Human Ethics Committee of the Nanjing Drum Tower Hospital. All participants were adults and they offered the written informed consents in this study.

## 3. Results

### 3.1. The dynamic NP-specific IgM and IgG responses among SFTS patients

From Jan 2016 to Oct 2018, a total of 43 laboratory-confirmed SFTSV infected patients were recruited in this study. From this cohort, 34 patients (79%) were survived, and 9 patients (21%) died ( Table 1). The NP-specific IgM responses among SFTS patients were determined every other day beginning from the moment they were hospitalized. We found that NP-specific IgM responses were elicited among 27 (63%) out of 43 patients before day 10. Positive NP-specific IgM responses were further found in 84% (36/43) of patients until their discharge or death, suggesting that the majority of patients had generated NP-specific IgM responses. Unfortunately, all 7 patients that did not generate any NP-specific IgM responses during the SFTS disease were died.

The natural history of SFTS disease is classified in three distinct stages known as the fever stage (day 0–6), multiple organ dysfunction syndrome (MODS) stage (day 7–13), and convalescence stage (after day 13) (Cui et al., 2014). Notably, 8 (19%) out of 43 patients generated NP-specific IgM responses during the fever stage (day0–6) from the disease onset ; 25 (58%) out of 43 patients generated NP-specific IgM responses during the MODS stage (day 7–13), and 3 (7%) out of 43 patients generated NP-specific IgM responses during the convalescence stage. As the first two weeks of the SFTS disease is the most critical period, we referred to patients who generated NP-specific IgM responses by the end of the first two weeks as “NP-specific IgM-positive patients,” while the rest of the SFTS patients were referred to as “NP-specific IgM-delayed patients”.

NP-specific IgG responses among 23 SFTS patients were also determined. Only 5 (17%) patients had positive NP-specific IgG responses by the end of day 13 of the disease onset. Furthermore, low levels of NP-specific IgG responses were observed in SFTS patients, compared to NP-specific IgM titer ($P < 0.05$) (Fig. 1B).

**Table 1**
Demographic and clinical characteristics of SFTS patients in our cohort.

| | total patients (n = 43) | NP-specific IgM-positive patients (n = 33) | NP-specific IgM-delayed patients (n = 10) | P |
|---|---|---|---|---|
| **demographic feature, n (%)** | | | | |
| sex, Male | 16 (37.2) | 13(39.4) | 3(30.0) | 0.719 |
| sex, Female | 27 (62.8) | 20 (60.6) | 7 (70.0) | 0.719 |
| age, y[#] | 56.5 (34, 82) | 56.5(3482) | 57 (4277) | 0.555 |
| **general symptoms, n (%)** | | | | |
| temperature > 39 °C | 25 (58.1) | 17 (51.5) | 8(80.0) | 0.153 |
| fatigue | 42 (97.7) | 32 (97.0) | 10 (100) | 1.000 |
| headache | 15 (34.9) | 11 (33.3) | 4 (40.0) | 0.719 |
| myalgia | 12 (27.9) | 10 (30.3) | 2 (20.0) | 0.698 |
| **gastrointestinal signs, n (%)** | | | | |
| lack of appetite | 43 (100) | 33(100) | 10 (100) | 1.000 |
| nausea | 22 (51.7) | 15 (45.5) | 7(70.0) | 0.281 |
| vomiting | 19 (44.2) | 12 (36.4) | 7 (70.0) | 0.079 |
| diarrhea | 11(25.6) | 8 (24.2) | 3(30.0) | 0.698 |
| lymphadenopathy | 10 (23.3) | 8(24.2) | 2 (20.0) | 1.000 |
| **consciousness change, n (%)** | | | | |
| apathy | 11 (25.6) | 6(18.2) | 5(50.0) | 0.092 |
| leghargy | 8(18.6) | 5 (15.2) | 3 (30.0) | 0.362 |
| muscular tremor | 1 (2.3) | 0 (0) | 1(10.0) | 0.233 |
| convulsions | 5(11.6) | 2(6.06) | 2(20.0) | 0.226 |
| coma | 7 (16.3) | 1 (3.03) | 6 (60.0)[b] | 0.001* |
| **hemorrhagic signs, n (%)** | | | | |
| petechiae | 9 (20.9) | 5 (15.2) | 4 (40.0) | 0.177 |
| haematemesis | 1 (2.3) | 0 (0) | 1(10.0) | 0.233 |
| **DIC, n (%)** | 5 (11.6) | 0 (0) | 5(50.0) | 0.000* |
| **MODS, n (%)** | 28 (65.1) | 21(63.6) | 10(100) | 0.040 |
| **fatality, n (%)** | 9 (20.9) | 2(6.06) | 7(70.0) | 0.000* |
| **severity, n (%)** | 14 (32.6) | 5(15.2) | 9(90.0) | 0.000* |

Data are presented as number of patients (percentage).
Continuous variables were compared using student t test, categorical variables using a Chi-square test.
[#] Data are presented as median (range).
* $p < 0.05$ was considered as statistically significant.

### 3.2. The clinical presentations of NP-specific IgM-positive patients and NP-specific IgM-delayed patients

The appearance of the central nervous system (CNS) symptoms, hemorrhagic signs, and MODS, were highly associated with the poor prognosis of SFTS patients (Chen et al., 2017c; Jia et al., 2017). We investigated whether NP-specific IgM-delayed SFTS patients would be at a high risk to suffer from severe clinical symptoms (Table 1). 60% of NP-specific IgM-delayed patients and only 3% of NP-specific IgM-positive patients fell into a coma. Hemorrhagic signs, such as petechiae, were found in 40% of NP-specific IgM-delayed patients, but only in 15% of NP-specific IgM-positive patients. 64% of NP-specific IgM-positive patients and all NP-specific IgM-delayed patients developed MODS. These findings suggested that NP-specific IgM-delayed patients tend to have severe clinical presentations compared to NP-specific IgM-positive patients. Notably, 70% of NP-specific IgM-delayed patients were died,

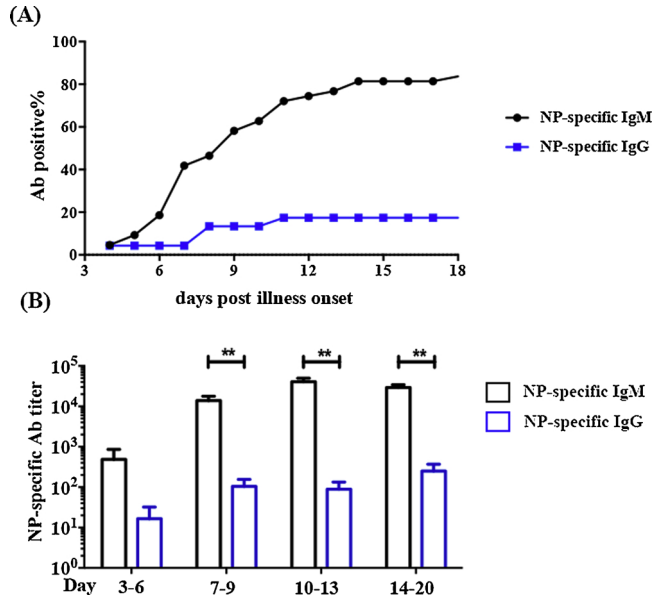


**Fig. 1.** The dynamic profile of NP-specific IgM and IgG responses in our cohort. (A) The accumulative percentage of patients with the presence of nucleocapsid protein (NP)-specific IgM and IgG responses during day 3 to day 18 since illness onset. The presence of IgM and IgG responses specific to NP was determined by ELISA. (B) The dynamic serological IgM and IgG titer from day 3 to day 18 since illness onset measured from 43 SFTS patients and 23 SFTS patients, respectively. The levels of IgM and IgG responses to NP were quantified by ELISA assay. The level of significance is indicated as follows: $^{**}P < 0.005$.

whereas only 6% of the NP-specific IgM-positive cases were fatal, indicating that the deficiency of NP-specific IgM could result in high risk of morality and severity of SFTS.

### 3.3. The dynamic clinical laboratory results between NP-specific IgM-positive patients and NP-specific IgM-delayed patients

Viral load is an important parameter in evaluating the viral replication *in vivo*. From day 7–9, NP-specific IgM-positive patients had significantly lower levels of viral load, compared to those in NP-specific IgM-delayed patients. From days 10–13, there was barely detectable level of SFTS viral RNA in IgM-positive patients, while remarkable high levels of viral loads were presented among NP-specific IgM-delayed patients (Fig. 2A). Moreover, by the end of the second week, 28 out of 33 (85%) NP-specific IgM-positive patients barely had detectable SFTS RNA copies whereas only 1 out of 10 patients fell into that category. This suggests that the induction of NP-specific IgM responses facilitates the clearance of SFTSV *in vivo*.

Since the degree of coagulopathy is significantly correlated with the severity of disease progression (Chen et al., 2017c), we determined the dynamic changes of coagulation parameters. First, NP-specific IgM-delayed patients had significantly reduced PLT when compared to NP-specific IgM-positive patients from day 7–9, 10–13 and 14–20 ($P = 0.001$, $P = 0.062$, and $P = 0.025$, respectively) (Fig. 2B). The activated partial thromboplastin time (APTT) in NP-specific IgM-delayed patients was prolonged substantially compared to NP-specific IgM-positive patients from day 7–9 and 10–13 of the disease onset ($P = 0.000$ and $P = 0.002$, respectively) (Fig. 2C). Consistently, NP-specific IgM-positive patients had a less prolonged prothrombin time (PT) compared to NP-specific IgM-delayed patients from day 7–9 and 10–13 (Fig. 2D) ($P = 0.004$ and $P = 0.003$, respectively). The thrombin time (TT) of NP-specific IgM-positive patients declined to the normal reference range and had a less significant prolongation on day 10–13 ($P = 0.005$ and $P = 0.017$, respectively) (Fig. 2E). Taken together, NP-specific IgM-positive patients suffered less exacerbated coagulation disturbance compared to NP-specific IgM-delayed patients.

The dynamic cardiac and liver enzymes among NP-specific IgM-positive patients and NP-specific IgM-delayed patients were measured. Remarkable elevated LDH and AST levels were demonstrated in IgM-

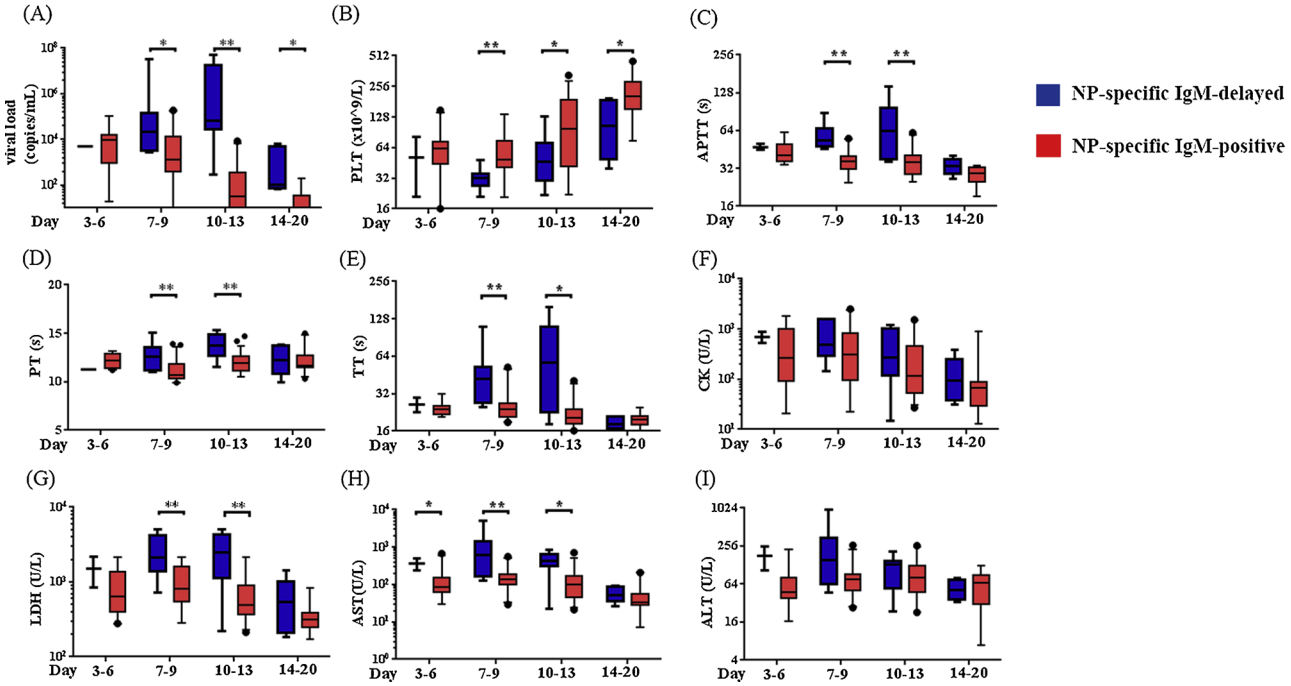


**Fig. 2.** Key clinical laboratory parameters between NP-specific IgM-positive and IgM-delayed SFTS patients from day 3 to day 20 after symptom onset. The dynamic laboratory parameters including (A) viral loads, (B) PLT, (C) APTT, (D) PT, (E) TT, (F) CK, (J) LDH, (H) AST, and (I) ALT were determined during day 3–6, day 7–9, day 10–13, and day 14–20. Abbreviation: PLT, platelet count; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; CK, creatine kinase; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine transaminase. The level of significance is indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.005$.

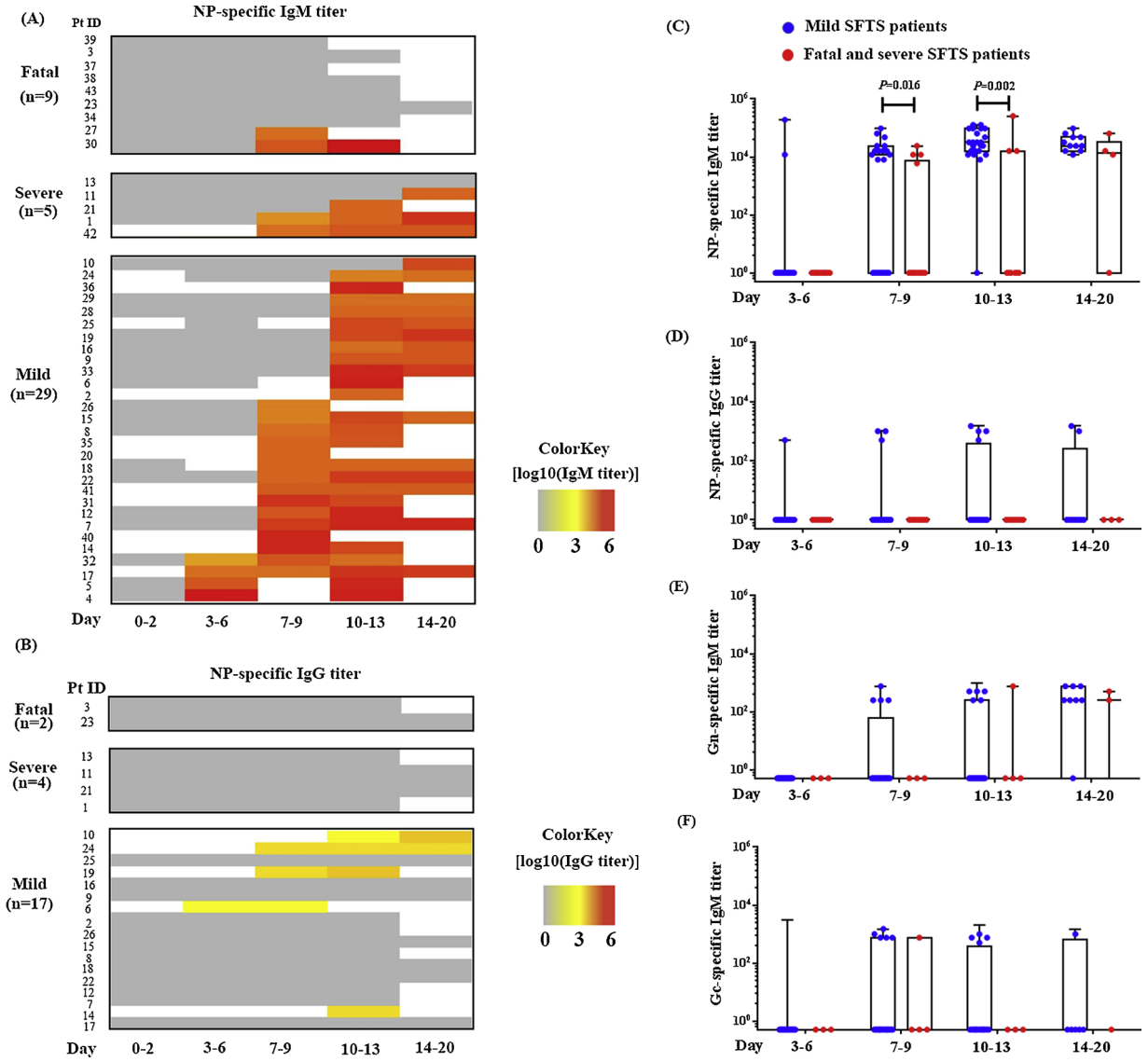


**Fig. 3.** Elevated levels of NP-specific IgM responses were present in mild SFTS patients, compared to severe and fatal SFTS patients. (A) The heatmap of NP-specific IgM titers among 43 SFTS patients with three different clinical outcomes (fatality, severity, and mildness) from day 0 to day 20 since SFTS illness onset. The color represents the magnitudes of NP-specific titer, in which grey color indicates no presence of NP-specific IgM response, yellow color indicates a titer of $1:10^3$, and red color represents a titer of 1:106, white color indicates no test performed since the serum was not available. Each row refers to an individual patient with a unique patient identification number (pt ID). (B) The heatmap of NP-specific IgG titers among 23 SFTS patients during SFTS disease progression. The color representation is same as in (A). (C) The comparison of NP-specific IgM titers between mild, severe, and fatal SFTS patients. (D) The comparison of NP-specific IgG titers between mild, severe, and fatal SFTS patients. (E) The comparison of Gn-specific IgM titers between mild, severe, and fatal SFTS patients. (F) The comparison of Gc-specific IgM titers between mild, severe, and fatal SFTS patients. Abbreviation: Pt ID, patient identification number. The *P* value with statistical significance is noted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

delayed patients during on day 7–9 and 10–13, compared to levels in NP-specific IgM-positive patients, especially at the MODS stage of SFTS ($P = 0.002$ and $P = 0.012$, respectively) (Fig. 2G and 2 H). NP-specific IgM-delayed patients also had slightly higher levels of ALT and CK than IgM-positive patients but without statistical significance (Fig. 2I and F). Our data indicate a greater extent of organ damages for NP-specific IgM-delayed patients when compared to NP-specific IgM-positive patients.

### *3.4. The magnitude of NP-specific IgM and IgG responses of SFTS patients with different disease progressions*

To further determine whether the magnitude of NP-specific IgM response could influence disease progression and clinical outcome, we divided the SFTS patients into three groups, fatal (n = 9), severe (n = 5), and mild patients (n = 29). As shown in the heat-map of Fig. 3A, the NP-specific IgM titer of individual patients during the SFTS disease progression was determined. Specifically, mild patients had significantly higher levels of NP-specific IgM titer on day 7–9 of the disease onset, compared to severe and fatal patients ($P = 0.016$). On day 10–13, substantially higher levels of IgM responses were shown in mild SFTS patients, compared to severe and fatal patients ($P = 0.002$) (Fig. 3C). Additionally, NP-specific IgG responses were also quantified in 23 SFTS patients, including 2 fatal cases, 4 severe cases, and 17 mild cases (Fig. 3B). Overall, a low level of NP-specific IgG response was found in SFTS patients. There was no significant difference for the NP-specific IgG titer between mild, fatal, and severe SFTS patients (Fig. 3D). Since Gn and Gc are two major antigenic components on the viral surface, we sought to assess the dynamic IgM responses targeting the Gn and Gc glycoprotein during the disease progression of 32

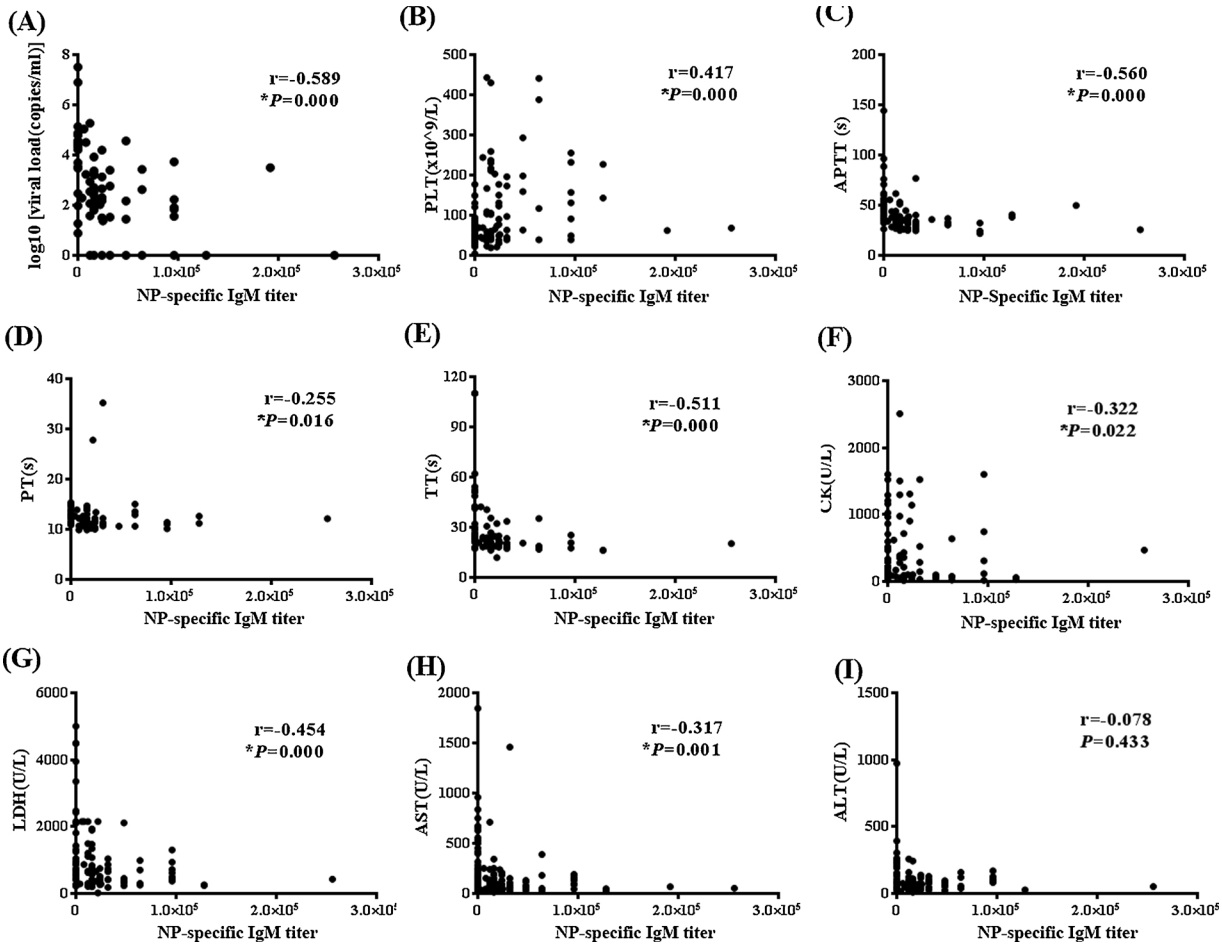


**Fig. 4.** The correlation analyses of the magnitude of NP-specific IgM responses and the clinical laboratory results from 43 SFTS patients, including (A) viral loads, (B) PLT, (C) APTT, (D) PT, (E)TT, (F) CK, (G) LDH, (H) AST, and (I) ALT. Correlation analysis was performed *via* a non-parametric Spearman correlation test. r indicates the correlation coefficient and P refers to the P-value of significance. Abbreviation: PLT, platelet count; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; CK, creatine kinase; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine transaminase.

patients using the ELISA assay. A comparable level of Gn or Gc IgM antibody responses was found between mild SFTS patients and fatal or severe SFTS patients (Fig. 3E-F). Our results indicate that absence of NP-specific IgM responses at MODS stage is highly associated with the fatal and severe clinical outcome of SFTS disease.

### *3.5. The correlation of the magnitudes of NP-specific IgM responses and the severity of clinical laboratory results*

To further illustrate the biological relevance of NP-specific IgM responses, correlation analyses of the magnitude of NP-specific IgM responses and clinical parameters were performed. The magnitude of NP-specific IgM responses correlated negatively with the level of serum SFTSV RNA ($r = -0.589$, $P = 0.000$), suggesting that a high abundance of NP-specific IgM responses could enhance the suppression of viral loads *in vivo*. Also, the NP-specific IgM titer correlated positively with PLT ($r = 0.417$, $P = 0.000$) but correlated negatively with APTT ($r = -0.560$, $P = 0.000$) and TT ($r = -0.511$, $P < 0.001$). Additionally, the magnitude of NP-specific IgM responses correlated weakly with the extent of cardiac and hepatic injuries, including CK ($r = -0.322$, $P = 0.022$), LDH ($r = -0.454$, $P < 0.001$), and AST ($r = -0.317$, $P = 0.001$). Taken together, we found that the magnitude of NP-specific IgM responses correlates negatively with the clinical severity of SFTS, in a weak manner (Fig. 4).

## 4. Discussion

Here, for the first time, we comprehensively analyzed the humoral response profile for the most immunogenic viral proteins, NP, in SFTSV, among SFTS patients with different clinical outcomes. Additionally, we compared the clinical outcomes and the disease progressions between NP-specific IgM-positive *versus* IgM-delayed patients. The clinical features among NP-specific IgM-positive and IgM-delayed patients were further characterized, revealed by the clinical presentations and the dynamic laboratory results. Our data strongly indicated that the absence of NP-specific IgM responses in the acute infection phase had a higher risk of fatal and severe clinical outcomes for SFTS patients. Specifically, compared to patients with NP-specific IgM-delayed responses, those with the induction of NP-specific IgM responses had a mild disease progression, revealed by the rapid clearance of SFTSV, less impaired hepatic and cardiac injuries, and less disturbed coagulopathy. More importantly, the magnitude of NP-specific IgM responses was also closely associated with the clinical recovery of SFTS disease.

NP is one of three major antigenic proteins encoded by the trisegmented single-stranded RNA genome of SFTSV. In our study, SFTSV-specific humoral responses focused on NP specific IgM responses during acute phase. First, N was identified as a major antigen during the acute infection phase and convalescent phase, which is consistent with other members of the *Phlebovirus* genus (Fafetine et al., 2007; Jansen van Vuren et al., 2007; Magurano and Nicoletti, 1999; Martin-Folgar et al., 2010; Yu et al., 2012). Consistent with a previous study reporting that

NP-specific IgM could be detected at the medium of 9–10 days (Lu et al., 2015), we also found that NP-specific IgM responses were readily detected before day 10 among 63% of patients. One intriguing aspect of this study was the discovery that NP-specific IgM responses were correlated with clinical recovery from SFTSV infection in our cohort. It is generally agreed that the NP of the bunyaviruses do not induce neutralizing antibodies; even the antibodies to the strongly immunogenic NP of RVFV had no neutralizing activity (Boshra et al., 2011a; Boshra et al., 2011b; Jansen van Vuren et al., 2011). For bunyavirus infection, it has been largely evidenced that almost all of the neutralizing polyclonal or mAbs are viral glycoprotein Gn- or Gc-specific (Arikawa et al., 1989, 1992), rather than NP-specific (Boshra et al., 2011b; Magurano and Nicoletti, 1999). Nevertheless, consistent with our findings, animal protection studies conducted *in vivo* demonstrated that NP-specific mAbs with no neutralizing activities partially protect animals form challenges with the Hantavirus and RVFV (Boshra et al., 2011b; Magurano and Nicoletti, 1999). The ongoing effort should be focus in the future on whether NP-specific IgM responses confer protection against SFTS *in vivo*.

Besides, NP, Gn, and Gc encoded by the M segment of SFTSV are responsible for virion attachment and membrane fusion, which are essential for host cell entry. Although the characteristic of the neutralizing antibodies against SFTSV has not been fully understood yet, it is speculated that Gn- and Gc-specific antibodies could be largely responsible for neutralization activities observed in the serum. Indeed, the neutralizing mAb identified thus far that recognizes a linear epitope in the ectodomain of the glycoprotein Gn, was able to block of the interaction between the GNP and the cellular receptor (Guo et al., 2013). In a 4-year follow up study, long-lasting neutralizing activities have been detected in recovered SFTS patients (Huang et al., 2016). Gn- and Gc-specific mAbs targeting the critical neutralizing epitopes need further investigation, which might be a feasible therapeutic intervention for severe SFTS patients. Interestingly, we did not identify any association between Gn- or Gc-specific antibody responses with the fatality and severity of the SFTS disease in our current study.

Our study also has several limitations. First, our sample size was relatively small. Second, although the absence of NP-specific IgM responses was shown to be closely associated with the fatality and severity of SFTS patients, our data demonstrated a weak correlation between NP-specific IgM responses and viral loads-, PLT-, APTT-, and TT-related clinical severity, suggesting that a multi-layer immune network might be involved in the host defense against SFTSV. Nevertheless, NP-specific IgM responses could be included as a scoring system for physicians to evaluate the clinical outcome of SFTS patients. Additionally, we could not affirm that NP-specific humoral responses directly contribute to the suppression of viral replication and clinical recovery of SFTS patients; this could be further investigated in the clinical trial of antibody-based therapy in SFTS patients.

## 5. Conclusions

Our study has demonstrated that NP-specific IgM-delayed SFTS patients tend to have severe or even fatal clinical outcomes, validated by remarkably high levels of viral loads and extensively disturbed laboratory findings observed in NP-specific IgM-delayed patients. Furthermore, our data revealed that compared to mild SFTS patients, fatal or severe SFTS patients had significantly lower levels of NP-specific IgM but not NP-specific IgG responses, Gn-specific IgM, nor Gc-specific IgG. Our data highlight distinct humoral profiles of NP-specific IgM responses among SFTS patients with different disease progression rates.

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## References

Arikawa, J., Schmaljohn, A.L., Dalrymple, J.M., Schmaljohn, C.S., 1989. Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. J. Gen. Virol. 70, 615–624. https://doi.org/10.1099/0022-1317-70-3-615.

Arikawa, J., Yao, J.S., Yoshimatsu, K., Takashima, I., Hashimoto, N., 1992. Protective role of antigenic sites on the envelope protein of Hantaan virus defined by monoclonal antibodies. Arch. Virol. 126, 271–281. https://doi.org/10.1007/BF01309700.

Bao, C.J., Guo, X.L., Qi, X., Hu, J.L., Zhou, M.H., Varma, J.K., Cui, L.B., Yang, H.T., Jiao, Y.J., Klena, J.D., Li, L.X., Tao, W.Y., Li, X., Chen, Y., Zhu, Z., Xu, K., Shen, A.H., Wu, T., Peng, H.Y., Li, Z.F., Shan, J., Shi, Z.Y., Wang, H., 2011. A family cluster of infections by a newly recognized bunyavirus in eastern China, 2007. Further evidence of person-to-person transmission. Clin. Infect. Dis. 53, 1208–1214. https://doi.org/10.1093/cid/cir732.

Boshra, H., Lorenzo, G., Busquets, N., Brun, A., 2011a. Rift valley fever: recent insights into pathogenesis and prevention. J. Virol. 85, 6098–6105. https://doi.org/10.1128/jvi.02641-10.

Boshra, H., Lorenzo, G., Rodriguez, F., Brun, A., 2011b. A DNA vaccine encoding ubiquitinated Rift Valley fever virus nucleoprotein provides consistent immunity and protects IFNAR(-/-) mice upon lethal virus challenge. Vaccine 29, 4469–4475. https://doi.org/10.1016/j.vaccine.2011.04.043.

Chen, Y., X., J, B, Liu, Y., Huang, R., Chen, J., Wu, C., 2017a. Risk factors associated with fatality of severe fever with thrombocytopenia syndrome: a meta-analysis. Oncotarget 8, 89119–89129. https://doi.org/10.18632/oncotarget.19163.

Chen, Y.X., J,B, Huang, R., Yan, X.M., Xiong, Y.L., Yong, L., Chao, Wu, 2017b. Occupational severe fever with thrombocytopenia syndrome following needle-stick injury. Infect. Control Hosp. Epidemiol. 38, 760–762. https://doi.org/10.1017/ice.2017.61.

Chen, Y.X., J,B, Yong, L., Huang, R., Chen, T., Chao, Wu, 2017c. Risk factors associated with fatality of severe fever with thrombocytopenia syndrome: a meta-analysis. Oncotarget 8, 89119–89129. https://doi.org/10.18632/oncotarget.19163.

Crowe Jr., J.E., 2017. Principles of broad and potent antiviral human antibodies: insights for vaccine design. Cell Host Microbe 22, 193–206. https://doi.org/10.18632/oncotarget.19163.

Cui, N., Bao, X.L., Yang, Z.D., Lu, Q.B., Hu, C.Y., Wang, L.Y., Wang, B.J., Wang, H.Y., Liu, K., Yuan, C., Fan, X.J., Wang, Z., Zhang, L., Zhang, X.A., Hu, L.P., Liu, W., Cao, W.C., 2014. Clinical progression and predictors of death in patients with severe fever with thrombocytopenia syndrome in China. J. Clin. Virol. 59, 12–17. https://doi.org/10.1016/j.jcv.2013.10.024.

Deng, B., Zhou, B., Zhang, S., Zhu, Y., Han, L., Geng, Y., Jin, Z., Liu, H., Wang, D., Zhao, Y., Wen, Y., Cui, W., Zhou, Y., Gu, Q., Sun, C., Lu, X., Wang, W., Wang, Y., Li, C., Wang, Y., Yao, W., Liu, P., 2013. Clinical features and factors associated with severity and fatality among patients with severe fever with thrombocytopenia syndrome Bunyavirus infection in Northeast China. PLoS One 8, e80802. https://doi.org/10.1371/journal.pone.0080802.

Ding, S., Niu, G., Xu, X., Li, J., Zhang, X., Yin, H., Zhang, N., Jiang, X., Wang, S., Liang, M., Wang, X., Yu, X.J., 2014. Age is a critical risk factor for severe fever with thrombocytopenia syndrome. PLoS One 9, e111736. https://doi.org/10.1371/journal.pone.0111736.

Eifan, S.A., Elliott, R.M., 2009. Mutational analysis of the Bunyamwera orthobunyavirus nucleocapsid protein gene. J. Virol. 83, 11307–11317. https://doi.org/10.1128/jvi.01460-09.

Fafetine, J.M., Tijhaar, E., Paweska, J.T., Neves, L.C., Hendriks, J., Swanepoel, R., Coetzer, J.A., Egberink, H.F., Rutten, V.P., 2007. Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. Vet. Microbiol. 121, 29–38. https://doi.org/10.1016/j.vetmic.2006.11.008.

Guo, X., Zhang, L., Zhang, W., Chi, Y., Zeng, X., Li, X., Qi, X., Jin, Q., Zhang, X., Huang, M., Wang, H., Chen, Y., Bao, C., Hu, J., Liang, S., Bao, L., Wu, T., Zhou, M., Jiao, Y., 2013. Human antibody neutralizes severe Fever with thrombocytopenia syndrome virus, an emerging hemorrhagic Fever virus. Clin. Vaccine Immunol. 20, 1426–1432. https://doi.org/10.1128/cvi.00222-13.

Huang, Y.T., Zhao, L., Wen, H.L., Yang, Y., Yu, H., Yu, X.J., 2016. Neutralizing antibodies to severe fever with thrombocytopenia syndrome virus 4 years after hospitalization. China Emerg. Infect. Dis. 22, 1985–1987. https://doi.org/10.3201/eid2211.160414.

Jansen van Vuren, P., Potgieter, A.C., Paweska, J.T., van Dijk, A.A., 2007. Preparation and evaluation of a recombinant Rift Valley fever virus NP for the detection of IgG and IgM antibodies in humans and animals by indirect ELISA. J. Virol. Methods 140, 106–114. https://doi.org/10.1016/j.jviromet.2006.11.005.

Jansen van Vuren, P., Tiemessen, C.T., Paweska, J.T., 2011. Anti-nucleocapsid protein immune responses counteract pathogenic effects of Rift Valley fever virus infection in mice. PLoS One 6, e25027. https://doi.org/10.1371/journal.pone.0025027.

Jia, B., Yan, X.M., Chen, Y.X., Wang, G.Y., Liu, Y., Xu, B., Song, P.X., Li, Y., Xiong, Y.L., Wu, W.H., Hao, Y.Y., Xia, J., Zhang, Z.P., Huang, R., Wu, C., 2017. A scoring model for predicting prognosis of patients with severe fever with thrombocytopenia syndrome. PLoS Negl. Trop. Dis. 11, e0005909. https://doi.org/10.1371/journal.pntd.0005909.

Kim, W.Y., Choi, W., Park, S.W., Wang, E.B., Lee, W.J., Jee, Y., Lim, K.S., Lee, H.J., Kim,

S.M., Lee, S.O., Choi, S.H., Kim, Y.S., Woo, J.H., Kim, S.H., 2015. Nosocomial transmission of severe fever with thrombocytopenia syndrome in Korea. Clin. Infect. Dis. 60, 1681–1683. https://doi.org/10.1093/cid/civ128.

Liu, W., Lu, Q.B., Cui, N., Li, H., Wang, L.Y., Liu, K., Yang, Z.D., Wang, B.J., Wang, H.Y., Zhang, Y.Y., Zhuang, L., Hu, C.Y., Yuan, C., Fan, X.J., Wang, Z., Zhang, L., Zhang, X.A., Walker, D.H., Cao, W.C., 2013. Case-fatality ratio and effectiveness of ribavirin therapy among hospitalized patients in china who had severe fever with thrombocytopenia syndrome. Clin. Infect. Dis. 57, 1292–1299. https://doi.org/10.1093/cid/cit530.

Lu, Q.B., Cui, N., Hu, J.G., Chen, W.W., Xu, W., Li, H., Zhang, X.A., Ly, H., Liu, W., Cao, W.C., 2015. Characterization of immunological responses in patients with severe fever with thrombocytopenia syndrome: a cohort study in China. Vaccine 33, 1250–1255. https://doi.org/10.1016/j.vaccine.2015.01.051.

Luo, L., Zhao, M.L., Wen, H.L., Zhang, Z.T., Liu, J.W., Fang, L.Z., Xue, Z.F., Ma, D.Q., Zhang, X.S., Ding, S.J., Lei, X.Y., Yu, X.J., 2015. Haemaphysalis longicornis ticks as reservoir and vector of severe fever with thrombocytopenia syndrome virus in China. Emerg. Infect. Dis. 21, 1770–1776. https://doi.org/10.3201/eid2110.150126.

Magurano, F., Nicoletti, L., 1999. Humoral response in Toscana virus acute neurologic disease investigated by viral-proteiNP-specific immunoassays. Clin. Diagn. Lab. Immunol. 6, 55–60. https://doi.org/10.1089/088922299311781.

Martin-Folgar, R., Lorenzo, G., Boshra, H., Iglesias, J., Mateos, F., Borrego, B., Brun, A., 2010. Development and characterization of monoclonal antibodies against Rift Valley fever virus nucleocapsid protein generated by DNA immunization. MAbs 2, 275–284. https://doi.org/10.4161/mabs.2.3.11676.

McMullan, L.K., Folk, S.M., Kelly, A.J., MacNeil, A., Goldsmith, C.S., Metcalfe, M.G., Batten, B.C., Albarino, C.G., Zaki, S.R., Rollin, P.E., Nicholson, W.L., Nichol, S.T., 2012. A new phlebovirus associated with severe febrile illness in Missouri. N. Engl. J. Med. 367, 834–841. https://doi.org/10.1056/NEJMoa1203378.

Nakamura, T., Yanagihara, R., Gibbs Jr., C.J., Gajdusek, D.C., 1985. Immune spleen cell-mediated protection against fatal Hantaan virus infection in infant mice. J. Infect. Dis. 151, 691–697. https://doi.org/10.1093/infdis/151.4.691.

Pekosz, A., He, B., Lamb, R.A., 1999. Reverse genetics of negative-strand RNA viruses: closing the circle. Proc. Natl. Acad. Sci. U. S. A. 96, 8804–8806. https://doi.org/10.1073/pnas.96.16.8804.

Pinschewer, D.D., Perez, M., de la Torre, J.C., 2003. Role of the virus nucleoprotein in the regulation of lymphocytic choriomeningitis virus transcription and RNA replication. J. Virol. 77, 3882–3887. https://doi.org/10.1128/JVI.77.6.3882-3887.2003.

Takahashi, T., Maeda, K., Suzuki, T., Ishido, A., Shigeoka, T., Tominaga, T., Kamei, T., Honda, M., Ninomiya, D., Sakai, T., Senba, T., Kaneyuki, S., Sakaguchi, S., Satoh, A., Hosokawa, T., Kawabe, Y., Kurihara, S., Izumikawa, K., Kohno, S., Azuma, T., Suemori, K., Yasukawa, M., Mizutani, T., Omatsu, T., Katayama, Y., Miyahara, M., Ijuin, M., Doi, K., Okuda, M., Umeki, K., Saito, T., Fukushima, K., Nakajima, K., Yoshikawa, T., Tani, H., Fukushi, S., Fukuma, A., Ogata, M., Shimojima, M., Nakajima, N., Nagata, N., Katano, H., Fukumoto, H., Sato, Y., Hasegawa, H., Yamagishi, T., Oishi, K., Kurane, I., Morikawa, S., Saijo, M., 2014. The first identification and retrospective study of Severe Fever with Thrombocytopenia Syndrome in Japan. J. Infect. Dis. 209, 816–827. https://doi.org/10.1093/infdis/jit603.

Walter, C.T., Bento, D.F., Alonso, A.G., Barr, J.N., 2011. Amino acid changes within the Bunyamwera virus nucleocapsid protein differentially affect the mRNA transcription and RNA replication activities of assembled ribonucleoprotein templates. J. Gen. Virol. 92, 80–84. https://doi.org/10.1099/vir.0.024240-0.

Yoshimatsu, K., Yoo, Y.C., Yoshida, R., Ishihara, C., Azuma, I., Arikawa, J., 1993. Protective immunity of Hantaan virus nucleocapsid and envelope protein studied using baculovirus-expressed proteins. Arch. Virol. 130, 365–376. https://doi.org/10.1007/BF01309667.

Yu, X.J., Liang, M.F., Zhang, S.Y., Liu, Y., Li, J.D., Sun, Y.L., Zhang, L., Zhang, Q.F., Popov, V.L., Li, C., Qu, J., Li, Q., Zhang, Y.P., Hai, R., Wu, W., Wang, Q., Zhan, F.X., Wang, X.J., Kan, B., Wang, S.W., Wan, K.L., Jing, H.Q., Lu, J.X., Yin, W.W., Zhou, H., Guan, X.H., Liu, J.F., Bi, Z.Q., Liu, G.H., Ren, J., Wang, H., Zhao, Z., Song, J.D., He, J.R., Wan, T., Zhang, J.S., Fu, X.P., Sun, L.N., Dong, X.P., Feng, Z.J., Yang, W.Z., Hong, T., Zhang, Y., Walker, D.H., Wang, Y., Li, D.X., 2011. Fever with thrombocytopenia associated with a novel bunyavirus in China. N. Engl. J. Med. 364, 1523–1532. https://doi.org/10.1056/NEJMoa1010095.

Yu, L., Zhang, L., Sun, L., Lu, J., Wu, W., Li, C., Zhang, Q., Zhang, F., Jin, C., Wang, X., Bi, Z., Li, D., Liang, M., 2012. Critical epitopes in the nucleocapsid protein of SFTS virus recognized by a panel of SFTS patients derived human monoclonal antibodies. PLoS One 7, e38291. https://doi.org/10.1371/journal.pone.0038291.

Zhang, Y.Z., He, Y.W., Dai, Y.A., Xiong, Y., Zheng, H., Zhou, D.J., Li, J., Sun, Q., Luo, X.L., Cheng, Y.L., Qin, X.C., Tian, J.H., Chen, X.P., Yu, B., Jin, D., Guo, W.P., Li, W., Wang, W., Peng, J.S., Zhang, G.B., Zhang, S., Chen, X.M., Wang, Y., Li, M.H., Li, Z., Lu, S., Ye, C., de Jong, M.D., Xu, J., 2012. Hemorrhagic fever caused by a novel Bunyavirus in China: pathogenesis and correlates of fatal outcome. Clin. Infect. Dis. 54, 527–533. https://doi.org/10.1093/cid/cir804.

Zhang, X., Liu, Y., Zhao, L., Li, B., Yu, H., Wen, H., Yu, X.J., 2013. An emerging hemorrhagic fever in China caused by a novel bunyavirus SFTSV. Sci. China Life Sci. 56, 697–700. https://doi.org/10.1186/s12985-016-0661-9.